CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells Protect the Proinflammatory Activation of Human Umbilical Vein Endothelial Cells

Shaolin He, Ming Li, Xuming Ma, Jing Lin, Dazhu Li

Objective—To investigate the role of CD4⁺CD25⁺forkhead box P 3 (Foxp3)⁺ T-regulatory cells (Tregs) in protecting the activation and function of human umbilical vein endothelial cells (HUVECs) induced by proinflammatory stimulus and the mechanisms of it.

Methods and Results—ECs play a major role in atherogenic initiation, changing their quiescence into activated phenotypes to support every phase of the inflammatory process. HUVECs were incubated alone, with Tregs or CD4⁺CD25⁻ T cells in the presence of anti–CD3 monoclonal antibodies for 48 hours, and then were stimulated with or without oxidized low-density lipoprotein/lipopolysaccharide for an additional 24 hours. Tregs are able to induce alternative expression of immune phenotypic markers of activated HUVECs by down modulating CD86 and to inhibit the adhesion molecule, such as vascular cell adhesion molecule-1 (VCAM-1) and proinflammatory cytokine (eg, monocyte chemoattractant protein-1 and interleukin 6), response of HUVECs to oxidized low-density lipoprotein/lipopolysaccharide. Moreover, Tregs downregulate proinflammatory factor nuclear factor-κB activation and induce resistance to suppression of anti-inflammatory factor Kruppellike factor 2 in HUVECs induced by a proinflammatory stimulus. Mechanism studies reveal that Treg-mediated suppression of HUVEC proinflammatory cytokines and adhesion molecule expression impaired by oxidized low-density lipoprotein/lipopolysaccharide require cell contact by cytotoxic T-lymphocyte antigen-4 and CD86 and by soluble factors (mainly interleukin 10 and transforming growth factor [TGF]-β).

Conclusion—Tregs may exert their protective effects against atherogenesis in part through inducing an immune-inhibitory phenotype of ECs involving cytotoxic T-lymphocyte antigen-4–dependent cell-to-cell contact and also requiring soluble factors (mainly interleukin 10 and TGF-β). (Arterioscler Thromb Vasc Biol. 2010;30:2621-2630.)

Key Words: atherosclerosis ■ regulatory T cells ■ endothelial cells adhesion molecules ■ endothelial function ■ immune system ■ oxidized lipids

Atherosclerosis is a chronic inflammatory disease of the arterial wall, in which both innate and adaptive immune responses contribute to disease initiation and progression.¹ ² The role of the immune system in atherosclerosis has received considerable interest in recent years. However, sufficient knowledge to justify the immune modulatory mechanisms has not yet been obtained.

Recent studies from several groups suggest that subtypes of T cells, called T-regulatory cells (Tregs), previously shown to maintain immunologic tolerance, inhibit the development and progression of atherosclerosis.³–⁶ Interestingly, the suppressive effects of these cells are not restricted to the adaptive immune system, including CD4⁺ T, CD8⁺ T, natural killer, or B cells. These cells can also affect the activation and function of innate immune cells (eg, monocytes, macrophages, and dendritic cells) and neutrophils⁷; however, their effects on endothelial cells (ECs) are less well-known.

Previous studies have shown that alloantigen-specific CD8⁺CD28⁻ T-suppressor cells can induce interleukin immunoglobulin-like transcript 3⁺ (ILT3⁺) ILT4⁺ tolerogenic ECs, inhibiting alloreactivity.⁸ ⁹ Because ECs are an important target of antigen-specific T-effector cells (in transplantation and in numerous autoimmune diseases), Tregs that inhibit EC activation and/or render ECs tolerogenic are likely to have a beneficial effect in the treatment of rejection, autoimmunity, and possibly atherosclerosis. Thus, the identification and characterization of Tregs regulating EC activation, inflammation, and dysfunction could be crucial in deciphering the effects and mechanisms of Tregs in atherosclerosis. Herein, we assessed the role of
CD4⁺CD25⁺Foxp3⁺ regulatory T cells in protecting the proinflammatory activation of human umbilical vein ECs (HUVECs) and the mechanisms of it.

**Methods**

**HUVEC Culture and Preparation of Low-Density Lipoprotein and Copper-Oxidized Low-Density Lipoprotein**

HUVEC culture was performed as previously described. Low-density lipoprotein (LDL) and copper-oxidized LDL were prepared as previously described. The supplemental information (available online at http://atvb.ahajournals.org) provides descriptions of the extended protocols.

**Isolation and Purification of Tregs and Coculture of T-Effector and Tregs**

Isolation and purification of Tregs were performed using the method of Dieckmann et al. The supplemental information provides descriptions of the extended protocols.

