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ORIGINAL RESEARCH

LAG3 Regulates T Cell Activation and Plaque Infiltration in Atherosclerotic Mice

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ABSTRACT

BACKGROUND The immune checkpoint receptor lymphocyte-activation gene 3 (LAG3) is a new target for immune checkpoint blockade (ICB), but the effects of LAG3 on atherosclerosis are not known.

OBJECTIVES The aim of the study was to evaluate the role of LAG3 on plaque inflammation using murine hypercholesterolemic models of atherosclerosis.

METHODS To study the role of LAG3 in atherosclerosis, we investigated both bone marrow chimeras lacking LAG3 in hematopoietic cells as well as global *Lag3*$^{-/-}$ knockout mice. Effects of anti-LAG3 monoclonal antibody monotherapy and combination therapy with anti-programmed cell death protein 1 (PD-1) were tested in hypercholesterolemic low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice and evaluated by histology and flow cytometry.

RESULTS LAG3-deficiency or treatment with blocking anti-LAG3 monoclonal antibodies led to increased levels of both interferon gamma–producing T helper 1 cells and effector/memory T cells, balanced by increased levels of regulatory T cells. Plaque size was affected by neither LAG3 deficiency nor LAG3 blockade, although density of T cells in plaques was 2-fold increased by loss of LAG3. Combination therapy of anti-PD-1 and anti-LAG3 had an additive effect on T cell activation and cytokine production and promoted plaque infiltration of T cells.

CONCLUSIONS Loss of LAG3 function promoted T cell activation and accumulation in plaques while not affecting plaque burden. Our report supports further clinical studies investigating cardiovascular risk in patients treated with anti-LAG3 ICB. (J Am Coll Cardiol CardioOnc 2022;4:635-645) © 2022 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Immune checkpoint blockade (ICB) targeting T cell-inhibitory pathways (cytotoxic T lymphocyte associated protein 4 [CTLA-4], programmed cell death protein 1 [PD-1] and programmed death-ligand 1 [PD-L1]) has proven successful as treatment for several types of tumors. The mechanisms of action of ICB are not completely understood, and likely varies between ICB targets, but converges on alleviating T cell inhibition in the tumor to allow for efficient antitumor responses.[1] Immune checkpoint

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ABBREVIATIONS AND ACRONYMS

CTLA-4 = cytotoxic T lymphocyte associated protein 4

HCD = high-cholesterol diet

ICB = immune checkpoint blockade

IFN = interferon

IL = interleukin

LAG3 = lymphocyte-activation gene 3

PD-1 = programmed cell death protein-1

PD-L1 = programmed death-ligand 1

TNF = tumor necrosis factor

Treg = regulatory T cell

WT = wild-type

molecules serve an important physiological role in regulating adaptive immune response, and ICB treatment has been shown to induce autoimmunity and tissue inflammation.[2-4]

Atherosclerosis is a chronic disease with involvement of both innate and adaptive immunity.[5] Previous work in hypercholesterolemic mice has shown that genetic loss of PD-1 or PD-L1 aggravates atherosclerosis and promotes vascular inflammation as indicated by increased vascular T cell accumulation.[6-8] Treatment with a combination of anti-PD-1 and anti-CTLA-4 antibodies likewise led to increased T cell accumulation in atherosclerotic plaques[9] and ICB treatment in cancer patients was associated with a 3-fold increased risk of cardiovascular disease.[10]

A new target for ICB in cancer is lymphocyte-activation gene 3 (LAG3) that is expressed on several leukocyte subsets, including T cells.[11] LAG3 is upregulated upon activation of T cells but proteolytically cleaved by ADAMT10/17 proteases,[12] and expressed at higher levels on exhausted T cells.[13] LAG3 interacts with several receptors including major histocompatibility complex II and galectin-3 and negatively modulates T cell activation and proliferation.[11]

Demonstrating an important immunoregulatory role of LAG3 in vivo, genetic knockdown of LAG3 in mice results in increased susceptibility to autoimmune diseases such as type 1 diabetes.[14] Recently, the RELATIVITY-047 (Study of relatlimab plus nivolumab versus nivolumab alone in participants with advanced melanoma) trial demonstrated that treatment with a monoclonal antibody against LAG3 (relatimab) in combination with anti-PD-1 (nivolumab) was superior to nivolumab monotherapy in patients with untreated advanced melanoma,[15] and the combination therapy of anti-LAG3/anti-PD-1 (Opdualag) is now approved by the U.S. Food and Drug Administration for clinical use. Given previous reports of ICB-induced vascular inflammation, a key outstanding question is what role LAG3 plays in regulating cardiovascular inflammation. In this report, we investigated the role of LAG3 in atherosclerosis and potential synergistic effects of dual LAG3/PD-1 blockade utilizing complementary mouse models of atherosclerosis.

METHODS

MICE. Mice in this study were bred and housed in the pathogen-free facility at the New Research Building (Harvard Medical School, Boston, Massachusetts, USA) or Lund University (Malmö, Sweden). All vertebrate animal related procedures were approved and were carried out in accordance with the Institutional Animal Care and Use Committee or approved by local government agencies in Sweden. Ethical permits were obtained before conducting any experiments. *Lag3*$^{-/-}$ mice originally derived by Miyazaki et al,[16] were generously provided by Dr Dario Vignali (University of Pittsburgh). Studies were conducted using both male and female mice.

BONE MARROW TRANSPLANTATION. Eleven-week-old male and female *Ldlr*$^{-/-}$ mice were subjected to 950 rad of total body irradiation and reconstituted with 1×10^6 bone marrow cells from *Lag3*$^{-/-}$ or wild-type (WT) mice. Bone marrow recipients received sulfamethoxazole/trimethoprim (Sulfatrim) treatment in drinking water for 1 week prior to and 4 weeks following bone marrow transplantation. All animals were allowed to recover on a chow diet for 4 weeks after bone marrow transplantation and then fed a high-cholesterol diet (HCD) containing 1.25% cholesterol (catalog no. D1218C; Research Diets) for 10 weeks until the end of the experiment.

