TRAM-Related TLR4 Pathway Antagonized by IRAK-M Mediates the Expression of Adhesion/Coactivating Molecules on Low-Grade Inflammatory Monocytes

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Low-grade inflammatory monocytes critically contribute to the pathogenesis of chronic inflammatory diseases such as atherosclerosis. The elevated expression of coactivating molecule CD40 as well as key adhesion molecule CD11a is a critical signature of inflammatory monocytes from both human patients with coronary artery diseases as well as in animal models of atherosclerosis. In this study, we report that subclinical superlow-dose LPS, a key risk factor for low-grade inflammation and atherosclerosis, can potently trigger the induction of CD40 and CD11a on low-grade inflammatory monocytes. Subclinical endotoxin-derived monocytes demonstrate immune-enhancing effects and suppress the generation of regulatory $CD8^+CD122^+$ T cells, which further exacerbate the inflammatory environment conducive for chronic diseases. Mechanistically, subclinical endotoxemia activates TRAM-mediated signaling processes, leading to the activation of MAPK and STAT5, which is responsible for the expression of CD40 and CD11a. We also demonstrate that TRAM-mediated monocyte polarization can be suppressed by IRAK-M. IRAK-M–deficient monocytes have increased expression of TRAM, elevated induction of CD40 and CD11a by subclinical-dose endotoxin, and are more potent in suppressing the CD8 regulatory T cells. Mice with IRAK-M deficiency generate an increased population of inflammatory monocytes and a reduced population of CD8 T regulatory cells. In contrast, mice with TRAM deficiency exhibit a significantly reduced inflammatory monocyte population and an elevated CD8 T regulatory cell population. Together, our data reveal a competing intracellular circuitry involving TRAM and IRAK-M that modulate the polarization of low-grade inflammatory monocytes with an immune-enhancing function. ***The Journal of Immunology*****, 2021, 206: 2980–2988.**

Persistent low-grade inflammation and associated chronic diseases such as atherosclerosis are leading health concerns with staggering economic tolls worldwide (1–4). Clinical and basic studies indicate that the recruitment and retention of polarized low-grade inflammatory monocytes to the vasculature followed by their interaction with adaptive immune T cells collectively contribute to the initiation and progression of atherosclerosis (5, 6). The elevated expression of costimulatory molecules such as CD40 and adhesion molecules such as CD11a on innate leukocytes is well-documented from human patients with coronary heart disease (7–9). However, molecular mechanisms responsible for the generation of low-grade inflammatory monocytes with elevated CD40 and CD11a are not well understood.

One of the key risk factors for atherosclerosis is the subclinical endotoxemia in circulation due to compromised mucosal barrier and gut leakage, a common complication in patients with low-grade inflammation, obesity, infection, and aging (10–12). Innate immune cells such as monocytes/macrophages can be potently polarized by subclinical low-dose endotoxin/LPS (SLD-LPS) into a nonresolving low-grade inflammatory state with preferential expression of inflammatory mediators, such as MCP-1, IL-12, CCR2, and CCR5 (13–16). We previously reported that polarized monocytes by subclinical-dose LPS contribute to the progression of atherosclerosis and unstable plaques with enlarged necrotic areas (13). However, the effects of subclinical low-dose LPS on the expression of CD40 and CD11a remain to be determined.

Mechanistically, LPS is recognized by the TLR-4 on monocytes and can activate either MyD88-dependent (MyD88/Mal) or MyD88-independent (TICAM-1/TRIF and TICAM-2/TRAM) pathways (17, 18). Although higher doses of LPS use MyD88 to induce robust yet transition monocyte activation, we and others recently documented that subclinical-dose LPS preferentially signals through TRAM instead of MyD88 to induce the expression of selected inflammatory mediators, such as IL-12 and CCR5 (15, 19). Consistent with our mechanistic observation, TRAM-deficient mice were shown to have reduced pathogenesis of atherosclerosis (20). The IL-1R–associated kinases (IRAKs) function further downstream of the TLR4 adaptor molecules such as MyD88/Mal, with IRAK-M serving as a potent inhibitor of the TLR4 pathway (21–23). We reported that IRAK-M–deficient monocytes have an elevated expression of MCP-1 and that IRAK-M–deficient mice develop more severe atherosclerosis (13). However, it is unclear

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Abbreviations used in this article: BMDM, bone marrow–derived monocyte; IRAK, IL-1R–associated kinase; KO, knockout; MFI, mean fluorescent intensity; NIH, National Institutes of Health; oxCAMKII, oxidized Ca^{2+}/calmodulin-dependent protein kinase II; ROS, reactive oxygen species; SLD-LPS, subclinical low-dose endotoxin/LPS; WT, wild type.

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whether IRAK-M may also modulate TRAM-mediated monocyte polarization and adhesion molecule expression.

Capitalizing on these studies, we aim to define whether subclinical low-dose LPS may potently induce the expression of costimulating molecule CD40 and adhesion molecule CD11a through TRAM or IRAK-M–mediated signaling processes in monocytes. Using primary bone marrow monocytes harvested from wild type (WT), TRAM, or IRAK-M knockout (KO) mice, we examined the expression of CD40 and CD11a expression through RT-PCR and flow cytometry analysis. We further characterized the regulation of downstream signaling molecules such as MAP kinase p38, tyrosine kinase c-Abl, and key transcription factor STAT5 involved in the expression of CD40 and CD11a (24–34). The effects of polarized monocytes by subclinical-dose LPS on the modulation of regulator CD8 T cells were examined both in vitro and in vivo.

Materials and Methods

Experimental mice and cell culture

WT C57BL/6 and IRAK-M$^{-/-}$ were purchased from The Jackson Laboratory. TRAM$^{-/-}$ mice were from Dr. Holger Eltzschig (University of Texas Houston) as we described previously (15). All experimental procedures were in compliance with guidance from the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University. Bone marrow cells were harvested as we described previously from the tibias and femurs of WT, IRAK-M$^{-/-}$, or TRAM$^{-/-}$ mice and cultured in complete RPMI 1640 (containing 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine) supplemented with 10 ng/ml M-CSF (PeproTech, Rocky Hill, NJ; no. 315-02). Cells were treated with either PBS (control) or SLD-LPS (100 pg/ml) and cultured for 5 d at 37°C in a humidified 5% CO_2 atmosphere. Culture medium was replaced with fresh media, and fresh PBS and LPS was added every 2 d, as we described previously (13, 14).