**Coculture of HUVECs and Tregs**

HUVECs that have grown to 90% confluence on 6-well plates (1×10⁶ cells per well) were washed 3 times with PBS and cultured in serum-free medium for 24 hours for synchronization. Then, nonadherent cells were removed and culture medium was changed. For coculture experiment, HUVECs were cultured without T cells, with Tregs (CD25⁺), or with CD4⁺CD25⁻ T cells (CD25⁻) for 48 hours in the presence of anti-CD3 monoclonal antibodies (mAbs) (50 ng/mL) and then with oxidized LDL (ox-LDL) (40 μg/mL) (Escherichia coli 0111:B4; Sigma, St. Louis, Mo) for an additional 24 hours; as a control group (control), HUVECs were cultured without anything but serum-free M199 medium for 24 hours. The concentrations of ox-LDL and LPS are those commonly used to investigate the inflammatory response in vitro. After the incubation period, floating T cells were aspirated, HUVECs were harvested, and supernatants were collected for further experiments.

**Detailed Procedures**

The supplemental information provides detailed descriptions of the following procedures: (1) flow cytometry for the detection of human leukocyte antigen (HLA) DR, CD86, CD80, and VCAM-1; (2) ELISA for the detection of monocyte chemotactic protein (MCP)-1, IL-6, IL-10, and TGF-β; (3) real-time RT-PCR for the detection of VCAM-1, MCP-1, IL-6, and Kruppellike factor (KLF) 2; and (4) electrophoretic mobility shift assay for the detection of nuclear factor (NF)-κB.

**Transwell Experiments and Fixation of Tregs**

Details of Transwell (TW) experiments are given in the supplemental information. The detailed methods for fixation of Tregs are performed as previously described, which may be seen in the supplemental data.

**Data Analysis**

All data are expressed as means of duplicate samples from 3 to 5 independently performed experiments. Data are presented as mean±SEM. The significance of differences was estimated by ANOVA, followed by Student-Newmann-Keuls multiple comparison tests. P<0.05 was considered significant. All statistical analyses were performed with computer software (SPSS, version 11.0; SPSS Inc, Chicago, Ill).

**Results**

**Identification of CD4⁺CD25⁺Foxp3⁺ T Cells**

We sorted CD4⁺CD25⁺ T cells from peripheral blood mononuclear cells of healthy volunteers. As expected, the purity of CD4⁺CD25⁺ T cells was >93% (Figure 1A) and most of the sorted CD4⁺CD25⁺ T cells were Foxp3⁺ (Figure 1B). To assess whether cells with a CD4⁺CD25⁺Foxp3⁺ T-cell (Treg) phenotype also displayed functional Treg char-

**Figure 1.** Identification of CD4⁺CD25⁺ Foxp3⁺ T cells. CD25⁺CD4⁺ T cells were sorted from peripheral blood mononuclear cells (PBMCs) of healthy volunteers. A and B, Fluorescence-activated cell sorter analysis shows the sort purity of CD25⁺CD4⁺ T cells (A) and Foxp3 expression in sorted subsets (B). The numbers display the frequency of cells within indicated populations. The bars in B indicate the marker gate for Foxp3⁺ cells. Different ratios of magnetic bead–sorted CD4⁺CD25⁺ T cells (Tregs) from PBMCs of healthy volunteers were incubated with effector CD4⁺CD25⁻ T cells (Tcon) in the presence of irradiated antigen presenting cells. C, Thymidine uptake was used for assessment of effector cell proliferative indexes. D, The comparative effect of CD4⁺CD25⁺ T cells (Tregs) expressed as percentage inhibition of CD4⁺CD25⁻ T cells (Tcon). cpm indicates counts per minute.
characteristics, we tested their capacity to suppress the proliferation of autologous cocultured CD4⁺CD25⁻ T cells (Tcon) after activation with anti–CD3 mAbs; we found that Tregs can efficiently suppress Tcon cell proliferation in a dose-dependent manner (Figure 1C and 1D).

**Effects of Tregs on the Expression of Immune Phenotypic Markers of HUVECs**

HUVECs were cocultured without T cells, with Tregs (CD25⁺), or with CD4⁺CD25⁻ T cells (CD25⁻) in the presence of anti–CD3 mAbs for 48 hours and then with or without (control) ox-LDL for an additional 24 hours. The expression of HLA DR, CD86, and CD80 in HUVECs was determined by flow cytometry. Ox-LDL impaired HUVECs (without T cells) displayed an activating phenotype by high expression of HLA DR (15.3±3.0%, *P*<0.001) and CD86 (39.4±5.1%, *P*<0.001) compared with a resting phenotype of HUVECs (control, 0.8±0.7%; without T cells, 1.7±0.7%; CD25⁺, 1.3±1.1%; CD25⁻, 1.6±1.2%; all *P*>0.05) (Figure 2). We also found that the effects of Tregs on the expression of immune phenotypic markers were seen with other proinflammatory agents, such as LPS (Figure 2).

