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Kasahara, Kazuyuki

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First Author's surname: Kasahara

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Kazuyuki Kasahara, MD; Naoto Sasaki, MD, PhD; Tomoya Yamashita MD, PhD;

Tomoyuki Kita, MD; Keiko Yodoi, MD; Yoshihiro Sasaki, MD;

Masafumi Takeda, MD, PhD; Ken-ichi Hirata, MD, PhD

Division of Cardiovascular Medicine, Department of Internal Medicine,

Kobe University Graduate School of Medicine, Kobe, Japan

Address for Correspondence:

1. Naoto Sasaki, MD, PhD. E-mail: sasakin@med.kobe-u.ac.jp

2. Tomoya Yamashita, MD, PhD. E-mail: tomoya@med.kobe-u.ac.jp

Division of Cardiovascular Medicine, Department of Internal Medicine,

Kobe University Graduate School of Medicine

7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.

TEL: +81-78-382-5846, FAX: +81-78-382-5859

Drs. Sasaki and Yamashita contributed equally to this work.

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Abstract

Background—Accumulating evidence suggests that the balance between pathogenic effector T cells (Teffs) and regulatory T cells (Tregs) may be important for controlling atherosclerotic disease. We hypothesized that a combination therapy with anti-CD3 antibody (CD3-Ab) and IL-2/anti-IL-2 monoclonal antibody complex (IL-2 complex) aimed at increasing the ratio of Tregs to Teffs would effectively inhibit atherosclerosis in mice.

Methods and Results—We treated apolipoprotein E-deficient mice fed a high-cholesterol diet with vehicle, CD3-Ab, IL-2 complex or their combination. Mice receiving the combination therapy had markedly reduced atherosclerotic lesions than mice treated with CD3-Ab or IL-2 complex alone. In addition, a striking increase in the Treg/Teff ratio of lymphoid organs and atherosclerotic lesions, along with plaque stabilization characterized by decreased macrophage content and increased collagen content was observed. The combination treatment also markedly reduced splenic $Ly6C^{high}$ inflammatory monocytes and might induce a favorable macrophage phenotype change in atherosclerotic lesions.

Conclusions—Our results indicate that in addition to suppressing Teff responses, enhancing Treg-mediated immune responses is more efficacious in preventing atherosclerosis, suggesting a novel therapeutic approach for atherosclerosis.

Keywords: atherosclerosis, inflammation, T cell, immune system

It is now widely accepted that inflammatory condition within the vessel wall is one of

the most important factors for atherosclerosis development,[1,2] and contributes toward plaque

instability, thrombotic arterial occlusion, and severe clinical events including acute coronary

syndrome and stroke. Following accumulation into the subendothelial space or intima

through the activated endothelium and differentiation into macrophages, monocytes take up

modified low-density lipoprotein (LDL) particles, and differentiate into foam cells, which

secrete pro-inflammatory cytokines causing activation of immune cells such as T cells.

After antigen presentation or activation by macrophages or dendritic cells (DCs), naïve CD4^{+}

T cells differentiate into effector T cells (Teffs) such as T helper type 1 (Th1), T helper type 2

(Th2), and T helper type 17 (Th17) lineages, which all play an important role in atherogenesis

in both humans and mice.[2] Th1 cells are known to promote atherosclerotic disease by

producing inflammatory cytokines such as interferon-γ.[3] However, the roles of Th2 or

Th17-mediated immune responses in atherosclerosis remain controversial. Recently,

immunoregulatory CD4^{+} T-cell subsets, namely regulatory T cells (Tregs) expressing CD25

(IL-2 receptor α-chain) molecule, have been shown to play a protective role in atherogenesis

by dampening Teff responses.[4-8] The transcription factor Foxp3 (forkhead box P3) is a

master regulator and the most reliable molecular marker for natural Tregs.[9] Recent study

demonstrated that genetic depletion of Foxp3^{+} Tregs increased atherosclerotic lesions in

atherosclerosis-prone mice by aggravating hypercholesterolemia.[10]   In consideration of these

previous studies, we believe that increasing the Treg/Teff ratio, by suppressing Teff responses

and promoting Treg responses, could be a promising therapeutic approach for atherosclerotic

disease.

Intravenous administration of anti-CD3-specific antibody (CD3-Ab) was shown to

suppress Teff immune responses and to be effective for suppressing atherosclerotic process in

mice,[11] autoimmune diabetes in mice and humans, and acute transplant rejection in humans.[12,]

[13]   Previous studies demonstrated induction of $CD4^+CD25^+$ Tregs, along with reduced

number of Teffs, following CD3-Ab treatment, explaining the long-term protective effects

observed in mouse models of autoimmune diseases,[12] although an increase in $CD4^+CD25^+$

Treg number was absent in atherosclerosis-prone mice.[11]   Despite these beneficial effects,

high doses of CD3-Ab cannot be used because of severe side effects such as low levels of

cytokine release from activated T-cells.[13]   Thus, in addition to this antibody treatment, other

therapeutic strategies to attain long-term therapeutic efficacy are needed.

Recent studies have demonstrated that injection of a recombinant mouse IL-2/anti-IL-2

monoclonal antibody complex (IL-2 complex) could be one promising avenue for the

expansion of $CD4^+CD25^+Foxp3^+$ Tregs[14] and studies have shown that this IL-2 complex

therapy suppressed the development and progression of atherosclerosis[15, 16] and experimental

autoimmune encephalitis[14] in mice without adverse effects.   However, it is reported that

Tregs cannot effectively suppress autoimmune reactions in mouse autoimmune disease model,

if inflammation in the target organ is not regulated,[17] suggesting that under inflammatory

conditions such as hypercholesterolemia, suppression of Teff immune responses before

expanding Tregs may result in an efficient reduction in atherosclerosis development via

augmenting regulatory immune responses.

In the present study, we determined if the combination therapy of CD3-Ab and IL-2

complex would effectively inhibit atherosclerosis in *ApoE*^{-/-} mice by enhancing regulatory

immune responses.   We propose the novel concept involving modulation of both effector

and regulatory arms of T-cell immune responses could be an attractive therapeutic approach

against atherosclerosis.

Methods

Animals and Experimental Design

Six-week-old *ApoE*^{-/-} mice were fed a high-cholesterol diet containing 0.2% cholesterol

and 21% fat (CLEA, Tokyo, Japan) and water ad libitum.   For blockade of CD3, 50 μg of

anti-CD3 antibody F(ab')2 (145-2C11; Bio X cell, West Lebanon, NH) or 50 μg of

isotype-matched hamster immunoglobulin G F(ab')2 (control IgG) (Bio X cell) was

intravenously injected into the mice for 5 consecutive days at 8 weeks of age. For IL-2

complex therapy, a recombinant mouse IL-2/anti-IL-2 mAb (JES6-1) complex (1μg IL-2 plus

5μg anti-IL-2 mAb) was given i.p. to the 9-week-old mice for 3 consecutive days, after which

they received once weekly from 10 to 16 weeks of age. Mice were anaesthetized with an

isoflurane and an intraperitoneal injection of pentobarbital (30 mg/kg body weight). Mice

were housed in specific pathogen-free animal facility at Kobe University, and all animal

experiments were conducted in accordance to the Guidelines for Animal Experiments at Kobe

University School of Medicine.