ADENO-ASSOCIATED VIRAL ADMINISTRATION OF MURINE PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9. AAV-PCSK9 ^{D374Y} was administered as previously described.[17] In brief, 9-week-old female and male WT (C57BL/6) or *Lag3*$^{-/-}$ (C57BL/6 background) mice were given a single dose of AAV-PCSK9 ^{D374Y} (5×10^{11} genome copies/injection) and fed an HCD for 10 weeks.

IMMUNE CHECKPOINT BLOCKADE. For studies investigating the effect of anti-LAG3 monotherapy on atherosclerosis, 9-week-old *Ldlr*$^{-/-}$ mice were fed an HCD for a total of 10 weeks, and anti-LAG3 (clone: C9B7W; BioXCell) or control immunoglobulin G1 (IgG1) (clone: HRPN; BioXCell) were administered (0.2 mg/dose) twice a week for the last 4 weeks. In an additional experiment, 8- to 12-week-old apolipoprotein E-deficient (*Apoe*$^{-/-}$ mice) were fed an HCD for 5 weeks and treated for the last 3 weeks with anti-LAG3 (clone: C9B7W; BioXCell) or control IgG1 (clone: HRPN; BioXCell) (0.2 mg/dose) twice a week. For studies investigating the effect of anti-LAG3/anti-PD-1 therapy, 7- to 9-week-old *Ldlr*$^{-/-}$ mice were fed an HCD for a total of 6 weeks, and 0.2 mg anti-PD-1 (clone: 29F.1A12; BioXCell), 0.2 mg anti-PD-1 and 0.2 anti-LAG3, or combined isotype control 0.2 mg IgG1/0.2 mg IgG2a (clones: HRPN and 2A3) were administered twice a week for the last 4 weeks.

HISTOLOGY. Oil Red O (Sigma-Aldrich) staining with hematoxylin counterstained was performed to visualize neutral lipids and allow for quantification of

atherosclerotic lesions. Van Gieson staining kit (Sigma-Aldrich) was utilized to stain plaque collagen. To quantify $CD4^+$ T cells and macrophages in lesions, sections were stained using anti-CD4 as primary antibody (Cat. No. 550280; BD Pharmingen) or anti-Mac-3 (M3/84, Cat. No. 550292; BD Pharmingen), respectively, and biotinylated rabbit anti-rat IgG (Cat. No. BA-4001; Vector Laboratories) as the secondary antibody. Sections were incubated with streptavidin-HRP (Cat. No. K0675; DAKO), and the reaction was visualized using AEC Substrate Chromogen (Cat. No. K3464; DAKO). To visualize the nuclei, sections were counterstained using Gill's hematoxylin. $CD4^+$ T cells were quantified by manually counting the stained cells. Adventitial tissue was identified as the area extending outward from the external elastic lamina not including myocardium. Assessment of plaque morphology was performed blinded using the Virmani classification.[18]

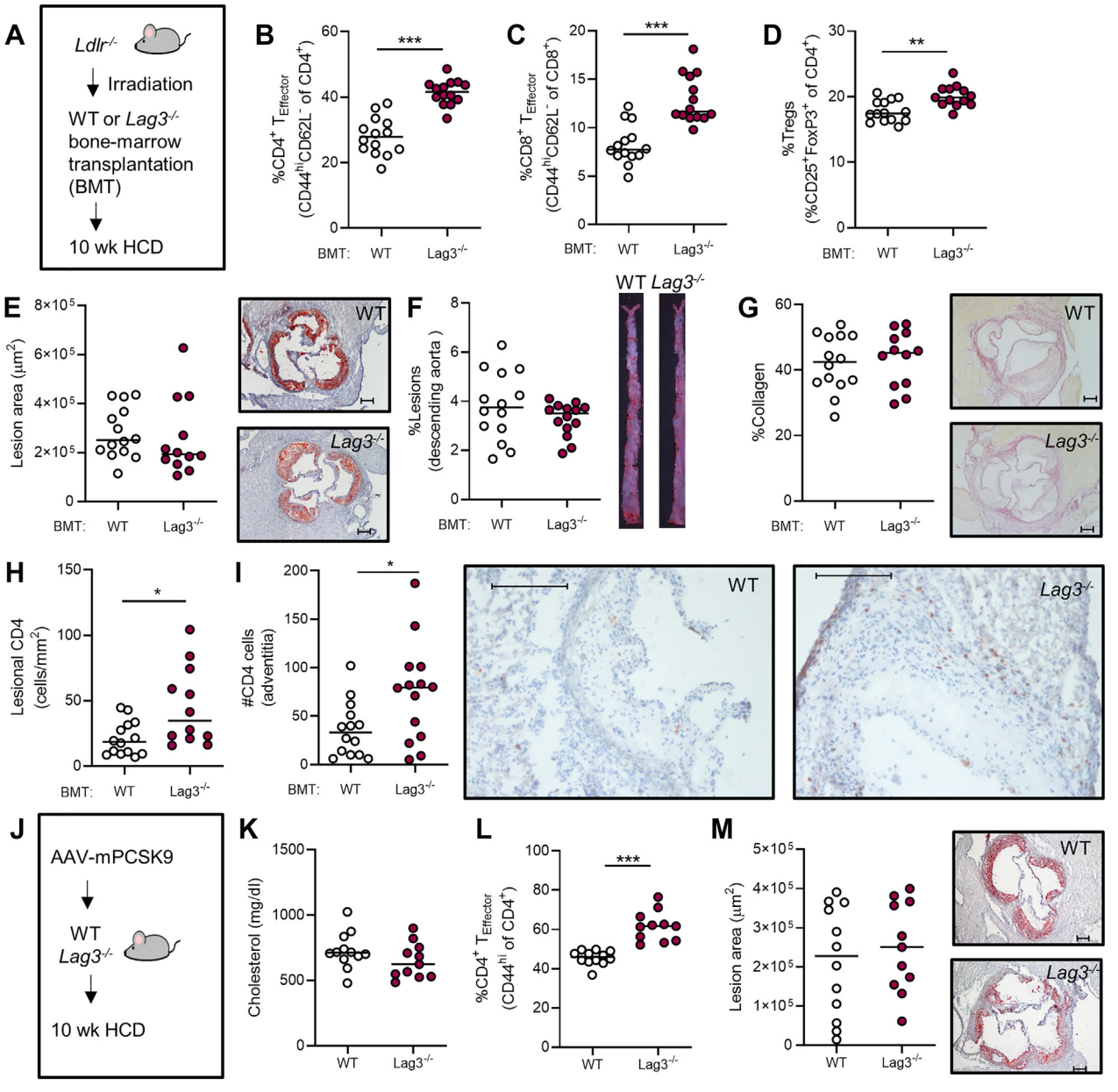
FLOW CYTOMETRY. Single-cell suspensions were analyzed by flow cytometry using the following antibodies: CD3 (17A2), CD4 (RM4-4), CD8 (53-6.7), CD44 (IM7), CD62L (MEL14) and CD25 (PC61), and PD-1 (29F.1A12). Zombie Aqua fixable viability dye (Cat. No. 423102) was used to remove dead cells. Anti-CD16/CD32 was added to antibody mix to block nonspecific monoclonal antibody binding. Fluorescence activated cell sorting buffer containing 0.5% bovine serum albumin and 0.02% sodium azide in phosphate-buffered saline without Ca^{2+} and Mg^{2+} was used for all the washing steps. To measure intracellular cytokines or other intracellular proteins, splenocytes or cells obtained from aortic digestion were stimulated with cell activation cocktail containing phorbol myristate acetate, ionomycin, and Brefeldin A (Cat No. 423303; BioLegend). Cells were fixed and permeabilized using a fixation and permeabilization kit (Cat. No. 88-8824-00; eBioscience) and subsequently stained with anti-interferon (IFN)-γ (XMG1.2), anti-interleukin (IL)-2 (JES6-5H4), and anti-tumor necrosis factor (TNF)-α (MP6-XT22). For T regulatory cell (Treg) and Ki67 proliferation analysis, unstimulated cells were fixed and permeabilized followed by Ki67 (16A8) and FoxP3 (FJK-16S) staining. Fluorescence activated cell sorting analysis was performed on a DxP11 or Gallios flow cytometer, and the data were analyzed using FlowJo software (FlowJo v10.8.0, LLC).