**Tregs Downregulate VCAM-1, MCP-1, and IL-6 Responses of HUVECs to ox-LDL/LPS**

HUVECs were cocultured without T cells, with Tregs (CD25⁺), or with CD4⁺CD25⁻ T cells (CD25⁻) in the presence of anti–CD3 mAbs for 48 hours and then with or without (control) ox-LDL/LPS for an additional 24 hours. The protein levels of VCAM-1, MCP-1, and IL-6 in HUVECs were determined by flow cytometry and ELISA. HUVECs exposed to ox-LDL have a significant increased expression of VCAM-1 (58.0±5.2%, *P*<0.001), MCP-1 (47.4±5.8 ng/mL, *P*<0.001), and IL-6 (10.8±1.4 ng/mL, *P*<0.001) compared with that from a control system (0.6±0.3%, 4.9±2.7 ng/mL, and 0.5±0.2 ng/mL, respectively). Treg-treated HUVECs significantly decreased VCAM-1 expression by 13.6±4.0% and MCP-1 (20.6±2.0 ng/mL), and IL-6 (3.9±0.8 ng/mL) expression compared with that from the system without T cells (VCAM-1, 58.0±5.2%, *P*<0.001; MCP-1, 47.4±5.8 ng/mL, *P*<0.01; and IL-6, 10.8±1.4 ng/mL, *P*<0.01) or the CD25⁻ system (VCAM-1, 58.3±5.1%, *P*<0.001; MCP-1, 47.7±4.0 ng/mL, *P*<0.001; and IL-6, 9.3±1.7 ng/mL, *P*<0.01) (Figure 3A and 3B). Similar results occurred in Treg-treated HUVECs cocultured to LPS (Figure 3A and 3B). To exclude the possible contributions of Treg-derived proinflammatory cytokines in these assays, HUVECs were precultured without T cells, with Tregs (CD25⁺), or with CD4⁺CD25⁻ T cells (CD25⁻) in the presence of anti–CD3 mAbs for 48 hours; T cells were depleted by using anti–CD2 beads, and the HUVECs were stimulated with or without (control) ox-LDL/LPS for an additional 24 hours. A similar result was observed (Figure 3C). More important, these latter experiments also demonstrate that the suppressive effects persist even once Tregs are removed from the assay.

The mRNA levels of VCAM-1, MCP-1, and IL-6 were also determined by real-time RT-PCR analysis. As shown in supplemental Figure, IA, GAPDH was used as endogenous control (C), and the amount of target was normalized to endogenous control gene. Coincident with the protein activity data previously given, VCAM-1, MCP-1, and IL-6 mRNA expression levels were reduced in CD25⁺ cultures relative to controls without T cells or CD25⁻.

To explore whether there was a threshold effect for Treg-mediated suppressive expression of VCAM-1, MCP-1, and IL-6 in mRNA levels in HUVECs, HUVECs (1×10⁶ cells per well) were cultured with or without various concentrations of anti–CD3 mAb–pretreated Tregs (1×10⁶, 2.5×10⁶, and 5×10⁶ cells per well, respectively) in the presence of ox-LDL for 24 hours; then, HUVEC proinflammatory cytokine expression levels were measured. A dose-dependent effect on HUVEC proinflammatory cytokine expression was noted in HUVECs (supplemental Figure, IB).

**Tregs Downregulate NF-κB Activation in HUVECs, Induced by a Proinflammatory Stimulus**

NF-κB is a ubiquitous transcription factor that regulates expression of proinflammatory and antiapoptotic genes; it is thought to play an important role in driving the inflammatory response. Therefore, we examined NF-κB activity in HUVECs cocultured with or without anti–CD3 mAb–activated Tregs after ox-LDL/LPS treatment. We performed an electrophoretic mobility shift assay with specific oligonucleotide probes for the NF-κB binding site regions. As shown in Figure 4, the reduced proinflammatory cytokines were reflected at the transcriptional level by a clear decreased NF-κB upregulation on ox-LDL/LPS stimulation from Treg-treated HUVECs (CD25⁺) (lane 4 or 7). In contrast, HUVECs cultured only with ox-LDL/LPS (without T cells) or HUVECs precultured with CD4⁺CD25⁻ T cells (CD25⁻) displayed an increase in NF-κB activation on ox-LDL/LPS triggering (lane 3, 5, 6, or 8). The identity of the NF-κB–specific band was confirmed by competition analyses with unlabeled oligonucleotide (competition) (lane 1).

**Tregs Induce Resistance to Suppression of KLF2 in HUVECs, Induced by a Proinflammatory Stimulus**

Because the transcription factor KLF2 is considered an important mediator of the anti-inflammatory properties of the endothelium, we next investigated the influence of proinflammatory stimuli, such as ox-LDL/LPS, on KLF2 mRNA levels in control cells for various periods and after coculture with Tregs. As depicted in Figure 5A, KLF2 mRNA expression was strongly suppressed at the point of 6 hours after treatment of HUVECs with ox-LDL/LPS, whereas the reduction observed in KLF2 mRNA levels induced by ox-LDL/LPS was significantly inverse regulated in the presence of Tregs (Figure 5B).
To investigate whether suppression of HUVEC activation and function depended on cell contact or soluble factors, we cultured HUVECs without T cells, with Tregs in the presence of anti-CD3 mAbs for 48 hours and then with or without ox-LDL/LPS for an additional 24 hours; as a control, HUVECs were cultured without anything but serum-free M199 medium for 24 hours. A through C, The expression of HLA DR, CD86, and CD80 in HUVECs was determined by flow cytometry (A, B, and C, respectively). D, Study data are representative of at least 3 separate flow cytometric analyses. + indicates CD25+ vs CD25-; $, no T vs control; $, CD25+ vs no T or CD25-; ***P<0.001; ##P<0.01; ###P<0.001; +P<0.05; ++P<0.01; §P>0.05; $P>0.05.