Atherosclerotic Lesion Assessments

Mice were anesthetized and the aorta was perfused with saline. The samples were cut

in the ascending aorta, and the proximal samples containing the aortic sinus were embedded

in OCT compounds (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Five consecutive sections

(10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and

stained with Oil Red O (Wako Pure Chemical Industries). Total plaque area and Oil Red O

stained areas were measured using Image J (National Institutes of Health, Bethesda, MD).

The volume of atherosclerosis and lipid accumulation in the aortic sinus was expressed as

mean size of the 5 sections for each mouse. Immunohistochemistry was performed on

acetone-fixed or formalin-fixed cryosections (10 μm) of aortic roots using antibodies to identify macrophages (MOMA-2, 1:400; BMA Biomedicals, Augst, Switzerland), CD4$^+$ T cells (CD4, clone H129.19, 1:100; BD Biosciences, San Jose, CA) and Foxp3$^+$ cells (Foxp3, clone FJK-16s, 1:100; eBioscience, San Diego, CA), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Staining with Masson's trichrome was used to delineate the fibrous area. Stained sections were observed under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence, Osaka, Japan) using the BZ Analyzer Software (Keyence). Stained sections were digitally captured, and the percentage of staining (the stained area per total atherosclerotic lesion area) was calculated. Quantitative analyses of CD4$^+$ T cells and Foxp3$^+$ cells in the atherosclerotic lesion were performed by counting the positive-stained cells, which was divided by total plaque area.

Flow Cytometric Analysis

Flow cytometry analysis was performed by Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA) using FlowJo software (Tree Star). For Intracellular cytokine staining, cells were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate (Sigma) and 1 mmol/L ionomycin (Sigma) for 5 hr in the presence of a GolgiStop (BD Bioscience). The antibodies used were as follows; anti-CD16/CD32 (clone 2.4G2; BD Bioscience), anti-CD4 (clone H129.19; BD Bioscience), anti-CD25 (clone PC61; BD Bioscience), anti-CD103

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(clone M290; BD Bioscience), anti-GITR (clone DTA1; BD Bioscience), anti-CTLA-4 (clone

UC10; BD Bioscience), anti-Foxp3 (clone FJK-16s; eBioscience), anti-CD11c (clone HL3;

BD Bioscience), anti-CD80 (clone 16-10A1; BD Bioscience), anti-CD86 (clone GL1; BD

Bioscience), anti-CD49b (clone HMa2; BD Bioscience), anti-LAG3 (clone C9B7W; BD

Bioscience), anti-CD11b (clone M1/70; BD Bioscience), anti-Ly6C (clone AL-21; BD

Bioscience), anti-CD115 (clone AFS98; eBioscience), anti-F4/80 (clone BM8; eBioscience),

anti-CD206 (clone C068C2; BioLegend, San Diego, CA), anti-IFNγ (clone XMG1.2;

eBioscience), anti-IL-4 (clone BVD4-1D11; eBioscience), anti-IL-10 (clone JES5-16E3;

eBioscience) and isotype-matched control antibodies.

Preparation of peritoneal macrophages

10-week-old *ApoE*^{-/-} mice of each group were treated with 3% thiogycollate broth i.p.

injection and sacrificed with isoflurane for peritoneal macrophage isolation after a 3-day

treatment as described previously.[18] Cells were plated onto culture dishes with RPMI

medium containing 10% FBS and incubated for 3-4h at 37°C and 5% CO^2. The adhesive

cells were used as macrophages for FACS analysis and RT-PCR analysis.

Real-Time RT-PCR Analysis

Total RNA was extracted from aortas or peritoneal macrophages after perfusion with

RNA later (Ambion, Austin, TX) using the TRIzol reagent (Invitrogen, Carlsbad, CA). For

RT, a PrimeScript RT reagent Kit (Takara, Shiga, Japan) was used. Quantitative PCR was performed using a SYBER Premix Ex Taq (Takara) and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) according to comparative threshold cycle method following manufacturer's protocol. The following primers were used to amplify CD4, CD25, Foxp3, CTLA-4, iNOS, MCP-1, CXCL10, Arg I, Fizz1, Ym1 and GAPDH: **CD4,** 5'-TCA CAC ATG AAG CAT GTC AGG-3' and 5'-GCA CTG GTT AGA ATG TGA GTC TGG-3'; **CD25,** 5'-CTG ATC CCA TGT GCC AGG AA-3' and 5'-AGG GCT TTG AAT GTG GCA TTG -3'; **Foxp3**, 5'-CTC ATG ATA GTG CCT GTG TCC TCA A-3' and 5'-AGG GCC AGC ATA GGT GCA AG-3'; **CTLA-4**, 5'-CCT CTG CAA GGT GGA ACT CAT GTA -3' and 5'-AGC TAA CTG CGA CAA GGA TCC AA-3'; **iNOS**, 5'-GCA GAG ATT GGA GGC CTT GTG -3' and 5'-GGG TTG TTG CTG AAC TTC AG TC-3'; **MCP-1**, 5'-GCA TCC ACG TGT TGG CTC A-3' and 5'-CTC CAG CCT ACT CAT TGG GAT CA-3'; **CXCL10**, 5'-TGA ATC CGG AAT CTA AGA CCA TCA A-3' and 5'-AGG ACT AGC CAT CCA CTG GGT AAA G-3'; **Arg I**, 5'-GGG AAT CTG CAT GGG CAA C-3' and 5'-GCA AGC CAA TGT ACA CGA TGT C-3'; **Fizz1,** 5'-CAG CTG ATG GTC CCA GTG AA-3' and 5'-CAA GCA CAC CCA GTA GCA CTC-3'; **Ym1,** 5'-GTA GGC CTC AAC CTG GAC TG-3' and 5'-CGT CAA TGA TTC CTG CTC CTG-3'; **GAPDH,** 5'-TGT GTC CGT CGT GGA TCT GA-3' and 5'-TTG CTG TTG AAG TCG

CAG GAG-3'. The amplification reactions were performed in duplicate, and the fluorescence curves were analyzed with the software included with the ABI PRISM 7500 system. GAPDH was used as an endogenous control reference.

Statistical Analysis

Data were expressed as the mean ± SEM. The Mann-Whitney U test was used to detect significant differences between 2 groups. The Kruskal-Wallis test was used to detect significant differences when comparing more than 3 groups, followed by post hoc Dunn's multi-comparison test. A value of $p<0.05$ was considered statistically significant. For statistical analysis, GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) was used.