STATISTICS. Data were tested for normal distribution using Kolmogorov-Smirnov normality test. Data comparing 2 groups were analyzed with unpaired 2-tailed Student's *t* test or Mann-Whitney *U* test. Data comparing 3 groups were analyzed with analysis of variance and Tukey's multiple comparison test or Kruskal-Wallis test and Dunn's multiple comparison tests. A P value of <0.05 was determined as significant. Statistical analysis was performed using GraphPad Prism v7 software (GraphPad Software). Dots represent individual mice and bars denote median in all graphs.

RESULTS

LAG3 REGULATES T CELL ACTIVATION AND ACCUMULATION OF PLAQUE T CELLS BUT DOES NOT AFFECT PLAQUE BURDEN. To evaluate the role of LAG3 in atherosclerosis, we performed several complementary models of experimental atherosclerosis. First, $Ldlr^{-/-}$ recipient mice were irradiated and reconstituted with bone marrow from WT C57BL/6 (WT) or $Lag3^{-/-}$ donors to generate bone marrow chimeras (n = 14/group). After 10 weeks of HCD feeding, mice were terminated and the cardiovascular and immunological phenotype investigated (**Figure 1A**). As compared with recipient mice reconstituted with WT donor bone marrow cells, recipient mice reconstituted with $Lag3^{-/-}$ donor cells showed increased levels of $CD44^{hi}CD62L^-$ $CD4^+$ and $CD8^+$ T effector cells, as well as increased levels of $CD25^+FoxP3^+$ Tregs (**Figures 1B to 1D**). Similarly, levels of circulating $CD44^{hi}CD62L^-$ $CD4^+$ and $CD8^+$ T effector cells were also increased in recipient mice reconstituted with $Lag3^{-/-}$ donor cells compared with WT donor cells (Supplemental Figures 1A and 1B). Weight, total cholesterol, low-density lipoprotein and high-density lipoprotein levels were not affected by LAG3 deficiency (Supplemental Figures 1C to 1F). Analysis of subvalvular and descending aortic atherosclerotic plaques revealed no difference in plaque burden (**Figures 1E and 1F**), and we observed no change in collagen (**Figure 1G**) or macrophage content (Supplemental Figure 1G). Categorization of plaque morphology (intimal thickening, intimal xanthoma, pathological intimal thickening, and fibrous cap atheroma) did not reveal any differences between groups (Supplemental Figure 1H). However, levels of $CD4^+$ T cell accumulation in aortic plaques and vascular adventitia were 2-fold ($P < 0.05$) increased in $Lag3^{-/-}$ bone marrow recipients compared with WT control mice (**Figures 1H and 1I**). To validate our findings using a complementary model of atherosclerosis, WT or $Lag3^{-/-}$ mice were injected with adeno-associated virus encoding a gain-of-function variant of proprotein convertase subtilisin/kexin