Figure 2. Tregs modulate expression of HLA DR and CD86 in HUVECs impaired by ox-LDL/LPS. HUVECs were cocultured without T cells (no T), with Tregs (CD25+), or with CD4+CD25+ T cells (CD25-) in the presence of anti-CD3 mAbs for 48 hours and then with or without ox-LDL/LPS for an additional 24 hours; as a control, HUVECs were cultured without anything but serum-free M199 medium for 24 hours. A through C, The expression of HLA DR, CD86, and CD80 in HUVECs was determined by flow cytometry (A, B, and C, respectively). D, Study data are representative of at least 3 separate flow cytometric analyses. + indicates CD25+ vs CD25-; $, no T vs control; $, CD25+ vs no T or CD25-; ***P<0.001; ##P<0.01; ###P<0.001; +P<0.05; ++P<0.01; §P>0.05; $P>0.05.
with that in a system without T cells (Figure 6A). The MCP-1 and IL-6 in supernatants from all of the described groups were also detected by ELISA assays; similar results were obtained (Figure 6B). This partial reversal of suppression could be because of the requirement of cell contact between activated Tregs and ox-LDL–impaired HUVECs.

Previous studies have shown that cytotoxic T-lymphocyte antigen-4 (CTLA-4) may be involved in the cell contact–mediated negative regulation of Tregs21,22; thus, we hypothesized that activated Tregs are able to downmodulate proinflammatory cytokine expression on ECs impaired by ox-LDL in a CTLA-4–dependent manner. Although Tregs are able to increase expression of CTLA-4 on stimulation,19 we need to confirm that this molecule was present in our cultures. Flow cytometry assays showed that, after being stimulated with anti–CD3 mAbs for 48 hours, viable Tregs and activated fixed Tregs were strongly positive in high expression of CTLA-4 (compared with resting Tregs or resting fixed Tregs: all \( P < 0.001 \)); this indicated that activated paraformaldehyde-fixed Tregs show a similar regulatory capacity as viable cells (Figure 6C). This was a fundamental study for further fixation and neutralization experiments. For fixation and neutralization experiments, HUVECs were cultured without T cells, with activated fixed Tregs, or with activated fixed Tregs added to anti–CTLA-4 mAbs (anti–CTLA-4, eBioscience) or isotype in the presence of ox-LDL for 24 hours; expression levels of VCAM-1, MCP-1, and IL-6 in HUVECs were then assayed. As shown in Figure 6D and 6E, VCAM-1, MCP-1, and IL-6 expression levels were significantly inhibited in the activated fixed Tregs group, which showed similar regulatory capacity as their norm viable counterpart compared with that in the group without T cells (\( P < 0.01 \)). However, the inhibitory effects of activated fixed Tregs were significantly reversed when anti–CTLA-4 mAbs were added (\( P < 0.01 \)). CD86 and CD80 are the only 2 known ligands for CTLA-4. Therefore, anti–CD86 mAbs

Figure 3. Tregs downregulate protein expression of VCAM-1, MCP-1, and IL-6 in HUVECs impaired by ox-LDL/LPS. Coculture was set up as described in the legend to Figure 2. A, HUVECs were harvested, and expression of VCAM-1 on them was observed by flow cytometry. B, Supernatants were collected, and MCP-1 and IL-6 were detected by ELISA. HUVECs were precultured without T cells (no T), with Tregs (CD25+), or with CD4+CD25– T cells (CD25–) in the presence of anti-CD3 mAbs for 48 hours; T cells were depleted by using anti–CD2 beads; and the HUVECs were stimulated with ox-LDL/LPS for an additional 24 hours. C, Supernatants from different groups were collected, and MCP-1 and IL-6 were determined by ELISA assays. Data are expressed as mean±SEM of at least 3 independent experiments. **P<0.001, ##P<0.01, +++P<0.01, ++++P<0.001.
(anti-CD86) or anti–CD80 mAbs (anti-CD80) were also added in HUVECs cultured with an activated fixed Tregs system. We found that anti-CD86, but not anti-CD80, has a similar reversion effect with the anti–CTLA-4 on the inhibitory effects of activated fixed Tregs. The isotype-matched control mAbs had no effect (Figure 6D and 6E).