Results

Effects of CD3 Ab, IL-2 Complex, or Combination Therapy on CD4^{+} T Cell Immune Responses

To compare the efficacy of CD3-Ab, IL-2 complex, and the combination therapy with CD3-Ab and IL-2 complex, 4 experimental groups were made as follows: IgG plus PBS-treated (control-treated), CD3-Ab plus PBS-treated (CD3-Ab-treated), IgG plus IL-2

complex-treated (IL-2 complex-treated), CD3-Ab plus IL-2 complex-treated (combination-treated) groups. As described in Figure 1A, *ApoE*^{-/-} mice were intravenously treated with CD3-Ab or control IgG daily for 5 consecutive days at 8 weeks of age, and then were intraperitoneally treated with IL-2 complex or control PBS for 3 consecutive days at 9 weeks of age. At 10 weeks of age, lymphoid cells from spleen and LNs were analyzed by flow cytometry. We first investigated the efficacy of CD3-Ab treatment on CD4+ T cells, and observed a marked reduction in CD4+ T cells in the spleen and LNs of CD3-Ab-treated mice, whereas the percentage of Foxp3+ Tregs and CD25+Foxp3+ Tregs within the CD4+ T cell population was not different between control-treated and CD3-Ab-treated mice (Figure 1B, 1C, 1D, and 1E). These results indicate that the atheroprotective effect of CD3-Ab treatment could be mainly due to the depletion of Teffs in our experiments, although short-term treatment with CD3-Ab has been shown to increase the proportion of Foxp3+ Tregs at later time points.[13] As treatment with IL-2 complex has been reported to systemically increase Tregs but not Teffs,[14, 15] we next investigated the impact of IL-2 complex on immune responses including Tregs and Teffs according to the protocol described in Figure 1A. The percentages of CD4+ T cells in the spleen and LNs were not different between control-treated and IL-2 complex -treated mice (Figure 1B and 1C). We found a trend towards increase in CD4+Foxp3+ Tregs and CD4+CD25+Foxp3+ Tregs in the spleen and LNs of IL-2

complex-treated mice compared to control-treated mice (Figure 1B, 1D and 1E). To examine the combined effects of Teff elimination and Treg induction on immune responses, we treated *ApoE*^{-/-} mice fed a high cholesterol diet with both CD3-Ab and IL-2 complex. We found a dramatic increase in CD4^{+}Foxp3^{+} Tregs and CD4^{+}CD25^{+}Foxp3^{+} Tregs in the spleen and LNs of CD3-Ab/IL-2 complex-treated mice, along with a trend towards decrease in CD4^{+} T cells in the spleen and LNs (Figure 1B, 1C, 1D, and 1E). Notably, the Treg/Teff ratio was much higher in the spleen and LNs of the mice with the combination therapy compared to control-treated or CD3-Ab-treated mice (Figure 1F). T regulatory type 1 (Tr1) cell is another Treg subset which does not express Foxp3 and can be clearly detected by surface markers CD49b and lymphocyte activation gene 3 (LAG-3).[19] We observed that the combination therapy markedly increased the number of splenic Tr1 cells (Figure 1G). Accordingly, these results indicate that the combination therapy can efficiently shift the Treg/Teff balance to Tregs.

Effects of CD3 Ab, IL-2 Complex, or Combination Therapy on Treg-associated Molecules, DC maturation and Th1/Th2 Balance

Next, the effects of each therapy on the expression of Treg-associated molecules in Foxp3^{+} Tregs were determined by flow cytometry. Notably, Foxp3^{+} Tregs from CD3-Ab/IL-2 complex-treated mice expressed higher levels of CD25, CD103,

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glucocorticoid-induced TNF receptor family-related gene/protein (GITR), cytotoxic T lymphocyte-associated protein 4 (CTLA-4) compared to those from control-treated or CD3-Ab-treated mice (Figure 2A), implying an activated phenotype of Tregs after the combination therapy. In addition, a trend towards increased expression of Treg-associated molecules was seen in mice with IL-2 complex alone. We also investigated the effect of each therapy on surface maturation markers CD80 and CD86 in splenic CD11c$^+$ DCs. Neither CD3-Ab nor IL-2 complex mono-therapy altered the expression of these maturation markers in splenic DCs, whereas we observed a modest but significant decrease in the expression of CD80, but not CD86, in splenic DCs of CD3-Ab/IL-2 complex-treated mice (Figure 2B). To determine whether the combination treatment changed T-cell responses and polarization, we examined cytokine secretion from CD4$^+$ T cells by intracellular cytokine staining. We found that IL-2 complex mono-therapy dramatically increased the percentage of splenic IFN-γ producing-Th1 cells, IL-4-producing Th2 cells, and IL-10-producing CD4$^+$ T cells compared to control-treated mice (Figure 2C). Notably, co-treatment with CD3-Ab canceled the increase in IFN-γ producing-CD4$^+$ T cells by IL-2 complex treatment, while the increase in IL-4 or IL-10-producing-CD4$^+$ T cells was still observed (Figure 2C). The Th1/Th2 ratio was much lower in the spleen of the mice with the combination therapy compared to mice in other groups (Figure 2D). Taken together, these results indicate that

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the combination therapy can induce activated phenotype of Tregs and the Th1/Th2 balance to Th2, which may contribute to a reduction in atherosclerosis development via suppressing inflammatory responses.

The Combination Therapy with CD3 Ab and IL-2 Complex Inhibits Atherosclerotic Lesion Formation and Induces Stable plaque Phenotype

To determine the effects of CD3-Ab, IL-2 complex, or combination therapy on atherosclerosis development, we assessed the atherosclerotic lesion formation of the 4 experimental groups. No adverse effects were observed in all groups throughout the experiments. The mice receiving combined therapy showed a decrease in plasma total cholesterol and LDL cholesterol levels and an increase in triglyceride levels compared to control-treated mice, whereas no statistical differences in plasma lipid profiles were detected between the other 3 groups (Table 1). CD3-Ab or IL-2 complex mono-therapy caused a modest but significant reduction in atherosclerotic lesion formation in the aortic root compared to control-treated mice ($61.6\pm2.8\times10^4$ μm^2 in control-treated mice, $50.6\pm1.7\times10^4$ μm^2 in CD3-Ab-treated mice, $50.7\pm1.9\times10^4$ μm^2 in IL-2 complex-treated mice; Figure 3A and 3B). Notably, CD3-Ab/IL-2 complex-treated mice showed a further reduction in atherosclerotic lesion formation ($37.2\pm1.9\times10^4$ μm^2, Figure 3A and 3B) compared to control-treated and each mono-therapy mice. Consistent with this, the lipid content of the

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plaques in the aortic sinus was also significantly decreased in the CD3-Ab/IL-2 complex-treated mice compared to control-treated and each mono-therapy mice (Figure 3C). In parallel with the cross-sectional studies, we performed en face analysis of thoracic aortas, revealing a significant reduction in aortic plaque burden in CD3-Ab/IL-2 complex-treated mice (3.74±0.12%) compared to control mice (5.53±0.23%) (Figure 3D).

To determine the effects of each therapy on plaque composition, immunohistochemical studies of atherosclerotic lesions in the aortic sinus were performed. Collagen contents in atherosclerotic lesions were significantly increased in CD3-Ab-treated and CD3-Ab/IL-2 complex-treated mice compared to control-treated mice (Figure 3A and 3E). Although CD3-Ab or IL-2 complex mono-therapy did not affect the recruitment of macrophages in atherosclerotic plaques, the lesions of CD3-Ab/IL-2 complex-treated mice showed a significant reduction in the macrophage accumulation compared to control mice (Figure 3A and 3F).