Coculture of monocytes with purified T cells

Bone marrow cells from WT, IRAK-M$^{-/-}$, or TRAM$^{-/-}$ mice were treated with PBS or SLD-LPS (100 pg/ml) and cultured for 5 d as described above. On day 5, cultured monocytes were harvested into a single cell suspension. T cells were purified from splenocytes via negative selection per the manufacturer's instructions (STEMCELL Technologies; no. 19851). A 1:1 ratio (10^6 cells/ml) of purified T cells to monocytes were cocultured for 48 h in the presence of 50 ng/ml recombinant murine IL-15 (Peprotech; no. 210-15) to preferentially maintain CD8 T cell population as described (35–38).

In vivo mouse model

ApoE$^{-/-}$ mice purchased from The Jackson Laboratory were crossed with IRAK-M$^{-/-}$ mice (provided by Dr. Rischard A. Flavell at Yale University School of Medicine) to obtain ApoE$^{-/-}$ IRAK-M$^{-/-}$ mice. The ApoE$^{-/-}$ and ApoE$^{-/-}$IRAK-M$^{-/-}$ mice (6–10-wk-old) were fed with a high-fat western diet (Harlan Teklad; 94059) for 2 mo with routine monitoring on a weekly basis. The spleen was then harvested for flow cytometry analysis.

Reagents

LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich. Primary anti-STAT5 (no. 94205S, 1:1000 in 5% BSA), anti-p38 (no. 921281, 1:1000 in 5% BSA), anti–phospho-p38 (no. 4511S, 1:500 in 5% BSA), anti–c-Abl (no. 28625, 1:500 in 5% BSA), anti–phospho-c-Abl (no. 2861S, 1:500 in 5% BSA), and anti-GAPDH (no. 2118, 1:1000) Abs were purchased from Cell Signaling Technology. Primary anti–oxidized Ca^{2+}/calmodulin-dependent protein kinase II (oxCAMKII) Ab (no. 36254, 1:1000, 5% BSA) was purchased from GeneTex. Primary anti-TRAM/TICAM2 Ab (no. AF4348, 1:200 in 5% BSA) was purchased from Novus Biologicals. Anti–β-actin (HRP) (no. 47778, 1:1000 in 5% BSA) was purchased from Santa Cruz Biotechnology. Secondary anti-rabbit IgG HRP-conjugated Ab (no. 7074, 1:2000 in 5% BSA) was purchased from Cell Signaling Technology. Primers for *musCD40, mus CD11a*, and *musBactin* were synthesized by Integrated DNA Technologies as follows: mus *CD40* forward: 5′ GCT ATG GGG CTG CTT GTT GA 3′; mus *CD40* reverse: 5′ ATG GGT GGC ATT GGG TCT TC 3′; mus *CD11a* forward: 5′ GAA GCT GAG CAG CCT TGT C 3′; mus *CD11a* reverse: 5′ CCC GTC ACT TGG ATG AGG AT 3′; mus *Bactin* forward: 5′ ACT GTC GAG TCG CGT CCA 3′; and mus *Bactin* reverse: 5′ ATC CAT GGC GAA CTG GTG G 3′.

Flow cytometry

The bone marrow cells at day 5 as well as cells obtained post-coculture as described above were harvested and blocked with Fc block (BD Biosciences; no. 553141) followed by staining with anti-CD40 (PE; BioLegend; no. 124609), anti-Ly6C (PE-Cy7; BioLegend; no. 128018), anti-CD11a (allophycocyanin; BioLegend; no. 101119), anti-CD11b (allophycocyanin-Cy7; BioLegend no. 101226), anti-CD8 (allophycocyanin-Cy7; BioLegend no. 100713), and anti-CD122 (allophycocyanin; BioLegend no. 123214) Abs. Samples were analyzed with a FACSCanto II (BD Biosciences), and data were analyzed with FlowJo (Ashland, OR).

Quantitative RT-PCR

Total RNA was isolated using TRIzol, cDNA strand was synthesized using reverse transcription kit (Applied Biosystems; no. 4368813), and real-time PCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA; no. 1725274) on CFX96 real-time PCR instrument (Bio-Rad Laboratories). Relative expression levels of different transcripts were calculated using double δ CT method and normalized to the expressions of a housekeeping gene *musBactin*. Graphs and statistical analysis were performed using GraphPad Prism v7.0.

Western blot

Western blot was performed as previously described (14). Briefly, cells were lysed in 2% SDS lysis buffer containing protease inhibitor mixture (Sigma; no. P8340), phosphatase inhibitor 2 (Sigma; no. P5726), and phosphatase inhibitor 3 (Sigma; no. P0044). Cell lysates were incubated on ice for 15 min and denatured at 95°C for 5 min. Protein concentration was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories; no. 5000112). Proteins were separated by size in 10% acrylamide, followed by transfer to PVDF membranes. Posttransfer, membranes were blocked with 5% milk for 1 h followed by probing with targeted protein Abs overnight. Finally, HRP-linked anti-rabbit IgG Ab and Advansta ECL detection kit (VWR; no. 490005-020) were used to detect the plots. ImageJ software (NIH) was used for relative protein quantification.

Statistical analysis

Graphs were made and statistical analyses were performed using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA). Student *t* test was used to determine the significance, where $p < 0.05$ was considered statistically significant.

Results

Low-grade inflammatory monocytes polarized by subclinical-dose LPS exhibit elevated levels of costimulatory molecule CD40 and adhesion molecule CD11a

Previous studies reveal that monocytes/macrophages subjected to a prolonged challenge with pathologically relevant subclinical low-dose SLD-LPS can potently express proinflammatory cytokines, chemokines, and chemokine receptors associated with chronic inflammatory diseases (11, 13, 14). In addition to cytokines and chemokines, costimulatory molecules such as CD40 and adhesion molecules such as CD11a are highly expressed in human monocytes collected from patients with coronary artery diseases (7–9). Given the significance of monocyte retention at inflamed tissues and their communication with adaptive T cells in disease pathogenesis, we, in this study, examined the expression levels of CD40 and CD11a in monocytes stimulated with SLD-LPS. Murine bone marrow–derived monocytes (BMDM) were cultured with or without 100 pg/ml LPS for 5 d to drive the polarization of low-grade inflammatory monocytes, as reflected in the expansion of Ly6C^{pos} monocyte populations (14). The expression levels of *Cd11a* and *Cd40* mRNAs were measured by real-time PCR. As shown in Fig. 1A and 1B, monocytes with prolonged LPS challenge exhibited significantly elevated levels of *Cd11a* and *Cd40* mRNAs as compared with control monocytes. We further corroborated our observation by examining the surface protein levels of CD11a and CD40 through flow cytometry analysis. As shown in Fig. 1C and 1D, prolonged SLD-LPS challenge significantly induced the population of inflammatory Ly6C^{++}CD11a^{+} as well as Ly6C^{++}CD40^{+} monocytes, respectively.