In the TW system, we found that inflammatory cytokine levels were still markedly reduced compared with those in HUVECs cultured alone (without T cells) \((P<0.01)\), indicating that cell contact was only partly necessary and that immunosuppressive cytokines may play a role in Treg-mediated suppression on HUVEC proinflammatory cytokine expression. To test the role of immunosuppressive cytokines in the mechanism of regulation by Tregs, we incubated the Tregs with anti–CD3 mAbs for 48 hours and then collected the supernatants, added them to ox-LDL–treated HUVECs supernatant (sup) for an additional 24 hours, and assayed the expression of VCAM-1, MCP-1, and IL-6 in HUVECs. We found that anti–CD3 mAb–treated Tregs can secrete immunosuppressive cytokines to inhibit VCAM-1 \((37.1\pm4.3\%\), \(P<0.001\)), MCP-1 \((19.75\pm1.9 \text{ ng/mL}, P<0.001\)), and IL-6 \((4.92\pm0.9 \text{ ng/mL}, P<0.01)\) expression in HUVECs compared with that in HUVECs cultured with ox-LDL alone \((62.3\pm4.9\%, 44.11\pm3.0 \text{ ng/mL}, \text{ and } 9.72\pm1.3 \text{ ng/mL}, \text{ respectively})\). These findings suggest that immune-suppressive cytokines were required in Treg-mediated suppression of
HUVEC proinflammatory cytokine expression impaired by ox-LDL (Figure 7B and 7C). Important recent advances in the comprehension of the mechanisms of atherosclerosis provided evidence that the immune-inflammatory response in atherosclerosis is modulated by regulatory pathways in which 2 anti-inflammatory cytokines (ie, IL-10 and TGF-β) play a critical role.2 Also, Tregs are able to produce immunosuppressive cytokines, such as IL-10 and TGF-β, upon stimulation.23 Therefore, we test whether IL-10 or TGF-β might be involved in the supernatant-mediated suppression of proinflammatory cytokine expression in HUVECs impaired by ox-LDL. First, we confirmed that these cytokines were present in our cultures. ELISA assays showed that supernatants from the coculture system were strongly positive in the expression of TGF-β (1.9±0.12 ng/mL, P<0.001) and IL-10 (678.1±27.8 pg/mL, P<0.001) compared with those in the control (0.8±0.1 ng/mL and 143.3±25.0 pg/mL, respectively), without T cell (0.7±0.09 ng/mL and 132.9±24.2 pg/mL, respectively), or CD25− system (0.6±0.14 ng/mL and 128.5±26.6 pg/mL, respectively) (Figure 7A). Second, neutralizing experiments were performed. Anti–IL-10, anti–TGF-β, or isotype mAbs were added to the supernatant system. After treatment with anti–TGF-β or anti–IL-10 mAbs, the inhibitory effects of supernatants were significantly reduced; moreover, the suppression of VCAM-1, MCP-1, and IL-6 expression in HUVECs was markedly abrogated when both neutralizing anti–IL-10 and neutralizing anti–TGF-β mAbs were added. The isotype-matched control mAbs had no effect. Furthermore, we cultured HUVECs with TGF-β mAbs (final concentration, 1.9 ng/mL) or IL-10 mAbs (final concentration, 678.1 pg/mL) for 48 hours and then added ox-LDL for another 24 hours. The expression of VCAM-1 in HUVECs and MCP-1 and IL-6 from supernatants was measured and showed a significant reduction in HUVECs from the TGF-β or IL-10 system compared with that from the no T-cell system (Figure 7B and 7C).

Discussion
Atherosclerosis is driven by a chronic inflammatory process within the arterial wall, initiated mainly in response to...
endogenously modified structures, particularly oxidized lipoproteins or LPS that stimulates both innate and adaptive immune responses, leading to further alteration of the vascular wall and promotion of disease progression and complications. The historical focus of immunologic studies on regulation of atherogenesis has been on the functions of infiltrating macrophages and T cells. However, recent reports demonstrated that ECs play a major role in atherogenic initiation, changing their quiescence into activated phenotypes to support every phase of the inflammatory process. ECs play an important role in many phases of immunologic events. In inflammation, ECs exhibit increased adhesiveness for host leukocytes and are involved in their recruitment to the interstitium of the tissue. Furthermore, ECs could effectively present alloantigens to lymphocytes, leading to T-cell activation. Concerns in recent studies have been focused on the mechanisms of costimulation for T-cell activation delivered by ECs. In the present study, we have examined the interactions of activated Tregs with ox-LDL/LPS–impaired HUVECs and found a critical role of Tregs in protecting the activation and function of HUVECs.