The Combination Therapy with CD3 Ab and IL-2 Complex Dramatically Enhances Regulatory Immune Responses in the Lesions

It has been reported that Tregs and Teffs migrate into atherosclerotic plaques and the lesional Treg/Teff balance may be important in controlling atherosclerotic process via diminishing inflammation within atherosclerotic plaques.[20] Immunohistochemical analysis

revealed that IL-2 complex mono-therapy did not affect the number of CD4$^+$ T cells in atherosclerotic plaques, whereas CD3-Ab mono-therapy or combination therapy showed a trend toward reduction in CD4$^+$ T cell infiltration compared to control mice (Figure 4A and 4B). Immunohistochemical studies of atherosclerotic lesions using anti-Foxp3 antibody demonstrated a marked increase in the number of Foxp3$^+$ Tregs within the plaque of IL-2 complex-treated or CD3-Ab/IL-2 complex-treated mice (Figure 4A and 4C). In addition, expression analyses of T cell and Treg-associated markers in the lesions by quantitative RT-PCR revealed that the combination therapy significantly decreased the relative mRNA expression of CD4 and markedly increased the expressions of Treg-associated markers such Foxp3, CD25, and CTLA-4 (Figure 4D), which is consistent with the results of immunohistochemical analyses. Collectively, these results indicate that this novel combination therapy with CD3-Ab and IL-2 complex inhibits the infiltration of effector CD4$^+$ T cells and promotes the migration of systemically expanded Tregs into the plaques in *ApoE$^{-/-}$* mice, and a subsequent increase in the Treg/Teff ratio in the atherosclerotic lesions, as well as in the lymphoid organs, may contribute to the reduction of macrophage accumulation, the increment of collagen content and the substantial inhibition of the atherosclerotic lesion formation by regulating systemic and local inflammatory responses.

The Combination Therapy Induces Macrophage Polarization toward a Less Inflammatory M2 Phenotype

To further reveal the mechanisms for reduced macrophage accumulation in the plaques, we examined the effects of the combination therapy on the monocyte number or macrophage phenotype change. We found that the number of $CD11b^+Ly6C^{high}$ monocytes were significantly decreased in the spleen of CD3-Ab/IL-2 complex-treated mice compared to control-treated mice (Figure 5A) and those monocytes expressed lower levels of CD115 (CSF-1 receptor), a growth factor receptor for CSF-1 involved in promoting atherosclerosis.[21] To examine whether the combination therapy affected the phenotype of macrophages *in vivo*, we induced inflammatory macrophage infiltration in the peritoneal cavity by thioglycollate injection as described previously.[18] We collected thioglycollate-induced peritoneal macrophages and performed flow cytometry and quantitative RT-PCR analysis. Flow cytometry analysis of peritoneal macrophages revealed that the combination therapy significantly reduced $CD11c^+$ M1 macrophages and increased $CD206^+$ M2 macrophages compared to control mice (Figure 5B). In contrast, there were no significant differences in the percentage of $CD11c^+$ M1 and $CD206^+$ M2 macrophages among the other 3 groups. Consistent with these results, quantitative RT-PCR analyses of peritoneal macrophages revealed that the combination therapy significantly decreased the expressions of M1

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macrophage markers including iNOS, MCP-1, and CXCL-10, and increased the expressions of M2 macrophage markers including Arg I, Fizz1, and Ym-1 (Figure 5C). Furthermore, we examined the mRNA expressions of iNOS and Arg I in atherosclerotic aortas by quantitative RT-PCR and found decreased expression of iNOS and increased expression of Arg I in CD3-Ab/IL-2 complex-treated mice compared to control-treated mice (Figure 5D). These results suggest that athero-protective effects of the combination therapy may be partly due to the reduced number of inflammatory monocytes or polarization of macrophages toward a less inflammatory phenotype, as a result of dramatic up-regulation of regulatory immune responses.

Discussion

In the present study, we examined the impact of Teff depletion, Treg expansion, and both intervention on the Teff and Treg immune responses in both lymphoid organs and atherosclerotic plaque and on the development of atherosclerosis, using CD3-Ab, IL-2 complex, or the combination of CD3-Ab and IL-2 complex, respectively. Monotherapy with CD3-Ab or IL-2 complex alone induced a modest but significant reduction in atherosclerotic plaque formation compared to untreated mice, whereas therapeutic intervention of both Teffs and Tregs using CD3-Ab/IL-2 complex combination therapy further prevented atherosclerosis

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development compared to each monotherapy group. The beneficial effects of the combination therapy were associated with a dramatic increase in the Treg/Teff ratio, up-regulation of Treg activation markers in lymphoid organs and atherosclerotic plaques, the shift of Th1/Th2 balance to Th2 in lymphoid organs, a reduced number of proinflammatory Ly6C^{high} monocytes in the spleen, and a conversion of the M1 to M2 macrophage state. This is the first report demonstrating that efficacy of IL-2 complex therapy in prevention of atherosclerosis can be greatly enhanced when combined with CD3-Ab. We believe that our findings are highly relevant for shaping future clinical strategies for preventing atherosclerotic diseases.

It is now clear that Teff immune responses are involved in accelerating atherosclerosis development and progression.[2] Furthermore, based on previous reports and our findings, Foxp3^{+} Tregs play a critical role in suppressing atherogenesis via attenuating inflammatory processes, suggesting that modulation of Treg-mediated immune responses represent a promising therapeutic approach for atherosclerosis. It has been demonstrated that local inflammatory responses in the target tissue should to be controlled to preserve the suppressive function of Tregs.[17] Because there may be many inflammatory cells including Teffs infiltrated in advanced atherosclerotic plaques, causing severe tissue inflammation, we should first eliminate such accumulated Teffs and down-regulate the Teff immune responses to

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effectively inhibit inflammatory responses before Treg therapy. To address this issue, we examined the impact of Teff depletion, Treg expansion, and both interventions on the Teff and Treg immune responses in both lymphoid organs and atherosclerotic plaque and on the development of atherosclerosis, using CD3-Ab, IL-2 complex, or the combination of CD3-Ab and IL-2 complex, respectively. The finding that the combination therapy led to a significant reduction in atherosclerosis compared to CD3-Ab or IL-2 complex monotherapy was very striking. The beneficial effects of the combination therapy included a dramatic change in the Treg/Teff ratio and up-regulation of Treg activation markers in lymphoid organs and atherosclerotic plaques. We found that the efficacy of IL-2 complex therapy to induce Tregs can be greatly enhanced when combined with CD3-Ab in $ApoE^{-/-}$ mice. Based on the above findings and previous data, we suppose that suppression of $CD4^+$ T cell-mediated immune responses before expanding Tregs may be essential for the induction of Tregs in plaques, maintenance of their anti-inflammatory effects, and subsequent reduction of atherosclerosis. Our observations suggest that therapeutic intervention aimed at enhancing Treg-mediated immune responses and limiting Teff-mediated immune responses might represent a novel therapeutic approach for atherosclerotic diseases.