FIGURE 1 Impact of LAG3 Deficiency on T Cell Activation and Atherosclerosis



(A) Irradiated low-density lipoprotein knockout mice (*Ldlr*$^{-/-}$) mice received wild-type (WT) (n = 14) or lymphocyte-activation gene 3 (LAG3) knockout (*Lag3*$^{-/-}$) (n = 14) bone marrow transplantation (BMT) and were fed a high-cholesterol diet (HCD) for 10 weeks to study atherosclerosis. **(B-D)** Levels of CD4^{+} and CD8^{+} CD44^{hi}CD62L^{-} T effector cells and CD25^{+}FoxP3^{+} regulatory T cells in spleen. **(E)** Histological quantification of subvalvular plaque area and representative Oil Red O-stained images. Scale bar = 100 μm. **(F)** En face analysis of descending aorta and representative images. **(G)** Van Gieson collagen staining and representative images. Scale bar = 100 μm. **(H, I)** Immunohistochemical staining for CD4 and quantification of lesional and adventitial CD4^{+} cells per mm^{2}. Scale bar =50 μm. **(J)** Adeno-associated viral administration of murine proprotein convertase subtilisin/kexin type 9 (AAV-mPCSK9^{D374Y}) to WT or *Lag3*$^{-/-}$ mice followed by 10 weeks of HCD. **(K)** Cholesterol levels in plasma. **(L)** Levels of CD4^{+} CD44^{+}CD62L^{-} T effector cells in spleen. **(M)** Histological quantification of subvalvular plaque area and representative Oil Red O-stained images. Scale bar =100 μm. An unpaired *t* test was used to analyze normally distributed data and a Mann-Whitney *U* test was used for non-normally distributed data. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

type 9 (AAV-PSCK9^{DY})[19] to induce hypercholesterolemia upon HCD feeding (**Figures 1J and 1K**). Similarly, in the context of AAV-PSCK9^{DY}-induced hypercholesterolemia, T cell activation was increased in *Lag3*$^{-/-}$ compared with WT mice, but plaque burden and plaque morphology remained unchanged (**Figures 1L and 1M**, Supplemental Figures 1I and 1J).

LAG3 BLOCKADE PROMOTES T CELL ACTIVATION AND PROMOTES PD-1 EXPRESSION. To test cardiovascular and immunoregulatory effects of LAG3 blockade, *Ldlr*$^{-/-}$ mice were injected with anti-LAG3 monoclonal antibodies for the last 4 weeks of a total 10 weeks on an HCD (n = 12/group) (**Figure 2A**). Similar as to what was observed in *Lag3*$^{-/-}$ mice, LAG3 blockade led to increased fractions of CD44^{hi}CD62L^{-} CD4^{+} T effector cells and elevated levels of FoxP3^{+} Tregs (**Figures 2B and 2C**) in spleen and aorta-draining lymph nodes, as well as increased levels of CD44^{hi}CD62L^{-} CD8^{+} T effector cells in spleen but not in aorta-draining lymph nodes (Supplemental Figure 2A). Similarly, both conventional T cells (FoxP3^{-}) and Tregs (FoxP3^{+}) displayed increased fractions of Ki67^{+} cells in anti-LAG3 treated mice (**Figure 2D**, Supplemental Figure 2B). Furthermore, LAG3 blockade resulted in increased levels of IFN-γ-producing T helper 1 cells (**Figure 2E**). Analysis of atherosclerotic plaque burden revealed no change in plaque burden or plaque morphology after anti-LAG3 treatment (**Figure 2F**, Supplemental Figure 2C).

LAG3 has been demonstrated to be expressed on exhausted PD-1-expressing T cells in the context of cancer and chronic infections.[20] In a separate experiment to evaluate if LAG3 blockade in atherosclerotic mice impacts expression of immunoregulatory PD-1, apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice were fed HCD for 6 weeks and treated with anti-LAG3 for the last 3 weeks (n = 7/group). Notably, LAG3 blockade expanded a population of CD44^{+}PD-1^{int} (intermediate) T cells of both CD4^{+} and CD8^{+} subsets (**Figures 3A to 3C**). ICB treatment (eg, anti-PD-1) has been shown to expand IFN-γ-producing CD8^{+} T cell subsets that retain capacity to produce multiple other cytokines besides IFN-γ.[21] Suggesting a similar immunostimulatory function of anti-LAG3 antibodies, we observed increased levels of IL-2/TNF-α coproduction by IFN-γ-producing CD8^{+} T cells after anti-LAG3 treatment (**Figures 3D and 3E**). Analyzing IL-2 and TNF-α separately, IL-2 production by IFN-γ-producing CD8^{+} T cells was upregulated, whereas TNF-α production was not affected (Supplemental Figure 3A). We did not observe any change in the ability of T helper 1 cells to produce IL-2 or TNF-α after anti-LAG3 blockade (Supplemental Figures 3B to 3D).