The important role of ECs in antigen presentation and immunogenicity in vascular inflammation and autoimmune responses has been recognized. ECs represent a highly heterogeneous population of cells with the ability to modulate the function of immune cells. The immunogenicity of ECs is significantly upregulated when ECs are activated and then can highly express HLA DR and costimulators, such as CD86 and CD80. Our studies showed that Tregs are able to induce alternative expression of immune phenotypic markers of activated HUVECs by down modulating CD86, which extends previous reports that costimulatory molecule expression on ECs can provide important supplementary signals for Tregs interacting with ECs. However, there is conflicting evidence in the literature regarding the expression of CD86 and CD80 molecules on ECs, with some reports showing their presence and others reporting their absence. These discrepancies may reflect different culture conditions and serial passages of cells or inherent differences between ECs derived from different vascular beds. Furthermore, it is possible that conflicting results regarding the tissue-specific phenotype of ECs could arise from the use of different isolation techniques or different selection of EC subpopulations during subculture. Moreover, Tregs inhibit the proinflammatory response of HUVECs to ox-LDL/LPS, accompanied by a reduction in the upregulation of NF-κB activation. All the data described demonstrated that Treg-treated ECs confer a quiescent EC state with reduced expression of costimulatory, adhesion, and chemokine molecules, indicating that Tregs may exert their protective effects against atherogenesis in part through inducing an immune-inhibitory phenotype of ECs to a greater extent than nontreated ECs. In recent years, KLF2 has emerged as a master regulator of endothelial quiescence, anti-inflammatory and antithrombotic properties, and vascular tone by activating atheroprotective and inhibiting atherogenic transcription. KLF2 acts as a central transcriptional switch point between the quiescent and activated states of the adult EC. Our study identified KLF2 as being inhibited by proinflammatory stimuli, such as ox-LDL/LPS, and inverse regulated by Tregs. Although the
molecular basis for Treg regulation of KLF2 remains unknown in HUVECs, recent studies by Kumar et al10 implicate p65 and histone deacetylases in repressing the expression of this factor. Whether similar mechanisms are operative in Treg-mediated suppression of HUVEC activation is unclear. However, the interesting possibility is raised that KLF2 and NF-κB may function in a mutually antagonistic manner and that the balance of these 2 factors may dictate the degree of cellular activation.

Several mechanisms of Treg-mediated suppression have been proposed, including secretion by the Tregs of immuno-suppressive cytokines, cell contact–dependent suppression, and functional modification or killing of activated protein C.7 To investigate the mechanisms of Treg-mediated suppression of ECs, TW experiments and neutralizing antibodies were used. By disrupting physical contact between HUVECs and Tregs (TW), the suppression of proinflammatory cytokine expression was only partly reversed, suggesting that cell-to-cell contact was required in Treg-mediated suppression. Activated-fixed and viable Tregs almost had a similar regulatory capacity as their normal viable counterpart. Moreover, blocking CTLA-4 on Tregs significantly decreased their suppression effects on activated ECs. Therefore, these experiments demonstrated that the suppression function of Tregs involved direct contact-dependent interactions with ECs and was, in part, mediated by CTLA-4. This underlines and extends prior findings on regulatory function, demonstrating that surface molecules induced after activation of Tregs are responsible for the regulatory capacity of these cells.

CTLA-4 belongs to the same family as CD28 and binds to the same ligands, CD80 and CD86. Recent reports have shown that the interaction of CTLA-4 with CD80/CD86, expressed by activated protein Cs, may modulate immune responses.40 This raises the possibility that anti–CTLA-4 mAbs may disrupt Treg function by preventing a CTLA-4–mediated signal through CD80/CD86 expressed on dendritic cells. The data show that binding of a CTLA-4–Ig fusion protein to the surface of dendritic cells induces expression of indoleamine 2,3 dioxygenase, leading to the depletion of tryptophan and the inhibition of T-cell function.41 CTLA-4 expressed by Tregs has similar effects, suggesting that this interaction may be important for the suppressive activity of these cells.42 Our findings that activated Tregs are able to down modulate CD86 and proinflammatory cytokine expression on ECs impaired by ox-LDL in a CTLA-4–dependent manner are in line with these data, raising the possibility that it is ligation of CD86 on ECs by CTLA-4 expressed on Tregs. Also requiring soluble factors, by IL-10 and TGF-β. This newly discovered ability of Tregs may help us to understand the mechanisms of Tregs in atherosclerosis processes and may also provide a novel tool to manipulate atherosclerosis development.

In conclusion, our study shows the following results. (1) Activated Tregs are able to down modulate costimulatory molecules CD86 and proinflammatory cytokine VCAM-1, MCP-1, and IL-6 expression on HUVECs impaired by ox-LDL/LPS. (2) Tregs downregulate proinflammatory factor NF-κB activation and induce resistance to suppression of anti-inflammatory factor KLF2 in HUVECs exposed to ox-LDL/LPS. (3) Tregs may exert their protective effect against atherogenesis in part through inducing an immune-inhibitory phenotype of ECs involving CTLA-4–dependent cell-to-cell contact and also requiring soluble factors, by IL-10 and TGF-β. This newly discovered ability of Tregs may help us to understand the mechanisms of Tregs in atherosclerosis development.

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Disclosures
None.

References
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Supplement Material: extended methods

**HUVECs culture**
HUVECs were isolated from human umbilical veins that were cannulated, perfused with Hanks’ solution to remove blood, and then incubated with 1% collagenase for 15 minutes at 37°C. After removal of collagenase (Sigma, USA), cells were cultured in MI99 medium (Gibco, USA) supplemented with 20% foetal calf serum (Gibco, USA), 100 ug/ml heparin (Sigma, USA), 50 ug/ml endothelial cell growth supplement (Gibco, USA), 25 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin, and grown at 37°C on tissue culture plates coated with 0.1% gelatin. Cells were passaged at confluence by splitting 1:4 and were used within the first eight passages. Use of human umbilical veins and the following blood from normal donors were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