Recent studies have demonstrated that treatment with IL-2 complex selectively increases $CD4^+CD25^+Foxp3^+$ Tregs without affecting other immune cells including $CD4^+$ T

cells, CD8^{+} T cells, or natural killer cells, although the immune response and activation status

of helper CD4^{+} T cells were not examined after treatment.[14, 15]   However, as shown in Figure

2A and 2B, we unexpectedly observed that IL-2 complex significantly induced the activation

of both Teffs and Tregs.   CD25 molecule is a component of the high-affinity IL-2 receptor

(IL-2R) and is functionally essential for Treg development by binding IL-2.[9]   However, this

molecule is expressed not only on Tregs but also on activated immune cells such as Teffs, for

proliferation through IL-2R signaling.[22]   Taken this into account, it is possible that IL-2

complex affects the activation and proliferation of both Teffs and Tregs.   Importantly, we

showed that the additional treatment with CD3-Ab in IL-2 complex-treated mice abolished

the up-regulation of atherogenic Th1 immune responses, while the up-regulation of the

immune responses such as IL-4 or IL-10 production from CD4^{+} T cells remained unchanged,

suggesting that addition of CD3-Ab may enhance the efficacy and reduce the risk of side

effects associated with IL-2 complex treatment.   Recent evidence suggests that several

subsets of Tregs differentiate from naïve T cells in the periphery under certain conditions and

have similar immunological properties with thymus-derived Foxp3^{+} Tregs.[9]   Previous studies

showed that peripherally generated Tregs such as Tr1 cells or CD4^{+}LAP^{+} (latency-associated

peptide) Tregs inhibit atherosclerosis in atherosclerosis-prone mice by producing IL-10 or

TGF-β, respectively.[7, 23] Our data indicate that induction of Tr1 cells may also contribute to the reduction of atherosclerosis following CD3-Ab/IL-2 complex combination therapy.

In the present study, we found that combination therapy dramatically increased not only the number of Foxp3^{+} Tregs but also the expressions of Treg activation markers in both lymphoid organs and atherosclerotic plaques. Our finding of marked up-regulation of CTLA-4, one of Treg activation markers, in Foxp3^{+} Tregs in CD3-Ab/IL-2 complex-treated mice is interesting, because CTLA-4-dependent suppression of antigen-presenting cell (APC) function including DCs by CD80 or CD86 down-regulation is supposed to be a key mechanism for Treg-mediated suppression.[24] Such DCs with low expression levels of CD80/CD86 are called 'tolerogenic DCs' and have been shown to contribute to the inhibition of atherosclerosis development and the regression of established plaques by inducing Tregs and inhibiting Teffs.[25, 26] Although we observed a modest decrease in the CD80 expression in splenic DCs of CD3-Ab/IL-2 complex-treated mice, whether the lesional suppression mechanisms through this pathway is involved in the reduction of atherosclerosis remains unclear and further studies are needed. To the best of our knowledge, we believe that this combination therapy is the most effective approach for specifically promoting regulatory immune responses.

A recent study has demonstrated that hypercholesterolemia decreases the Treg/Teff ratio in atherosclerotic lesions via inhibiting Treg accumulation within plaques.[20] This implies the possibility that increasing proportion of Tregs in atherosclerotic lesions could be a hopeful strategy to dampen plaque inflammation and prevent atherosclerosis, although further extensive experiments will be required to identify the exact role of intraplaque Tregs in atherogenesis. In this study, we showed that the marked reduction of atherosclerosis from combination therapy was associated with a dramatic increase in the Treg/Teff ratio and up-regulation of Treg activation markers not only in lymphoid organs but also in atherosclerotic plaques, implying that the beneficial effects of the combination therapy may partly be due to the induction of Tregs with activated phenotype in atherosclerotic plaques.

Monocytes infiltration from the peripheral blood to the subendothelial space or intima followed by macrophage differentiation is believed to be critical in initiation of atherosclerosis in humans and animals. The extent of macrophage recruitment into atherosclerotic plaques may depend on the cholesterol levels or monocyte number in the blood.[27] In the present study, we found that the combination therapy significantly reduced proinflammatory Ly6C^{high} monocytes compared to untreated or monotherapy groups, suggesting a possible contribution to decreased macrophage accumulation in atherosclerotic plaques. We found that thioglycollate-induced peritoneal macrophages from CD3-Ab/IL-2

complex-treated mice showed lower mRNA expressions of M1 markers and higher expressions of M2 markers.   Regarding macrophage markers, a similar trend was observed in the atherosclerotic aortas of CD3-Ab/IL-2 complex-treated mice.   Th1 cells are reported to contribute to generation of M1 macrophages, whereas Th2 cells promote generation of M2 macrophages.[28]   In addition, IL-10 derived from Tregs induces STAT3 (Signal Transducer and Activator of Transcription 3) activation and drives M2 polarization in mice with severe combined immunodeficiency.[29]   Collectively, we suppose that in CD3-Ab/IL-2 complex-treated mice, phenotype change of thioglycollate-induced peritoneal macrophages toward M2 phenotype may be attributable to dramatic expansion of Tregs as well as a shift from Th1 to Th2 immune responses, which represents one possible explanation for the athero-protective effects of the combination therapy.

In conclusion, we have demonstrated that combined intervention using CD3-Ab and IL-2 complex induced a remarkable inhibition of atherosclerosis via dramatically increasing the Treg/Teff ratio in lymphoid organs and atherosclerotic plaques, which caused macrophage polarization toward a less inflammatory M2 phenotype.   Our data imply that therapeutic intervention aimed at enhancing Treg-mediated immune responses and limiting Teff-mediated immune responses might represent a novel therapeutic approach for atherosclerotic diseases.

Conflict of Interest Disclosures

None.

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Figure Legends

Figure 1. Effect of a novel combination therapy on Teffs and Tregs. **A,** Experimental design is shown. Arrows with circle represent intravenous injection with 50 µg anti-CD3 antibody and arrows with square represent intraperitoneal injection with IL-2/anti-IL-2 mAb complex (1µg IL-2 plus 5µg anti-IL-2 mAb). For analysis of systemic immune responses, lymphocytes from spleen and LNs were prepared at 10 weeks of age. **B,** Representative results of CD4 and Foxp3 expressions in the spleen assessed by FACS. The graphs represent the percentage of CD4$^+$ cells within the total splenocytes and the percentage of Foxp3$^+$ cells within the CD4$^+$ population. **C-F,** The percentage of (**C**) CD4$^+$ T cells within total cells, (**D**) Foxp3$^+$ Tregs and (**E**) CD25$^+$ Foxp3$^+$ Tregs within the CD4$^+$ T cell population were determined by FACS. (**F**) The ratio of CD4$^+$Foxp3$^+$ Tregs to CD4$^+$Foxp3$^-$ Teffs was also determined as Treg/Teff ratio. **G,** The percentage of $^+$CD49b$^+$LAG-3$^+$ cells within CD4$^+$ T cells were determined by FACS. n=5 mice per group. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. Effects of the combination therapy on Treg-associated molecules, DC maturation and Th1/Th2 responses. **A,** The expression levels of Treg-associated markers were analyzed by FACS gating on CD4$^+$Foxp3$^+$ Tregs in the spleen. Histograms show mean fluorescence intensity (MFI). **B,** The expression levels of CD80 and CD86 were analyzed gating on

CD11c$^+$ DCs in spleens.   **C,** Splenocytes from 10-week-old mice in each group were prepared and intracellular cytokine staining was performed.   The graphs represent the frequencies of IFN-γ, IL-4, and IL-10-producing CD4$^+$ T cells.   **D,** The ratio of IFN-γ-producing CD4$^+$ T cells to IL-4 or IL-10-producing CD4$^+$ T cells in the spleen was determined as Th1/Th2 ratio.   n=5 mice per group.   *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 3.   Effects of the combination therapy on the development of atherosclerosis and plaque compositions.   **A,** Representative photomicrographs of Oil Red O staining, Masson's trichrome staining, MOMA-2 staining in the aortic sinus of mice in each group.   The black bar on Oil Red O staining represents 200 μm and black bars on the others represent 100 μm.