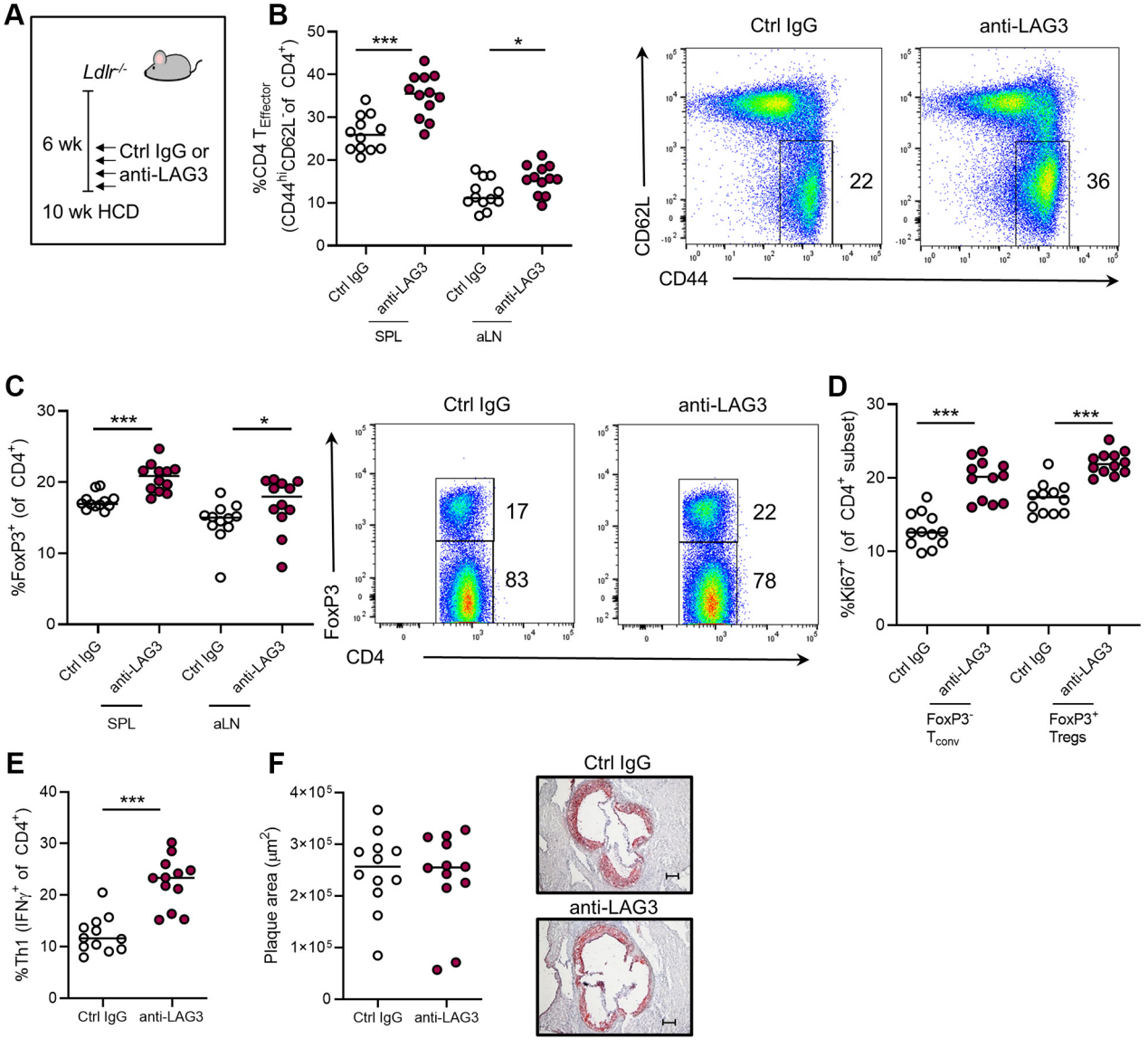
ADDITIVE EFFECT OF LAG3 AND PD-1 BLOCKADE IN ACTIVATING T CELLS IN ATHEROSCLEROTIC MICE. Of relevance to the recently approved anti-LAG3/anti-PD-1 combination therapy in patients with melanoma,[15] we tested the immunological and cardiovascular outcomes of combination therapy in atherosclerotic mice. *Ldlr*$^{-/-}$ mice were fed HCD for 6 weeks and treated with control IgG, anti-PD-1 monotherapy, or anti-PD-1/anti-LAG3 combination therapy for the last 4 weeks (n = 7-9/group) (**Figure 4A**). Results showed a striking additive effect of anti-LAG3 combined with anti-PD-1 for promoting CD44^{hi}CD62L^{-} T effector cell expansion and IFN-γ production of both CD4^{+} and CD8^{+} T cells (**Figures 4B to 4F**, Supplemental Figure 4A), compared with anti-PD-1 monotherapy or control IgG. Levels of Tregs were also expanded by anti-PD-1/anti-LAG3 combination therapy (**Figure 4G**). Cholesterol levels were not affected by ICB treatment (**Figure 4H**). Analyzing the atherosclerotic plaques, neither anti-PD-1 monotherapy nor anti-PD-1/anti-LAG3 combination therapy influenced plaque burden or plaque morphology (**Figure 4I**, Supplemental Figure 4B). However, accumulation of lesional and adventitial CD4^{+} T cells were increased by anti-LAG3/anti-PD-1 combination therapy (**Figures 4J and 4K**, Supplemental Figure 4C).

DISCUSSION

ICB has been shown to promote cardiovascular disease and aggravate atherosclerosis in patients.[10] Recently, combination therapy of PD-1- and LAG3-blocking antibodies has been approved for use in patients with unresectable or metastatic melanoma. Our study demonstrates that LAG3 regulates T cell activation in atherosclerotic mice and that loss of LAG3 function promotes T cell infiltration in atherosclerotic plaques and surrounding vascular adventitia (see **Central Illustration**).

The effects of loss of LAG3 on immune function and atherosclerosis observed in our study is in line with that observed for studies of other immune checkpoint molecules. Similar to atherosclerotic *Lag3*$^{-/-}$ mice, both PD-1 and PD-L1 deficiency promoted influx of T cells to atherosclerotic lesions and increased levels of T cell activation and cytokine production.[6-8] Antibody-mediated blockade of the PD-1 pathway promoted accumulation of T cells in lesions without any effect on plaque size.[6] However, atherosclerotic mice lacking PD-1 or PD-L1 displayed