**Preparation of LDL and copper-oxidized LDL**
Blood for lipoprotein isolation was collected in EDTA (1 mg/mL) from normal lipidemia donors after 12 hours of fasting. LDL (density=1.03 to 1.063 g/L) was isolated from the plasma after density adjustment with KBr, by preparative ultracentrifugation at 50 000 rpm/min for 22 hours, using type 50 rotor. They were dialyzed against phosphate buffered saline (PBS) containing 0.3 mM EDTA, sterilized by filtration through a 0.22 mm filter, and stored under nitrogen gas at 4°C. Protein content was determined by the method of Lowry et al. Copper oxidation of LDL was performed by incubation of post-dialyzed LDL (1 mg of protein/ml in EDTA-free PBS) with copper sulfate (10 mM) for 24 hours at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (36.8±2.5 nmol of malondialdehyde equivalents/mg protein, mean±S.D. n=5), and relative electrophoretic mobility was 4.0±0.3 (mean±S.D. n=5).² ³

**Isolation and purification of Tregs**
Isolation of Human peripheral blood mononuclear cells (PBMCs) which obtained from normal volunteers was performed using Ficoll-Hypaque density gradient
centrifugation following the manufacturer’s protocol (Human lymphocyte separation medium from Chinese Academy of Medical Sciences Institute of Biomedical Engineering, China). CD4\(^+\)T cells were isolated from total PBMCs by negative selection using LD column (Miltenyi Biotec, Germany). Purified CD4\(^+\)T cells incubated with anti-human CD25 magnetic beads (Miltenyi Biotec, Germany), and separated into CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) fractions by positive selection using MS column (Miltenyi Biotec, Germany). The positively selected CD25\(^+\) cell fractions were separated again over an MS column to achieve higher purities. As assessed by using fluorescence-activated cell sorting (FACS) (Becton-Dickinson, Oxnard, NJ), purities of CD4\(^+\)CD25\(^+\)T cells and CD4\(^+\)CD25\(^-\)T cells were >93% and >99%, respectively. To further confirm the identity of these freshly Tregs, the primers (5’-ACA CCA CCC ACC GCC ACT-3’ and 5’-TCG GAT GCC ACA GAT GAA GC-3’) were used to measure the expression of Foxp3 mRNA, the intracellular Foxp3 staining was performed with the APC-anti-human Foxp3 staining set (bioscience, USA) according to the manufacturer’s instructions\(^4\).

**Co-culture of T-effector and Tregs**

CD4\(^+\)CD25\(^-\) (Tcon) and CD4\(^+\)CD25\(^+\)T cells (Treg) (10\(^4\) cells/well) were cultured in RPMI medium supplemented with 10% fetal calf serum at different Tcon: Treg ratios (0:1, 1:0, 1:1, 2:1, 4:1 and 8:1) in the presence of anti-CD3 mAbs (50 ng/ml) in 96-well plates (Nun, Germany). All cells were cultured in a final volume of 200 µL in the presence of irradiated 10\(^4\) T cell–depleted accessory cells (APC) per well. After 48 hours, \([^3H]\) thymidine (1 µCu/well) was added for 16 hours before proliferation was assayed by scintillation counting (MicroBeta1450 Liquid Scintillation Counter, Perkin Elmer, USA). Percent inhibition of proliferation was determined from the following formula: (1–\(^{[^3]H}\)thymidine uptake (cap) of co-culture of Treg with Tcon divided by cpm of Tcon alone)\(\times\)100%. Triplicate wells were used in all suppression experiments\(^5\).

**Flow cytometry for detection of HLA DR, CD86, CD80 and VCAM-1**

After the incubation period, HUVECs from different groups were incubated with human leukocyte antigen DR (HLA DR)-PE antibody solution, CD86-PE antibody solution, CD80-PE antibody solution and VCAM-1-PE antibody solution (eBioscience,
USA) at 4°C for 0.5 h, respectively. And then we washed them two times with PBS which contains 2% bovine serum albumin (BSA) and 0.1% NaN₃. For isotype control, PE-conjugated human anti-IgG₁ antibodies (eBioscience, USA) were used. Cells were re-suspended in 500 μL PBS with 1% paraformaldehyde and immediately processed using FACS LSR II (Becton Dickinson, USA). After correction for unspecific binding (isotype control), the percentage of positive cells were analyzed by the Cell Quest program (Becton Dickinson, USA).

**Enzyme-linked immunosorbent assay (ELISA) for detection of MCP-1, IL-6, IL-10 and TGF-β**

Supernatants from different groups were collected and kept frozen at -80°C until the cytokine levels (MCP-1, IL-6, IL-10 and TGF-β) were determined by ELISA assays according to the manufacturer’s instructions (R&D Systems, USA). The results were compared with a standard curve. Each assay was carried out in triplicate for each sample. Absorbance was measured at 450 nm by means of a spectrophotometer.