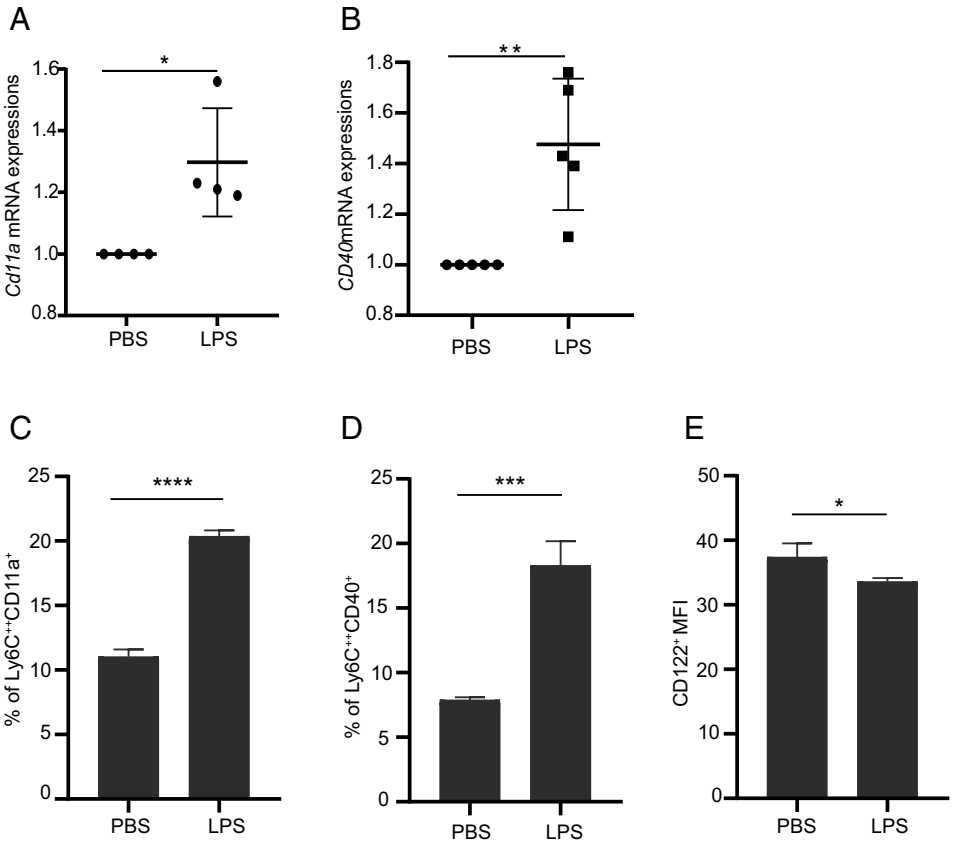

FIGURE 1. SLD-LPS induces expressions of CD40 and CD11a in BMDM. BMDMs were treated with SLD-LPS (100 pg/ml) or PBS for 5 d. Relative mRNA expressions of *Cd1a* (**A**) and *Cd40* (**B**) normalized to *Bactin* were determined by real-time RT-PCR. Frequency of $Ly6C^{++}CD11a^{+}$ (**C**) and $Ly6C^{++}CD40^{+}$ (**D**) populations was determined and quantified by flow cytometry. (**E**) Purified T cells were cocultured for 48 h with monocytes previously primed by SLD-LPS. The MFI of CD122 in $CD8^{+}$ T cells were examined and quantified by flow cytometry. Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Student *t* test.

Frequent interactions between innate monocytes and T cells occur during disease pathogenesis and contribute to either T cell activation or regulation (39, 40). With particular relevance, enhanced CD8 T cell activation and reduced $CD8^{+}CD122^{+}$ T regulatory cells are closely related to enhanced pathogenesis of atherosclerosis, both in animal models and human patients with coronary artery diseases (41–43). Although monocytes have been implicated in the modulation of $CD8^{+}CD122^{+}$ T regulatory cells, molecular mechanisms were not well understood (41, 43). Given our observation that low-grade inflammatory monocytes express elevated levels of CD11a and CD40, we then tested whether low-grade inflammatory monocytes may selectively reduce the $CD8^{+}CD122^{+}$ T regulatory cell population in vitro. Monocytes polarized with either PBS or 100 pg/ml LPS were cocultured with purified T cells in the presence of IL-15 for two additional days, which preferentially sustains CD8 T cells (35–38). As shown in Fig. 1E, CD8 T cells cocultured with LPS-polarized monocytes exhibited significantly reduced levels of CD122 as compared with ones cocultured with control monocytes.

SLD-LPS activates intracellular signaling processes involved in the expression of CD40 and CD11a

To further understand the underlying molecular mechanisms, we examined the involvement of key signaling molecules involved in the expression of CD11a and CD40 in polarized monocytes. As previous studies suggest that low-grade inflammatory monocytes accumulate reactive oxygen species (ROS) and cellular stress (11, 44), we tested the activation status of stress kinase p38 as well as oxCAMKII. oxCAMKII is a downstream molecule oxidized and activated by ROS (45, 46). Studies in other cellular systems suggest that LPS treatment leads to an increase in oxidative activation of Ca^{2+}/calmodulin-dependent protein kinase II, further enhancing inflammation signaling (45, 47). Thus, we tested the levels of activated p38 (p-p38) and oxCAMKII in monocytes polarized by SLD-LPS as compared with PBS control. We observed that SLD-LPS–polarized monocytes exhibited significantly elevated levels of p-p38 and ox-CAMKII as compared with control monocytes, as shown in Fig. 2A and 2B, respectively.

Previous studies in other cellular systems also suggest that ROS-mediated activation of c-Abl and STAT5 may be critically involved in the expression of CD40 and CD11a (25, 28, 31–34, 48). Although higher doses of LPS were shown to activate STAT5 (24–27), potentially through JAK2-independent and c-Abl–dependent pathway (28–30, 49), it is not known whether pathologically relevant SLD-LPS may similarly activate c-Abl and STAT5. We observed that SLD-LPS can potently induce the activated form of c-Abl (phospho-cAbl) as well as STAT5 in monocytes stimulated with SLD-LPS (Fig. 2C, 2D, respectively). Collectively, our data suggest that SLD-LPS may induce cellular adhesion and costimulatory molecules through activating both the p38 and STAT5 signaling networks.