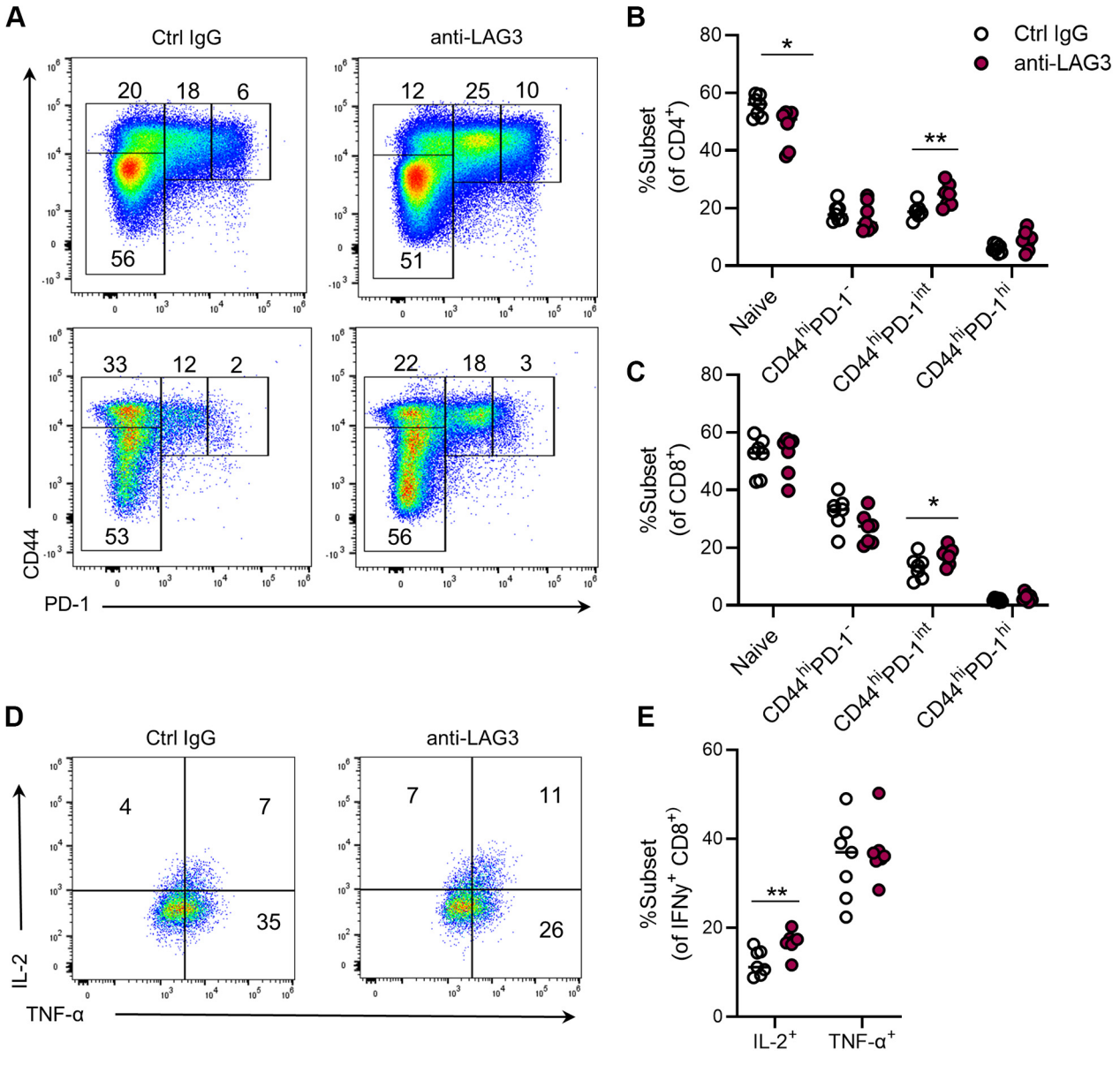
FIGURE 2 Anti-LAG3 Treatment Expands T Helper 1 Cells and Regulatory T Cells



(A) Low-density lipoprotein knockout mice (*Ldlr*$^{-/-}$) were fed an HCD for 10 weeks and injected with 0.2 mg isotype control (ctrl) immunoglobulin G (IgG) (n = 12) or anti-LAG3 (n = 12) twice a week for 4 weeks. **(B, C)** CD4^{+} CD44^{hi}CD62L^{-} effector cells or CD4^{+}CD25^{+}FoxP3^{+} regulatory T cells (Tregs) were quantified in spleen (SPL) or aortic lymph nodes (aLN). **(D)** Levels of the proliferation marker Ki67 were measured in CD4^{+} conventional T cells (FoxP3^{-} T_{conv}) or Tregs (FoxP3^{+} Tregs) in SPL. **(E)** Levels of interferon (IFN)-γ–producing T helper 1 (Th1) cells in SPL. **(F)** Histological quantification of subvalvular plaque area and representative Oil Red O–stained images. Scale bar = 100 μm. An unpaired *t* test was used to analyze normally distributed data and a Mann-Whitney *U* test was used for non-normally distributed data. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations as in Figure 1.

increased lesion size. A recent study investigating PD-1/CTLA-4 combination therapy observed a similar phenotype of increased T cell accumulation in lesions without changes in plaque size, although demonstrating an increase in plaque necrotic core area.[9] In our study, we observed an additive effect of PD-1 and LAG3 blockade on T cell activation and cytokine production. However, we did not observe any difference in plaque T cell infiltration comparing anti-PD-1 monotherapy and anti-PD-1/anti-LAG3 combination therapy, although the anti-PD-1/anti-LAG3 combination therapy treatment group displayed levels