**Real-time RT-PCR for detection of VCAM-1, MCP-1, IL-6 and KLF2**

Total RNA from different groups of HUVECs were isolated using Trizol reagent (Invitrogen, USA) according to manufacturer’s instruction, respectively. One microgram of total RNA was reverse transcribed using RNA PCR Kit (Takara Biotechnology, Japan) and the resulting cDNA was used as a PCR template. The mRNA levels were determined by real-time PCR with Applied Biosystems Step One Real-Time PCR System (Applied Biosystem, USA) according to the manufacturer’s instructions. GAPDH was used as endogenous control. Primers for human VCAM-1 (5'-CAT CCA CAA AGC TGC AAG AA-3’ and 5'-CCT GGA TTC CCT TTT CCA GT-3’); MCP-1(5’-CTC ATA GCA GCC ACC TTC ATT C-3’ and 5’-CAA GTC TCC GGA GTT TGG GTT T-3’); IL-6(5’-CAA ATT CGG TAC ATC CTC GAC GAC GGC-3’ and 5’-GGT TCA GGT TGT TTT CTG CCA GTG C-3’); KFL2 (5’-GCA CGCAC ACAG GTGAGAAG-3’ and 5’-ACCAGTCACAGTTTGGGAGGG-3’) and GAPDH(5’-CCA CCC ATG GCA AAT TCC ATG GCA-3’ and 5’-TCT AGA CCG CAG GTC AGG TCC ACC-3’).PCR reaction mixture contained the SYBR Green I (Takara Biotechnology, Japan), cDNA, and the primers. Relative gene expression level (the
amount of target, normalized to endogenous control gene) was calculated using the comparative Ct method formula $2^{-\Delta\Delta Ct}$.

**Electrophoretic mobility shift assay (EMSA) for detection of NF-κB**

For the preparation of nuclear extracts, HUVECs from different groups were gently lysed in a NP-40 containing sucrose buffer while the nuclei remain intact. After a washing step, the nuclei are suspended in a hypotonic "low salt buffer": the nuclei swell. Then a "high salt buffer" is added slowly: the nucleoplasm is extracted into the buffer while the nuclear envelop stays intact and retains the genomic DNA. The extract is separated from the nuclear envelop/DNA by centrifugation. Light Shift Chemiluminescent EMSA kit (Pierce, USA) was used to detect DNA-protein interaction. The sequences of NF-κB consensus oligonucleotides were: forward 5'-AGTTGAGGGGACTTTCCCAGGC-3'; reverse 5'-GCCTGGGAAAGTCCCCTC-AACT-3'. For the EMSA, the binding reactions were performed for 20 minutes in 10mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 50 ng/uL poly (dI-dC), 0.05% NP-40, 2.5% glycerol, biotin 3'-end -labeled double-stranded oligonucleotide, and nuclear protein extract. Samples were electrophoresed on a native polyacrylamide gel and then transferred to a nylon membrane. The biotin end-labeled DNA was detected by chemiluminescence. To check if the observed shifted bands are specific for NF-κB, competition tests are run: additionally to the labeled NF-κB probe a 200-fold excess of non-labeled ("cold") oligonucleotide is added in.

**Transwell experiments**

Transwell (0.4 um pore size, Corning, Acton, MA) experiments were performed by culturing HUVECs (1×10⁶ cells/well) in the lower well and Tregs (5×10⁵ cells/well) with anti-CD3 mAbs in the inserts. After 48 hours of co-culture, the top compartments (inserts) were removed, and ox-LDL (40 ug/ml) was added for 24h. After the incubation period, HUVECs were collected for further experiments.

**Fixation of Tregs**

For fixation experiments Tregs were isolated and divided into four fractions. One part was activated with anti-CD3 mAbs for 48h and fixed with 2% paraformaldehyde for 1
h at 4°C (activated-fixed Tregs); the second part was fixed with 2% paraformaldehyde for 1 h at 4°C without activation by ox-LDL (resting-fixed Tregs); the third part was stimulated with anti-CD3 mAbs for 48h (viable Tregs); and the last part was left untreated (resting Tregs). Thereafter, fixated cells were washed extensively and used in regulation assays together with the untreated fraction. This procedure prevents any cytokines production from Tregs yet maintains their cell contact dependent suppressive functions.  

References:
Supplementary Information: legend for figures I

Figure I Tregs down-regulate mRNA expression of VCAM-1, MCP-1 and IL-6 in HUVECs impaired by ox-LDL/LPS.

Co-culture system was set up as described in Figure legend 2. Total RNA from HUVECs were collected and VCAM-1, MCP-1, IL-6 mRNA were analyzed by Real-Time PCR. GAPDH was used as endogenous control (A). To explore whether there was a threshold effect for Tregs mediated expression of VCAM-1, MCP-1 and IL-6 in mRNA levels in HUVECs, HUVECs (1×10^6 cells/well) were cultured without or with various numbers of anti-CD3 mAbs activated Tregs (1×10^5, 2.5×10^5, 5×10^5 cells/well), then with or without(control) ox-LDL for an additional 24h, VCAM-1, MCP-1 and IL-6 mRNA in HUVECs were measured (B). Data are presented as mean±SEM of triplicate wells, and are representative of at least three independent experiments. (* is indicated for V.S. control; # is indicated for V.S. no T; + is indicated for CD25+ V.S.CD25-.*: p<0.05, **: p<0.01, ***: p<0.001; #: p<0.05, ##: p<0.01, ###: p<0.001; +: P<0.05, ++: P<0.01, +++: P<0.001)