B, Quantitative analysis of atherosclerotic lesion size in the aortic sinus was performed using Oil red O-stained sections.   Horizontal bars represent mean.   n=10 to 12 mice per group.

C, Quantitative analysis of Oil red O-stained positive area (lipid content) in the aortic sinus. Horizontal bars represent mean.   n=10 to 12 mice per group.   **D,** Representative photomicrographs of Oil red O staining and quantitative analysis of atherosclerotic lesion size in the thoracic aortas in each group.   Horizontal bars represent mean.   n=3 to 4 mice per group.   **E-F,** Quantitative analyses of Masson's trichrome staining (**E,** n=8 mice per group) and MOMA-2 staining (**F,** n=10 to 12 mice per group) in the aortic sinus of mice in each

28

group.   *P<0.05, **P<0.01, ***P<0.001.

Figure 4.   Effects of the combination therapy on aortic Teffs and Tregs.   **A,** Representative photomicrographs of CD4 staining and Foxp3 staining in the aortic sinus of mice in each group.   The black bar on each staining represents 100 μm.   **B-C,** Quantitative analyses of CD4$^+$ T cells (**B**) and Foxp3$^+$ Tregs (**C**) in the aortic sinus of mice in each group.   n=8 mice per group.   **D,** Messenger RNA expressions of CD4, Foxp3, CD25 and CTLA-4 in atherosclerotic aortas were quantified by quantitative RT-PCR and normalized to GAPDH. n=5 to 6 mice per group.   *P<0.05, **P<0.01, ***P<0.001.

Figure 5.   The combination therapy polarizes macrophages from the M1 to M2 state.   **A,** The percentage of Ly6C^{hi} monocyte subset (**left**) and the expression levels of CD115 (CSF-1 receptor) (**right**) in the spleen of 10-week-old mice in each group were examined by FACS. n=5 mice per group.   **B,** The percentage of CD11c or CD206 in thioglycollate-induced peritoneal macrophages (F4/80$^+$) was analyzed by FACS.   n=5 mice per group.   **C,** Messenger RNA expressions of M1 macrophage markers (iNOS, MCP-1, CXCL10) and M2 macrophage markers (Arg I, Fizz1, Ym-1) in thioglycollate-induced peritoneal macrophages were quantified by quantitative RT-PCR and normalized to GAPDH.   n=4 to 5 mice per