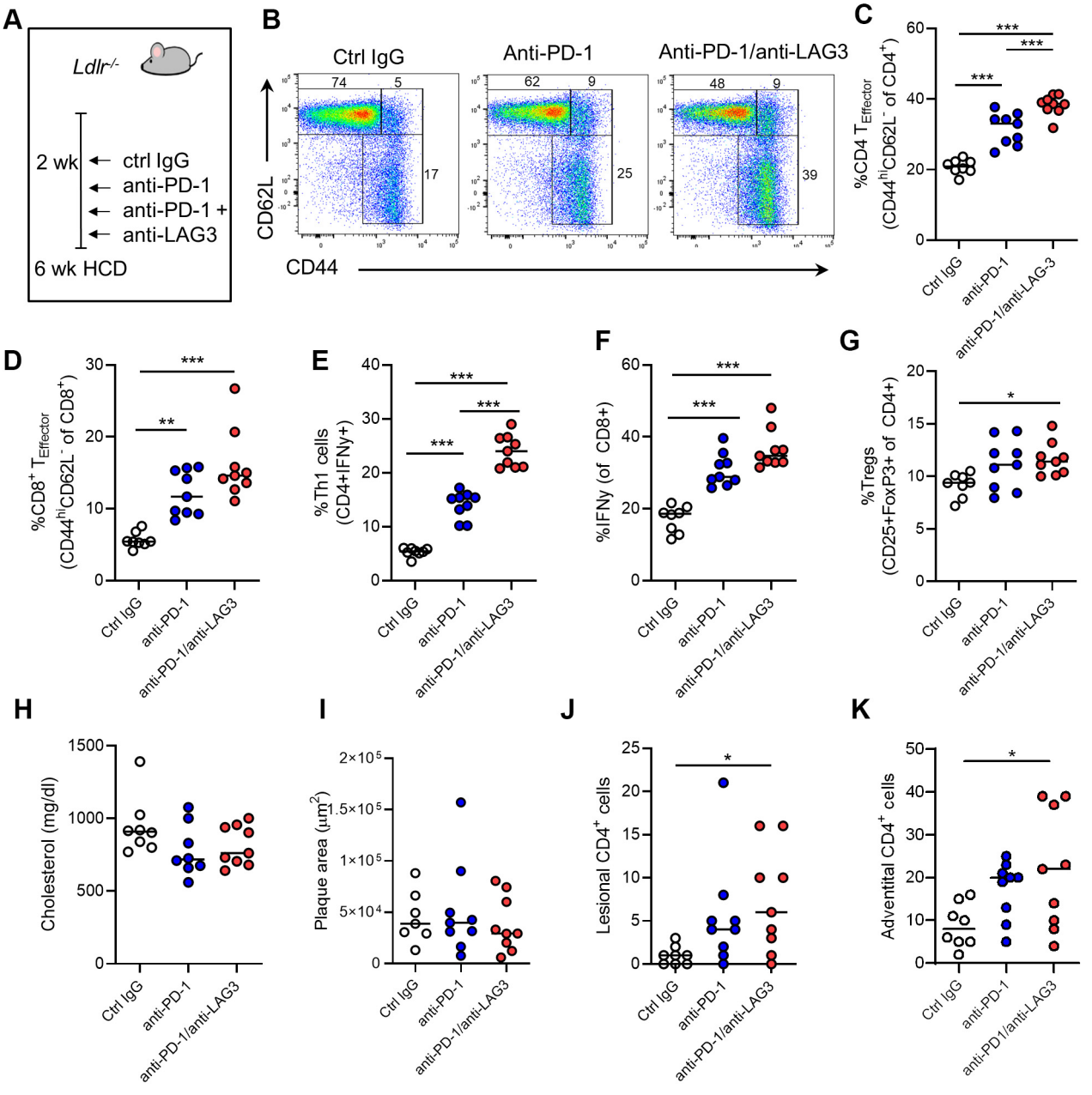
FIGURE 3 **Anti-LAG3 Treatment Promotes PD-1 Expression and IL-2 Production**



Apolipoprotein E–deficient mice (*Apoe*$^{-/-}$) fed an HCD for 6 weeks and injected with 0.2 mg isotype ctrl IgG (n = 12) or anti-LAG3 (n = 12) twice a week for 3 weeks and SPLs were analyzed. **(A)** Representative flow cytometry plots of CD4$^+$ **(top)** and CD8$^+$ **(bottom)** T cells analyzed for CD44 and programmed cell death protein 1 (PD-1). **(B, C)** Quantification of CD4$^+$- and CD8$^+$-naive (CD44$^-$PD1$^-$), CD44$^+$PD-1$^-$, CD44^{hi}PD-1^{int} (intermediate), and CD44$^+$PD-1^{hi} (high) populations. **(D)** Representative flow cytometry plots of interleukin (IL)-2 and tumor necrosis factor (TNF)-α gated on IFN-γ$^+$ CD8$^+$ T cells. **(E)** Quantification of IL-2 and TNF-α production of IFN-γ$^+$CD8$^+$ T cells. An unpaired *t* test was used to analyze normally distributed data and a Mann-Whitney *U* test was used for non-normally distributed data. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations as in Figures 1 and 2.

significantly above the control group. This suggests that treatment with LAG3-blocking antibodies in patients may promote infiltration of highly inflammatory T cells to the atherosclerotic lesions and potentially promote plaque destabilization leading to cardiovascular events. Long-term follow-up studies are needed to evaluate whether combined PD-1 and LAG3 blockade has cardiovascular side effects

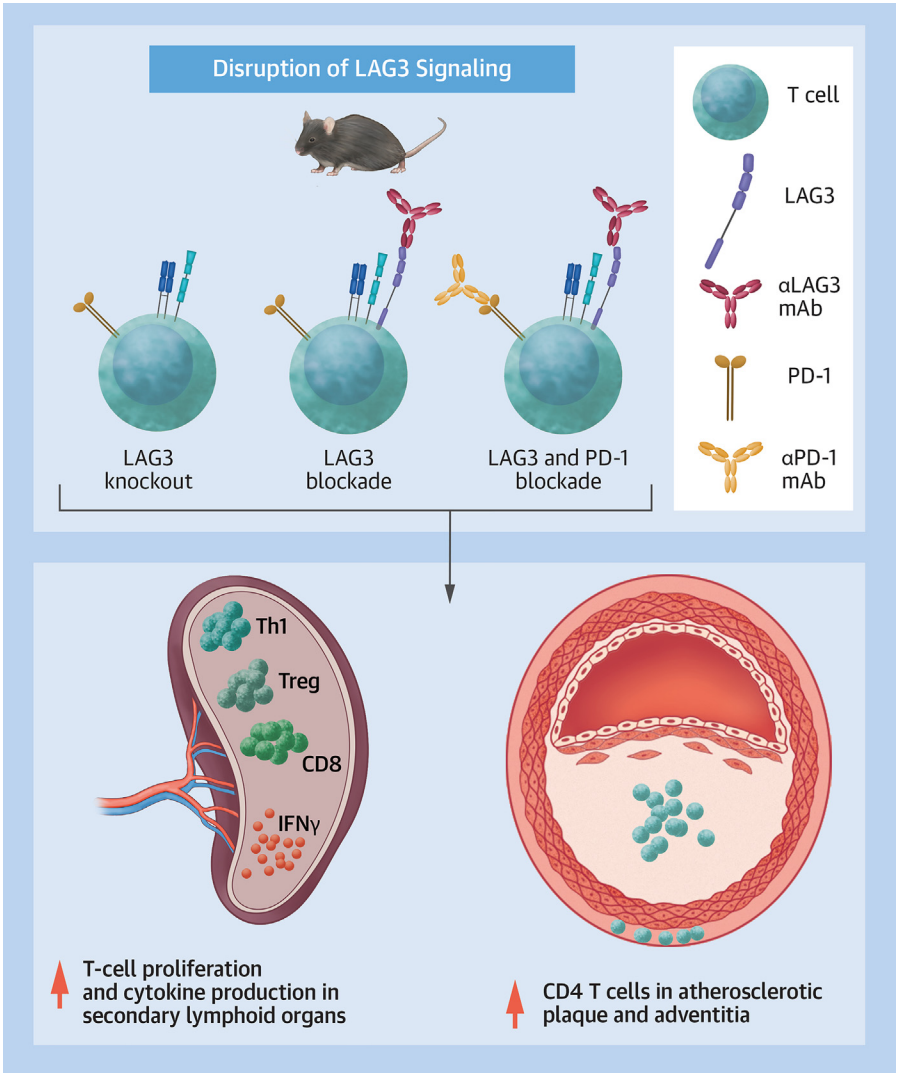
FIGURE 4 Additive Effects of Anti-LAG3/Anti-PD-1 Combination Therapy on Immune Activation in Atherosclerotic Mice