IRA-M deletion preferentially expand the population of inflammatory $Ly6C^{++}$ monocytes

IRAK-M, also known as IRAK-3, is an immune suppressor of innate leukocytes (21–23). Macrophages from IRAK-M KO mice exhibit reduced tolerance and enhanced proinflammatory responses as compared with WT controls when treated with LPS (21). IRAK-M KO mice develop severe atherosclerosis with polarized monocytes expressing elevated levels of MCP-1 (13). However, it is still unclear which subpopulation of monocytes are predominantly modulated by IRAK-M. We therefore compared the activation profiles of WT and IRAK-M KO monocytes challenged with SLD-LPS. Consistent with our previous findings (13, 50), prolonged superlow-dose LPS challenge led to the expansion of both inflammatory $CD11b^{+}Ly6C^{+}$ as well as $CD11b^{+}Ly6C^{++}$ WT monocytes (Fig. 3A, 3B). Interesting, IRAK-M deletion preferentially expanded the

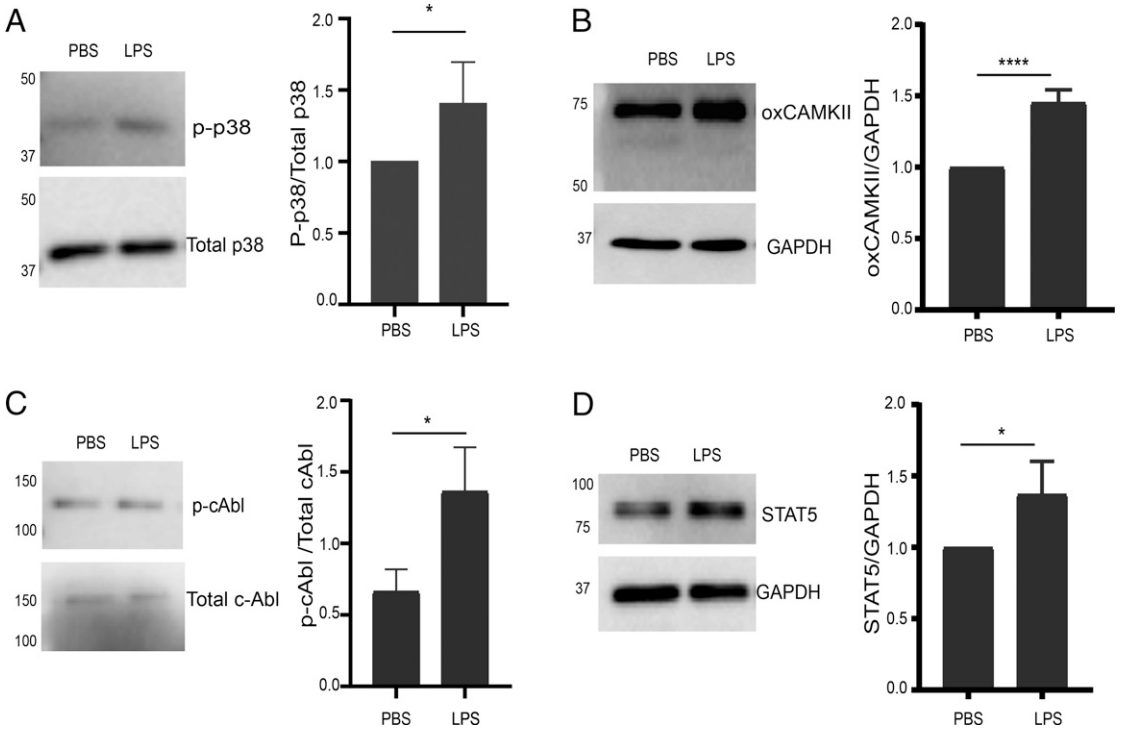

FIGURE 2. Activation of key signaling molecules in monocytes by SLD-LPS. BMDMs were treated with SLD-LPS (100 pg/ml) or PBS for 5 d. The levels of p-p38, total p38, oxCaMKII, p-cAbl, total cAbl, and STAT5 were determined by Western blot (**A**–**D**, respectively). Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). *$p < 0.05$, ****$p < 0.0001$, Student t test.

classical $CD11b^{+}Ly6C^{++}$ inflammatory monocytes as compared with WT cells treated with SLD-LPS (Fig. 3).

IRAK-M deficiency further enhances the expression of CD40 and CD11a on inflammatory monocytes

IRAK-M KO mice exposed to OVA exhibit elevated infiltration of airway inflammatory cells, higher proinflammatory cytokine levels in lung homogenates, and enhanced macrophages activation represented by elevated expression of costimulatory molecules (51, 52). Based on our findings in Fig. 3, we tested whether IRAK-M may also modulate the expression of CD11a and CD40, specifically in $Ly6C^{++}$ monocytes polarized by SLD-LPS. Indeed, as measured by flow cytometry, we observed that the population of $Ly6C^{++}$ $CD11a^{+}$ and $Ly6C^{++}CD40^{+}$ monocytes were significantly expanded in WT BMDMs cultured by SLD-LPS for 5 d. Strikingly, the population of $Ly6C^{++}CD11a^{+}$ and $Ly6C^{++}CD40^{+}$ monocytes were further expanded in IRAK-M KO BMDMs cultured with SLD-LPS as compared with WT BMDMs (Fig. 4A, 4B). Given the expansion of $Ly6C^{Pos}$ monocytes due to IRAK-M deletion, we further tested whether IRAK-M KO monocytes may further reduce the

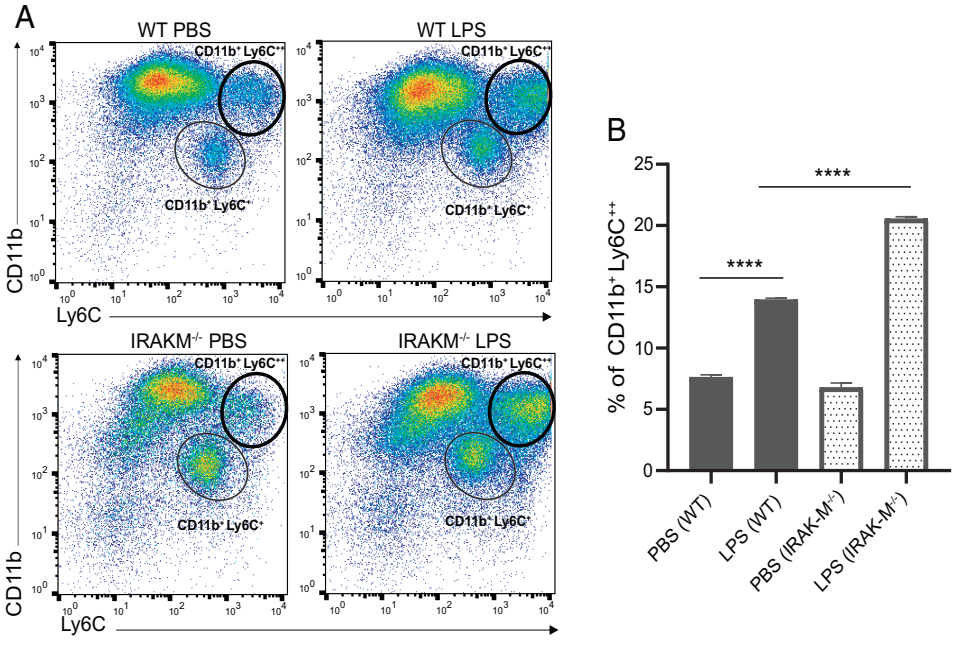

FIGURE 3. IRAK-M deletion leads to the expansion of inflammatory $CD11b^{+}Ly6C^{++}$ monocytes. WT and IRAK-$M^{-/-}$ monocytes were treated with SLD-LPS (100 pg/ml) or PBS for 5 d, and the $CD11b^{+}Ly6C^{++}$ population was determined and quantified by flow cytometry (**A** and **B**, respectively). Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). ****$p < 0.0001$, Student t test.