group.   **D,** Messenger RNA expressions of iNOS and Arg I in atherosclerotic aortas were quantified by quantitative RT-PCR and normalized to GAPDH.   n=5 to 6 mice per group. *P*<0.05, **P*<0.01, ***P*<0.001.
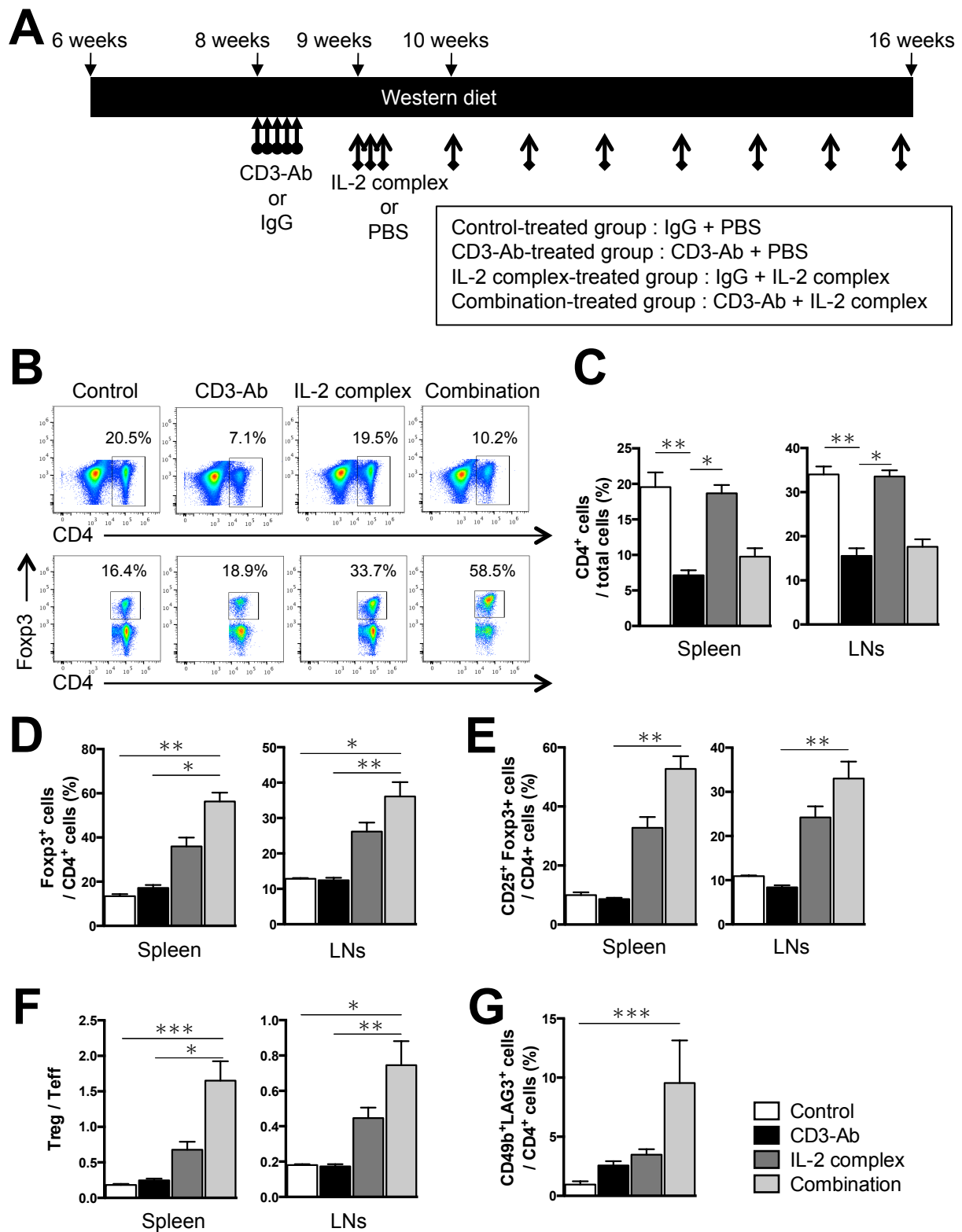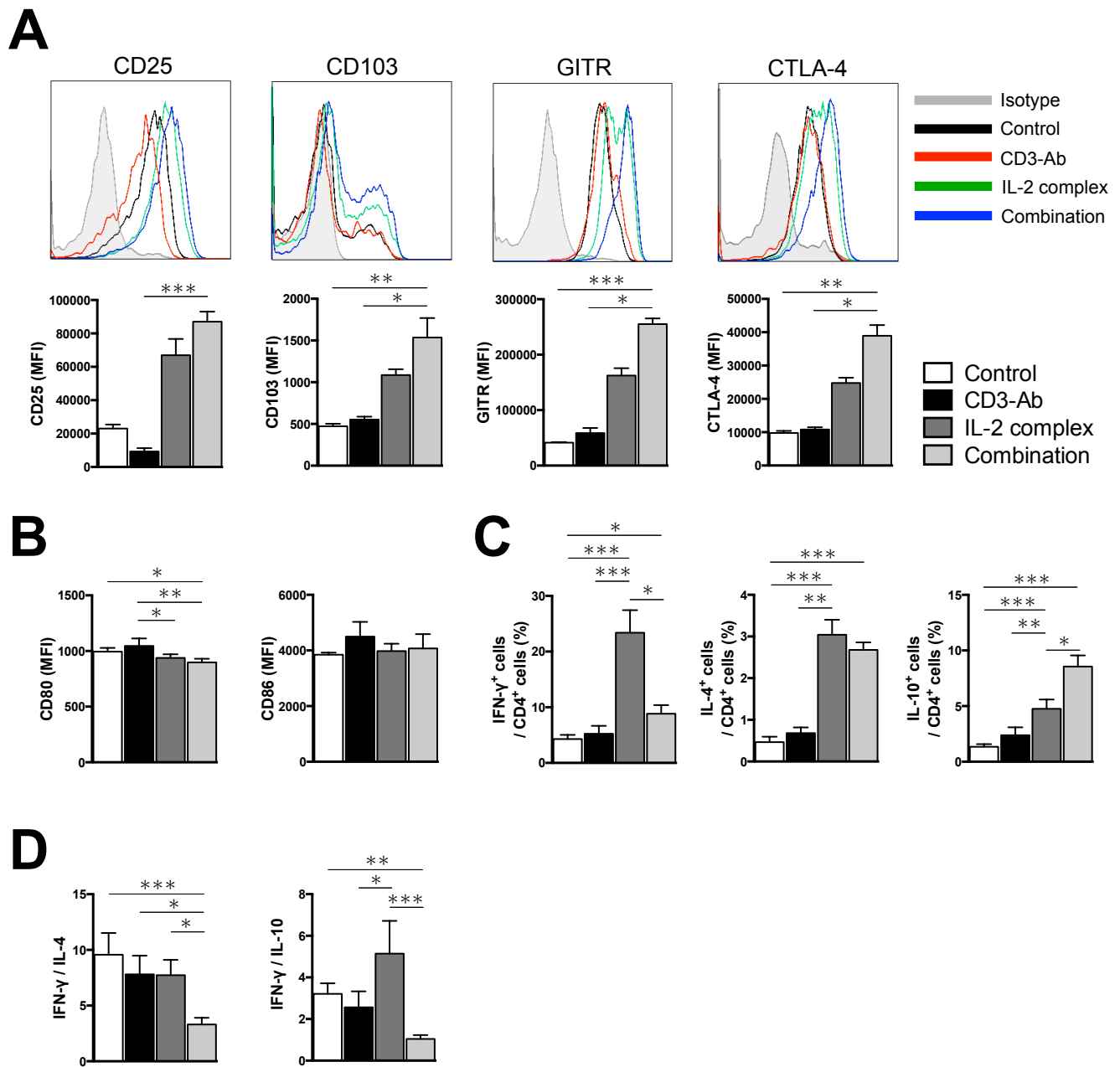
Table 1. Body weight and plasma lipid profiles

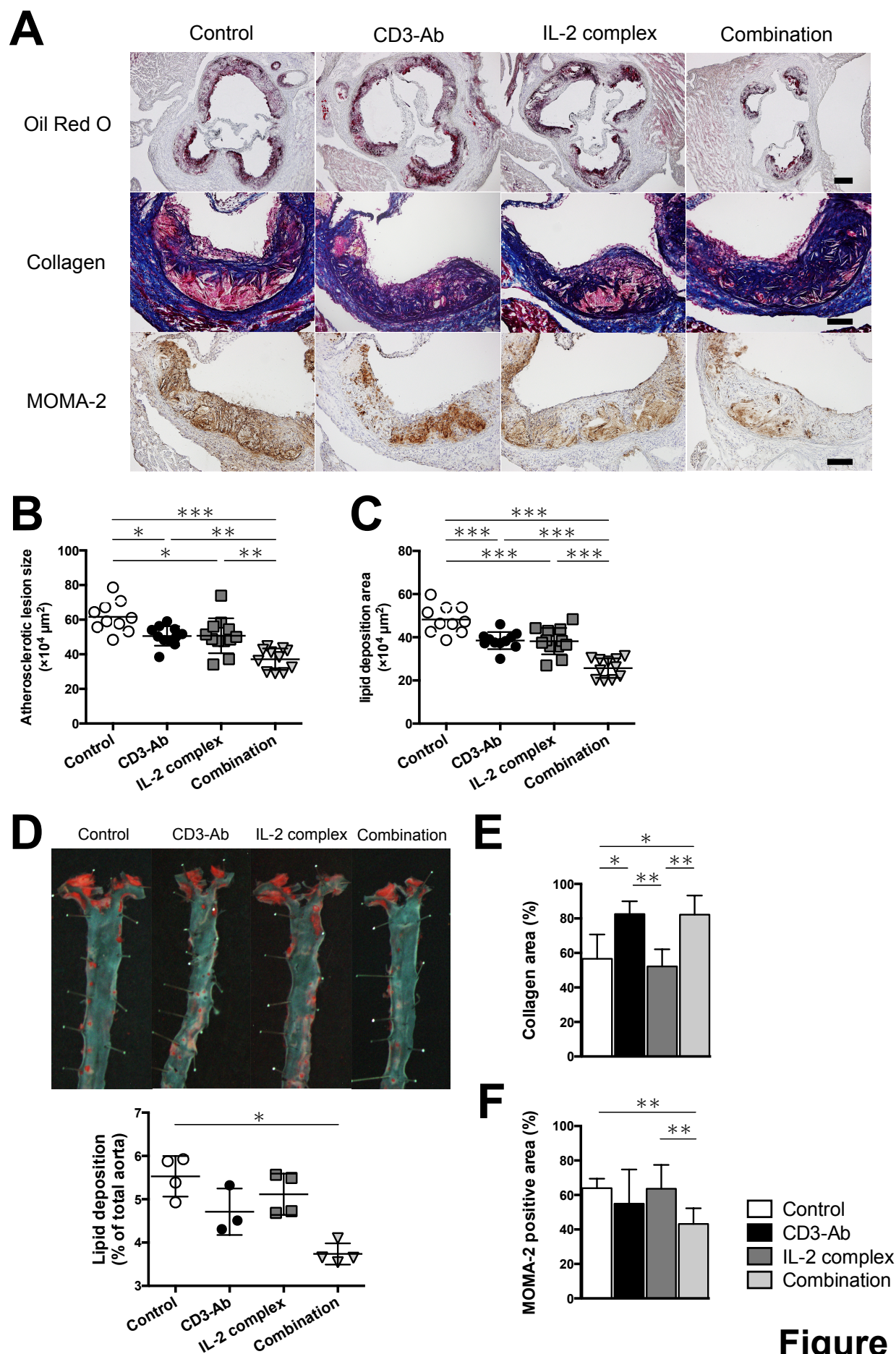|	Control	CD3-Ab	IL-2 complex	Combination
Body weight (g)	21.0 ± 0.4	21.6 ± 0.5	21.4 ± 0.3	22.2 ± 0.5
Total-cholesterol (mg/dl)	854.7 ± 52.1	716.3 ± 45.7	696.2 ± 34.1	568.1 ± 37.5*
HDL-cholesterol (mg/dl)	5.1 ± 0.7	7.0 ± 0.6	5.5 ± 0.3	7.1 ± 0.6
LDL-cholesterol (mg/dl)	196.9 ± 12.6	168.8 ± 12.7	165.2 ± 9.0	137.5 ± 12.8*
Triglycerides (mg/dl)	26.0 ± 3.8	37.6 ± 4.0	35.7 ± 4.8	42.5 ± 4.7