(A) Low-density lipoprotein knockout mice (*Ldlr*$^{-/-}$) were fed an HCD for 6 weeks and injected with 0.2 mg isotype ctrl IgG (n = 7), anti-PD-1 (n = 9) or anti-PD-1 + anti-LAG3 (n = 9) twice a week for 4 weeks. **(B, C)** Representative flow cytometry plots and quantification of splenic CD4$^+$ CD44^{hi}CD62L$^-$ effector cells. **(D)** Levels of CD8$^+$ CD44^{hi}CD62L$^-$ effector cells. **(E, F)** Quantification of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells. **(G)** Levels of CD4$^+$CD25$^+$FoxP3$^+$ Tregs. **(H)** Levels of plasma cholesterol. **(I)** Quantification of subvalvular plaque area. **(J, K)** Quantification of CD4$^+$ T cells in lesions and adventitia. Analysis of variance and Tukey's multiple comparison test or Kruskal-Wallis test and Dunn's multiple comparison tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations as in Figures 1 to 3.

and whether increased cardiovascular monitoring in these patients is warranted. Notably, in the MESA (Multi-Ethnic Study of Atherosclerosis) study, levels of soluble LAG3 were inversely associated with risk of coronary heart disease.[22] Our findings suggest potential cardiovascular implications in individuals with deficiencies in LAG3 production.

CENTRAL ILLUSTRATION **LAG3 Regulates T Cell Activation and Plaque Infiltration in Atherosclerotic Mice**



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Genetic loss of lymphocyte-activation gene 3 (LAG3) or LAG3 blockade in combination with programmed cell death protein-1 (PD-1) blockade in atherosclerotic mice promotes T cell activation and accumulation of T cells in plaques and adventitia. mAb = monoclonal antibody.

One consistent finding in both LAG3-deficient mice and anti-LAG3-treated mice is expansion of both conventional effector T cells and Tregs. It has previously been demonstrated that loss of LAG3 promotes Treg expansion and function.[23] Similarly, studies have shown that PD-1 blockade promotes Treg function, and that Tregs may promote progression of cancer in a subset of cancer patients that respond to anti-PD-1 therapy by expansion of Tregs.[24] It is possible that in our model, proatherogenic effects of loss of LAG3 leading to activated cytokine-producing T cells are partially countered by antiatherogenic Treg expansion, leading to a net-zero effect on plaque size. We propose that the observed increased accumulation of T cells in plaques of LAG3 deficient mice or after LAG3/PD-1 blockade is due to a combination of factors including increased generation of tissue-homing effector T cells as well as possibly augmented autoimmune responses against putative self-antigens such as apolipoprotein B. Further

studies are required to evaluate expansion of autoreactive T cells after ICB treatment.

STUDY LIMITATIONS. First, we cannot rule out differences in functionality between the anti-LAG3 antibody used in our study compared with human antibodies used for blocking LAG3 (eg, relatimab). Second, it is possible that the age of mice used for our studies may have affected the impact of LAG3 blockade, as expression of markers of exhaustion are known to increase in aged mice.[25] A similar limitation is that we were only able to treat mice with anti-PD-1 or anti-LAG3 antibodies for 4 weeks because these were rat antibodies, which can induce a mouse-anti-rat immunoglobulin response that would impair the blocking function. If a longer period of effective treatment were possible, it may have further aggravated plaque inflammation or plaque burden. In addition, further studies are needed to evaluate if plaque inflammation is reversed is upon cessation of ICB treatment. Third, the mechanisms underlying T cell activation and plaque accumulation after LAG3 deficiency or blockade are beyond the scope of this study. However, recent work suggests that LAG3 exerts its immunoregulatory effects by reducing T cell receptor signaling, and that T cells lacking LAG3 have a lower threshold for proliferation.[26] This is consistent with our results demonstrating increased proliferation of T cells in LAG3-deficient mice or mice given LAG3-blocking antibodies. We have also recently reported that bone marrow-derived dendritic cells lacking LAG3 showed enhanced antigen-induced $CD4^+$ T cell activation and proliferation, strongly suggesting that LAG3 in non-T cells significantly influences T cell function.[27]

CONCLUSIONS

We demonstrate that LAG3 deficiency promotes activation and recruitment of proatherogenic T cells to atherosclerotic plaques and that LAG3 and PD-1 blockade have an additive effect on T cell activation. Although atherosclerotic lesion size was not affected by either LAG3 deficiency or blockade, increased T cell activation and lesional infiltration of T cells suggests risk of plaque inflammation and destabilization. These findings encourage follow-up studies of cardiovascular events in patients treated with LAG3/PD-1-blocking antibodies.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: LAG3, a novel target for next-generation ICB, regulates T cell activation and infiltration of T cells in atherosclerotic plaques in a mouse model of atherosclerosis.

TRANSLATIONAL OUTLOOK: This study adds to the growing evidence of ICB therapies promoting plaque inflammation. Clinical studies are required to evaluate potential adverse effects of novel anti-LAG3 therapeutics on cardiovascular disease.

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KEY WORDS **atherosclerosis, cardiovascular disease, immune checkpoint blockade, inflammation, T cells**

APPENDIX **For supplemental figures, please see the online version of this paper.**