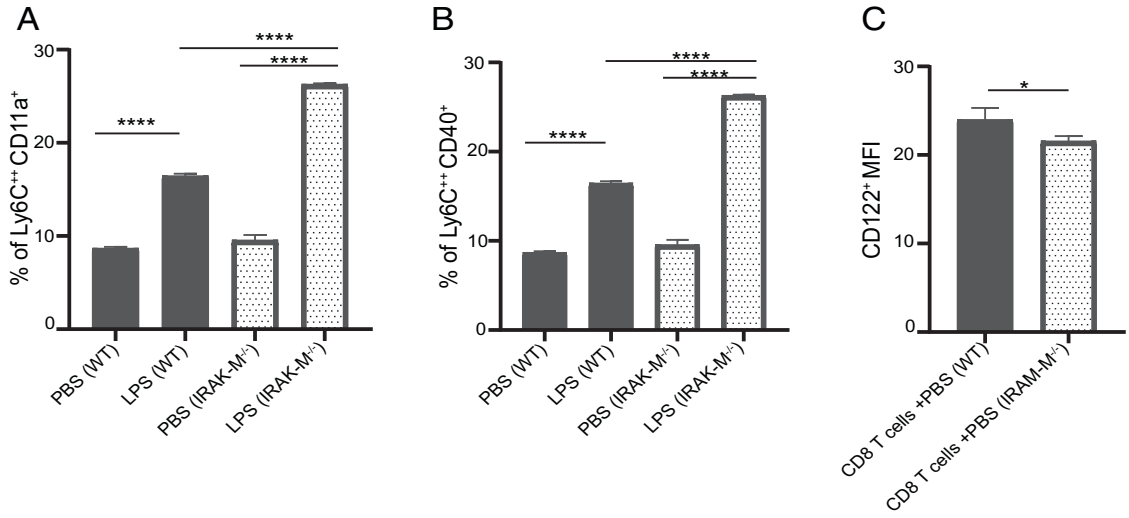

FIGURE 4. IRAK-M deficiency enhances the expression of CD40 and CD11a on inflammatory monocytes. WT and IRAK-M$^{-/-}$ BMDMs were treated with SLD-LPS (100 pg/ml) or PBS for 5 d. Frequencies of Ly6C^{++}CD11a^{+} (**A**) and Ly6C^{++}CD40^{+} monocytes (**B**) in WT and IRAK-M$^{-/-}$ BMDM were quantified via flow cytometry. (**C**) Purified T cells were cocultured for 48 h with either WT or IRAK-M$^{-/-}$ monocytes. The MFI of CD122 in CD8 T cells were examined and quantified by flow cytometry. Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). *$p < 0.05$, ****$p < 0.0001$, Student t test.

CD8^{+}CD122^{+} T regulatory cell population. Through the coculture experiment, we observed that the mean fluorescent intensities (MFI) of CD122 were significantly reduced within CD8^{+} T cells (representative of CD8 T regulatory cells) following coincubation with IRAK-M KO monocytes as compared with T cells coincubated with WT monocytes (Fig. 4C). Our data extend previous observations regarding the inhibitory role of IRAK-M during the polarization of low-grade inflammatory monocytes mediated by pathologically relevant SLD-LPS.

IRAK-M negatively modulates monocyte polarization by SLD-LPS through suppressing TLR4 adaptor molecule TRAM

Mechanistically, IRAK-M negatively regulates the MAPK and inflammatory signaling pathway following TLR4 activation (21, 53). We, in this study, confirmed the effects of IRAK-M in low-grade inflammatory monocytes polarized by SLD-LPS. We observed that IRAK-M KO monocytes challenged with SLD-LPS exhibited elevated levels of phosphorylated p38 (Fig. 5A). IRAK-M was also shown to suppress STAT5 in neutrophils (54). We found that IRAK-M KO monocytes polarized with SLD-LPS exhibited elevated levels of phospho–c-Abl as well as STAT5 as compared with their WT counterparts (Fig. 5B, 5C, respectively). Because emerging studies suggest that TRAM instead of MyD88 is critically involved in mediating the effects of SLD-LPS in monocytes (15, 50), we further tested whether IRAK-M may also modulate TRAM levels. Interestingly, we observed that TRAM protein levels were elevated in IRAK-M KO monocytes as compared with WT monocytes following the stimulation of SLD-LPS (Fig. 5D). Our data suggest a novel mechanism: IRAK-M deletion may enhance monocyte polarization by SLD-LPS through increasing TRAM-mediated intracellular signaling processes.

TRAM deficiency reduces the population of CD11b^{+}Ly6C^{++} inflammatory monocytes

With emerging evidence suggesting TRAM as a key mediator for sustaining nonresolving inflammation in monocytes, we examined the impact of TRAM deficiency on the monocyte polarization by SLD-LPS. As shown in Fig. 6, we observed that TRAM deficiency completely ablated the expansion of CD11b^{+}Ly6C^{++} population upon stimulation with SLD-LPS. At the mechanistic level, we observed that STAT5 induction by LPS was also completely ablated in TRAM KO monocytes (Fig. 6C). Our data further confirm that TRAM is critically responsible for mediating the effects of subclinical superlow-dose LPS. Collectively, our data reveal a novel toggle switch involving IRAK-M and TRAM, involved in modulating the expansion of inflammatory CD11b^{+}Ly6C^{++} monocytes.

TRAM deficiency dampens the expression of CD40 and CD11a

Because we observed that TRAM deficiency significantly reduces the population of CD11b^{+}Ly6C^{++} inflammatory monocytes, we further tested whether TRAM deficiency may also impact the levels of CD11a and CD40 post–SLD-LPS stimulation. We observed that Ly6C^{++}CD11a^{+} as well as Ly6C^{++}CD40^{+} populations were significantly reduced in TRAM KO BMDM as compared with the WT counterparts (Fig. 7A, 7B, respectively). We further tested whether monocytes with TRAM deletion may modulate CD122 levels on cocultured CD8^{+} T regulatory cells. Purified T cells were cocultured with TRAM KO monocytes as described in *Materials and Methods*. We observed that the levels of CD122 were significantly elevated in CD8 T cells cocultured with TRAM-deficient monocytes as compared with WT monocytes (Fig. 7C). Together, our data suggest that TRAM deletion could potentially dampen low-grade inflammation by reducing the expression of adhesion and coactivating molecules on monocytes while enhancing regulatory CD8 T cells.