Results are expressed as means ± SEM.   n=10 to 12 mice per group.   *$P<0.05$ vs Control

A

6 weeks   8 weeks   9 weeks   10 weeks   16 weeks

Western diet

CD3-Ab or IgG

IL-2 complex or PBS

Control-treated group : IgG + PBS
CD3-Ab-treated group : CD3-Ab + PBS
IL-2 complex-treated group : IgG + IL-2 complex
Combination-treated group : CD3-Ab + IL-2 complex

B

Control   CD3-Ab   IL-2 complex   Combination

20.5%   7.1%   19.5%   10.2%

CD4

16.4%   18.9%   33.7%   58.5%

Foxp3

CD4

C

CD4+ cells / total cells (%)

Spleen   LNs

D

Foxp3+ cells / CD4+ cells (%)

Spleen   LNs

E

CD25+ Foxp3+ cells / CD4+ cells (%)

Spleen   LNs

F

Treg / Teff

Spleen   LNs

G

CD49b+LAG3+ cells / CD4+ cells (%)

Control
CD3-Ab
IL-2 complex
Combination

Figure 1.

Figure 2.

A

	Control	CD3-Ab	IL-2 complex	Combination
Oil Red O				
Collagen				
MOMA-2				

B Atherosclerotic lesion size (×10⁴ μm²)

C lipid deposition area (×10⁴ μm²)

D Control CD3-Ab IL-2 complex Combination

Lipid deposition (% of total aorta)

E Collagen area (%)

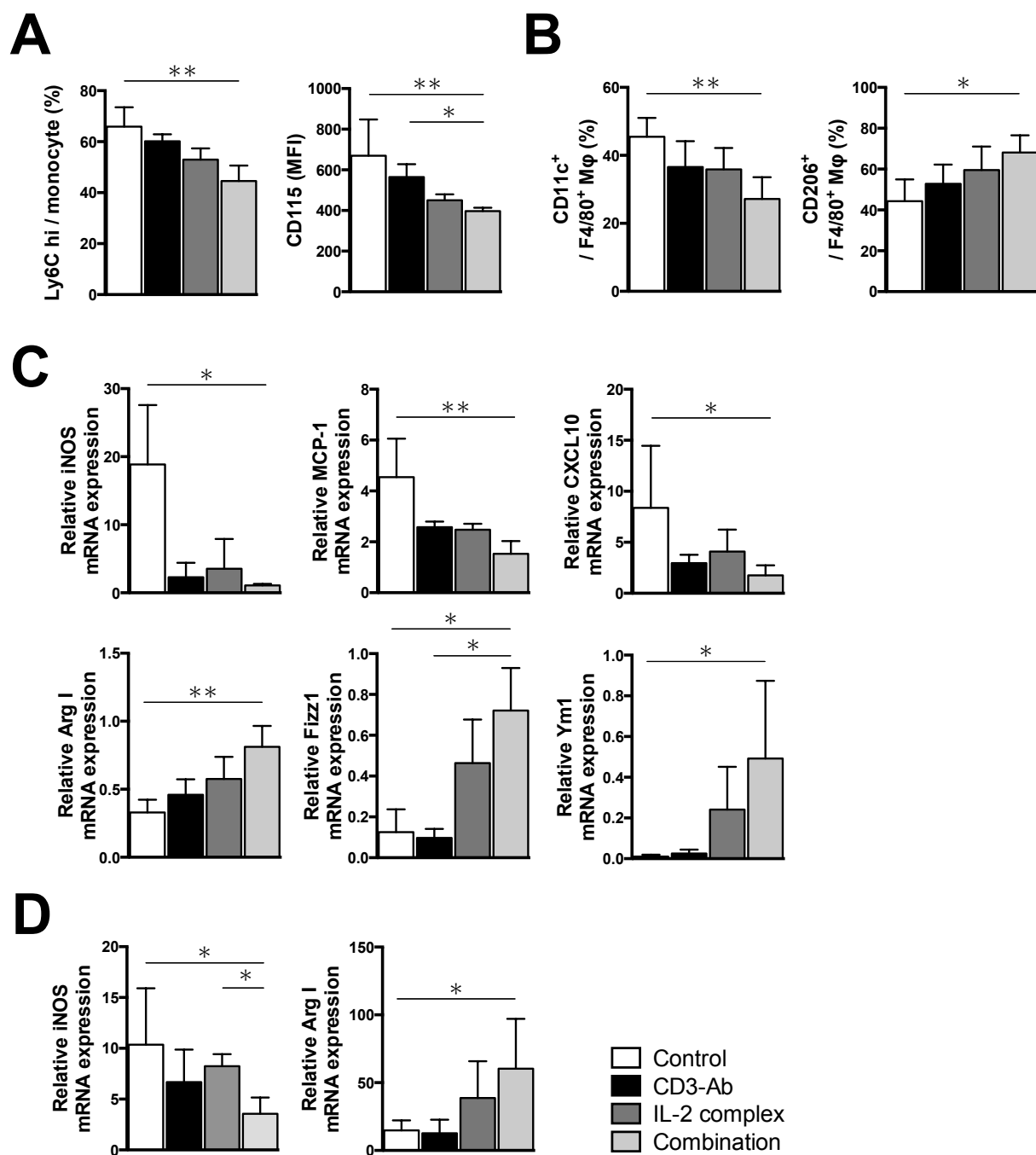**F** MOMA-2 positive area (%)

☐ Control
■ CD3-Ab
▨ IL-2 complex
▨ Combination

Figure 3.

Figure 4.

Figure 5.