CD40 and CD11a levels are elevated in monocytes collected from IRAK-M KO mice and reduced in monocytes from TRAM KO mice

We next examined the in vivo relevance of our in vitro observations. We used the ApoE-deficient mouse model commonly used in the atherosclerosis study. ApoE$^{-/-}$, ApoE$^{-/-}$/IRAK-M$^{-/-}$, and ApoE$^{-/-}$/TRAM$^{-/-}$ mice were fed with a high-fat diet for 2 mo as previously described to induce the pathogenesis of atherosclerosis (13, 55–57). At the end of the feeding regimen, splenic monocytes were analyzed for the expression levels of CD11a through flow cytometry. As shown in Fig. 8, we observed expansions of CD11b^{+}Ly6C^{++} monocytes as well as Ly6C^{++}CD11a^{+} monocytes from ApoE$^{-/-}$/IRAK-M$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (Fig. 8A, 8B). We further examined the levels of

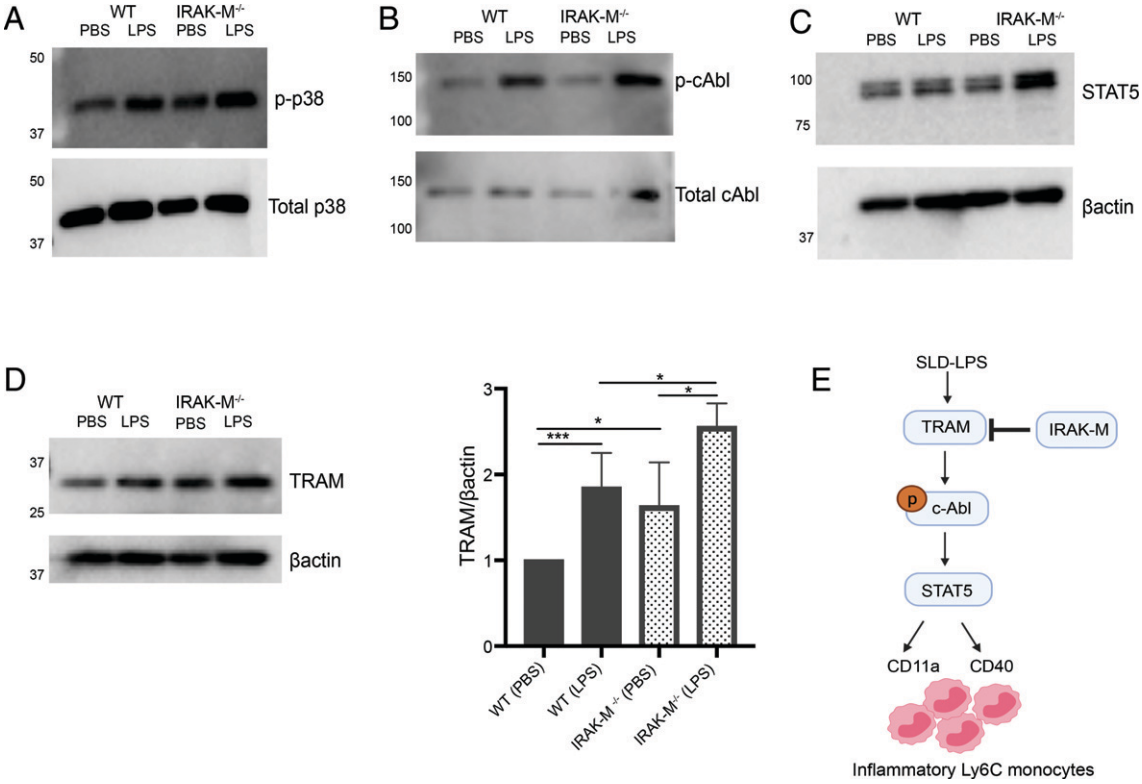

FIGURE 5. Enhanced molecular signaling and elevated TRAM levels in IRAK-M–deficient monocytes. BMDMs from WT and IRAK-M$^{-/-}$ mice were treated with SLD-LPS (100 pg/ml) or PBS for 5 d, and the levels of p-cAbl, total cAbl, STAT5, p-p38, and total p38 were determined by Western blot (**A**–**C**). Protein levels of TRAM were determined by Western blot and quantified using ImageJ (NIH) (**D**). The proposed mechanism was represented in the illustration (**E**). Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). $^{*}p < 0.05$, $^{***}p < 0.001$, Student t test.

CD8^{+}CD122^{+} T regulatory cells. As shown in Fig. 8C, the levels of CD122 within the CD8 T cell population (representative of CD8 T regulatory cells) were significantly reduced in ApoE$^{-/-}$/IRAK-M$^{-/-}$ mice as compared with ApoE$^{-/-}$ mice.

In sharp contrast, the populations of CD11b^{+}Ly6C^{++} monocytes as well as Ly6C^{++}CD11a^{+} monocytes were significantly reduced in splenocytes harvested from ApoE$^{-/-}$/TRAM$^{-/-}$ mice as compared with ApoE$^{-/-}$ mice (Fig. 8D, 8E). Correspondingly, the levels of CD122 within the CD8 T cells were significantly elevated in ApoE$^{-/-}$/TRAM$^{-/-}$ mice as compared with ApoE$^{-/-}$ mice (Fig. 8F). Together, our in vivo data suggest that IRAK-M negatively modulates TRAM-mediated monocyte polarization and communication with adaptive T cells in vivo, potentially contributing to the pathogenesis of atherosclerosis.

Discussion

This study reveals the potent induction of adhesion molecule CD11a and coactivating molecule CD40 on low-grade inflammatory monocytes by subclinical superlow-dose LPS, regulated by a competing intracellular circuitry involving TRAM and IRAK-M. IRAK-M–deficient monocytes have elevated CD11a and CD40 induction by SLD-LPS and exhibit an elevated immune-enhancing effect by suppressing CD8 T regulatory cells. Deficiency in IRAK-M, the TLR4 pathway suppressor, causes an increase in TLR4 signaling adaptor TRAM as well as downstream signaling processes, leading to the elevated expression of CD11a and CD40. In contrast, the induction of CD11a and CD40 is significantly attenuated in TRAM-deficient monocytes.

Our data better clarify the phenotypic nature of low-grade inflammatory monocytes related to the pathogenesis of chronic inflammatory disease. The generation of low-grade inflammatory monocytes under chronic inflammatory conditions is well recognized as a crucial contributor for the pathogenesis of diseases such as atherosclerosis through secreting inflammatory mediators as well as accumulating lipids and forming foam cells (13, 58). Monocytes further contribute to the pathogenesis of atherosclerosis through enhanced retention within atherosclerotic plaques and amplification of local inflammatory environment by interacting with adaptive immune cells (6, 13, 59, 60). Elevated expression of key adhesion molecule CD11a as well as coactivating molecule CD40 are critical signatures of inflammatory monocytes from both human patients with coronary artery diseases as well as in animal models of atherosclerosis (7–9). Subclinical endotoxemia is a key risk factor for low-grade inflammation and atherosclerosis (10, 12, 13, 61). Extending previous reports that demonstrated an induction of soluble inflammatory mediators in monocytes challenged with subclinical superlow-dose LPS, our current data reveal that monocytes polarized by subclinical superlow-dose LPS adopt a low-grade inflammatory state with potent immune-enhancing effects, characterized by an elevated expression of adhesion molecule CD11a as well as coactivating molecule CD40, which is capable of amplifying immune activation through suppressing the CD8 T regulatory cells both in vitro and in vivo.

Mechanistically, our data identify a competing intracellular network involving IRAK-M and TRAM that modulates the expression of CD11a/CD40 in low-grade inflammatory monocytes challenged with subclinical superlow-dose LPS. Previous studies suggest that IRAK-M is a potent suppressor of TLR4 signaling in monocytes and macrophages, primarily through suppressing MyD88-mediated assembly of IRAK1 activation complex (21). IRAK-M–deficient monocytes have elevated expression of proinflammatory cytokines

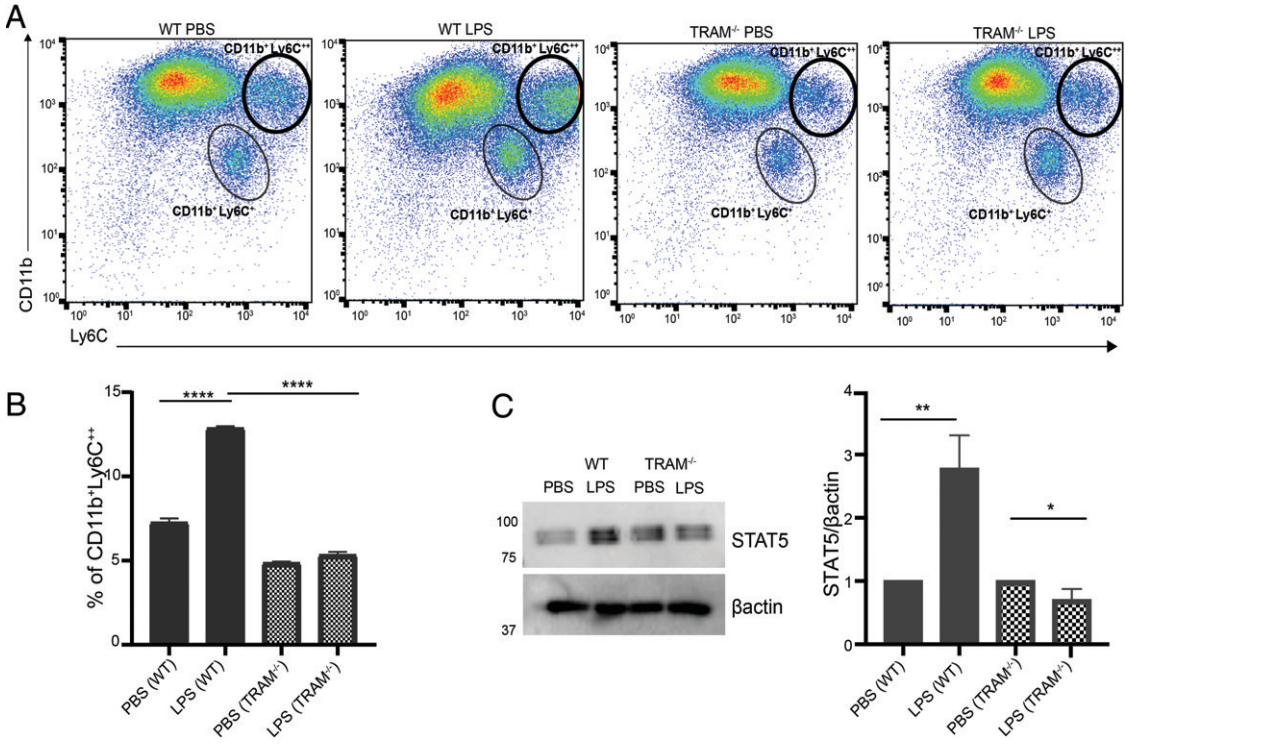

FIGURE 6. TRAM deficiency abolishes the expansion of Ly6C monocytes. WT and TRAM$^{-/-}$ BMDMs were treated with SLD-LPS (100 pg/ml) or PBS for 5 d, and the CD11b^{+}Ly6C^{++} population was determined and quantified by flow cytometry (**A** and **B**, respectively). Protein levels of STAT5 was determined by Western blot and quantified using ImageJ (NIH) (**C**). Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, Student t test.

when challenged with LPS (21, 52). Extending these observations, our current study further reveals that IRAK-M specifically suppresses the CD11b^{+}Ly6C^{++} inflammatory monocytes as well as the expression of CD40 and CD11a in low-grade inflammatory monocytes induced by SLD-LPS. Together with previous studies reporting that infiltrating monocytes within inflamed tissues were largely of Ly6C^{pos} origin (62–64), our current study helps to explain increased monocytes/macrophage contents observed in atherosclerotic plaques of experimental animals with IRAK-M deletion (13). The suppression of anti-inflammatory CD8 T regulatory cells by the expanded population of Ly6C^{++} monocytes due to IRAK-M deficiency may further exacerbate the inflammatory environment propagating the pathogenesis of atherosclerosis. Our current study provides an initial clue for intervening in the complex interaction of innate and adaptive immune cells through altering either IRAK-M or TRAM in monocytes. Based on our limited phenotypic observation, future in-depth analyses are clearly needed to better define molecular and cellular dynamics involved in the cross-talk among monocytes and adaptive immunity.

Intriguingly, we observed that IRAK-M deficiency in the low-grade inflammatory monocytes exhibits elevated expression of TRAM, a unique adaptor molecule downstream of TLR4. This is consistent with previous reports that TRAM, instead of MyD88, is the key adaptor that mediates the low-grade inflammatory responses to superlow-dose LPS (15, 50). We observed that TRAM-deficient monocytes have a significantly reduced population of CD11b^{+}

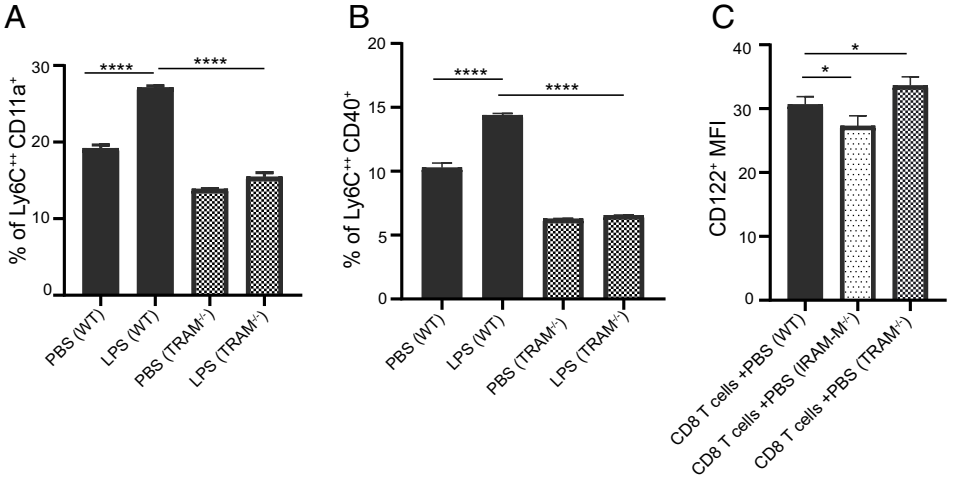

FIGURE 7. SLD-LPS fails to induce CD40 and CD11a in TRAM-deficient monocytes. Frequency of Ly6C^{++} CD11a^{+} (**A**) and Ly6C^{++}CD40^{+} (**B**) population in WT and TRAM$^{-/-}$ BMDM after 5-d SLD-LPS (100 pg/ml) treatment was analyzed and quantified using flow cytometry. Purified T cells were cocultured for 48 h with either WT or TRAM$^{-/-}$ monocytes. The MFI of CD122 in CD8 T cells were examined and quantified by flow cytometry (**C**). Data are representative of at least three independent experiments, and error bars represent means ± SEM ($n = 3$ for each group). *$p < 0.05$, ****$p < 0.0001$, Student t test.

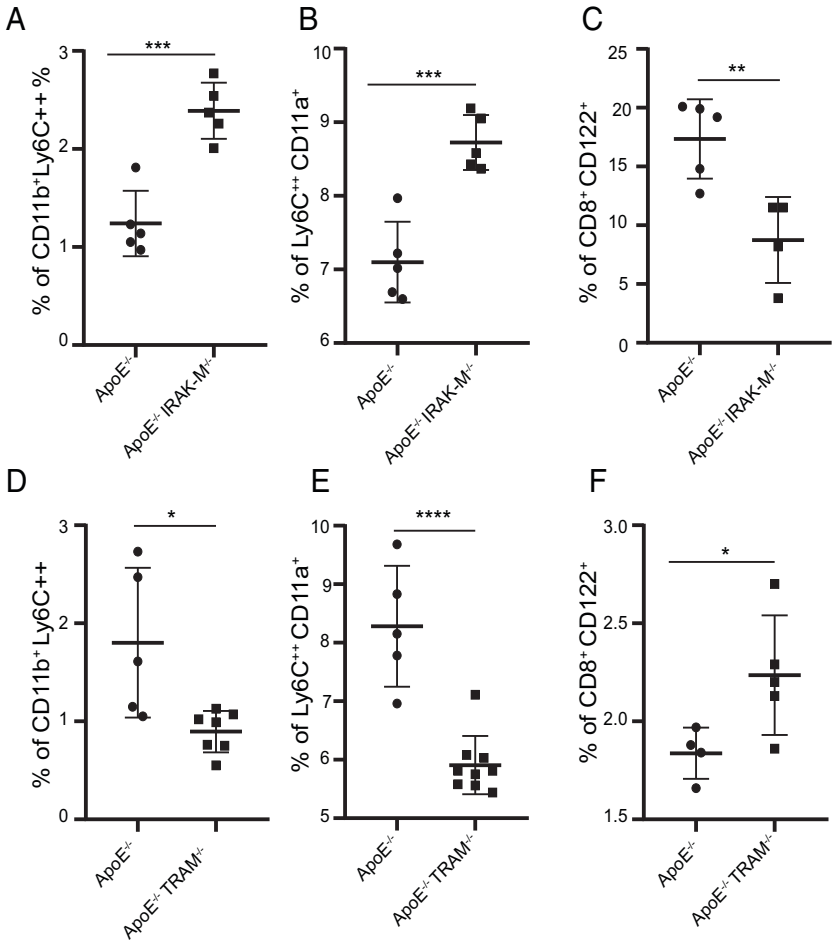

FIGURE 8. IRAK-M deficiency enhances whereas TRAM deficiency reduces inflammatory phenotypes in atherosclerotic mice. ApoE$^{-/-}$, ApoE$^{-/-}$ IRAK-M$^{-/-}$, and ApoE$^{-/-}$ TRAM$^{-/-}$ mice were fed with a high-fat diet for 2 mo. The frequencies of CD11b^{+}Ly6C^{++} cells (**A** and **D**), Ly6C^{++}CD11a^{+} cells (**B** and **E**), and CD8^{+}CD122^{+} T cells (**C** and **F**) in splenocytes were analyzed and quantified by flow cytometry. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, Student *t* test.

Ly6C^{++} inflammatory monocytes as well as a reduced expression of CD11a and CD40 following stimulation with superlow-dose LPS. Our current finding complements a previous in vivo study showing reduced atherosclerosis pathogenesis in mice with myeloid deletion of TRAM instead of MyD88 (20). Together with previous findings that subclinical-dose LPS can sustain the polarization of low-grade inflammatory monocytes through reducing the expression of IRAK-M (13), our data reveal a mutually inhibitory toggle switch composed of IRAK-M and TRAM that modulates the polarization of inflammatory monocytes. Subclinical endotoxemia may switch on the inflammatory polarization through reducing IRAK-M and enhancing TRAM.

Collectively, our study reveals an immune-enhancing phenotype of low-grade inflammatory monocytes with an elevated expression of adhesion molecular CD11a and coactivating molecule CD40. Our data also clarifies the unique role of IRAK-M in modulating TRAM-mediated polarization of low-grade inflammatory monocytes capable of interacting with an adaptive immune environment within the inflamed tissues. These findings provide important clues for future translational studies toward systems characterization of pathogenic monocytes involved in the pathogenesis of atherosclerosis.

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Disclosures

The authors have no financial conflicts of interest.

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