CD4⁺CD25⁺Foxp3⁺Regulatory T Cells Protect Endothelial Function Impaired by Oxidized Low Density Lipoprotein via the KLF-2 Transcription Factor

Ming Li^{1*}, Xiang Wang^{1*}, Wenbo Fu^{3*}, Shaolin He¹, Dazhu Li^{1†} and Qinmei Ke^{2†}

¹Department of Cardiology, Institute of Cardiovascular Diseases, ²Department of Geriatrics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, ³Department of Cardiology, Wuhan General Hospital of PLA, Wuhan, *M.L, X.W. and W.B.F. contributed equally to this work, [†]D.Z.L and Q.M.K. are joint corresponding authors

Key Words

CD4⁺CD25⁺Regulatory T cells • Human umbilical vein endothelial cells • Oxidized Low Density Lipoprotein • Krüppel-Like Factor-2

Abstract

Objective: To investigate the regulation of CD4⁺CD25⁺Regulatory T cells (Tregs) on pro-inflammatory adhesion molecules, Krüppel-Like Factor-2 (KLF-2) and its downstream transcriptional targets in human umbilical vein endothelial cells (HUVECs) impaired by ox-LDL and the mechanisms of it. **Methods and results:** HUVECs were cultured in the continuous presence of ox-LDL (0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L) for 4, 6, 12 and 24 hours to allow identification of early- and late-induced genes, respectively, whereas non-stimulated controls were taken at 0 hours. The expression of pro-inflammatory adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, KLF-2 and its target genes eNOS, PAI-1 were determined by real time RT-PCR and/or western-blot analysis. Expression of pro-inflammatory adhesion

molecules, KLF-2, eNOS and PAI-1 in HUVEC cultured alone or with anti-CD3 mAbs activated Tregs, followed by addition of ox-LDL (50 mg/L) for 6 hours, are compared to expression levels in control cultures. Ox-LDL treated HUVECs increased pro-inflammatory adhesion molecules expression, as well as increased PAI-1 but decreased eNOS expression accompanied with significant downregulating of KLF-2 at a dose and time dependent manner. Furthermore, ox-LDL increased pro-inflammatory adhesion molecules but inhibited KLF2 expression was reversed by addition of Tregs. Small interfering RNA reduced endogenous KLF-2 expression and partly reversed the suppressive effect of Tregs on HUVECs activation, which strongly implicate KLF-2 as a transcriptional regulator of the Tregs-mediated effects in endothelial cells. Mechanism studies reveal that Treg-mediated KLF2 expression in HUVECs impaired by ox-LDL requires cell contact as well as soluble factors. **Conclusions:** Tregs could protect endothelial function that is largely dependent on KLF2 and its downstream transcriptional targets regulation involving cell-to-cell contact and soluble factors.

Copyright © 2011 S. Karger AG, Basel

Introduction

Recent studies from several groups suggest that subtypes of T cells, called Tregs, previously shown to maintain immunological tolerance, inhibit the development and progression of atherosclerosis [1-4]. Interestingly, the suppressive effects of these cells are not restricted to the adaptive immune system including CD4⁺T cells, CD8⁺T cells, natural killer (NK) cells, or B cells, but can also affect the activation and function of innate immune cells (monocytes, macrophages, dendritic cells) and neutrophils [5], but their effects on ECs are less well known.

EC activation with the expression of cell adhesion molecules on the cell surface is considered to be an early event which subsequently leads to EC dysfunction and ultimately to vascular injury, key events associated with acute and chronic inflammation, including sepsis, atherosclerosis and acute vascular and chronic allograft rejection [6]. So identification and characterization of Tregs regulating the ECs activation, inflammation and dysfunction could be crucial in deciphering the effects and mechanisms of Tregs in atherosclerosis.

Considering the role of the endothelium in the initiation and propagation of vascular wall injury, there is a need for the discovery of molecular targets to serve as inhibitors of EC activation, dysfunction and vascular injury [7]. In recent years, Kruppel-like factor 2 (KLF-2) is emerging as a master regulator of endothelial quiescence, anti-inflammatory, anti-thrombotic properties, and vascular tone by activating atheroprotective and inhibiting atherogenic transcription [8]. Expression of KLF-2 in endothelial cells can be suppressed by proinflammatory cytokines such as TNF- α , interleukin (IL)-1 β , lipopolysaccharide (LPS) and thrombin, which are important in the pathogenesis of atherosclerosis. Contrary to the down-regulation of KLF-2 by the above cytokines, expression of KLF-2 in endothelial cells is highly promoted by laminar flow shear stress or 3-hydroxy-3-methylglutaryl coenzyme A inhibitors (statins) [9].

In previous studies, we investigated the role of CD4⁺CD25⁺Foxp3⁺ T regulatory cells (Tregs) in protecting the activation and function of human umbilical vein endothelial cells (HUVECs) induced by pro-inflammatory stimulus ox-LDL, which has come to be viewed as a core, central element in the pathogenesis of atherosclerosis. Of particular interest, we have shown that Tregs could strongly up-regulate the expression of anti-inflammatory factor KLF2 at mRNA level in ECs activated with ox-LDL [10], suggesting KLF2 maybe a

new “molecular switch” for Tregs to regulate EC phenotype and functions. In this study, we further explored whether KLF2, a biomechanically activated gene recently identified as part of the endothelial “atheroprotective phenotype,” and its downstream transcriptional targets is regulated by Tregs.

Materials and 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 M199 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 [11]. 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 μ m 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 $\pm$ 2.5 nmol of malondialdehyde equivalents/mg protein, mean $\pm$ S.D. n=5), and relative electrophoretic mobility was 4.0 $\pm$ 0.3 (mean $\pm$ S.D. n=5) [12, 13].

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 >99% and >93%, respectively. To further confirm the identity of these Tregs activated by anti-CD3 mAbs (50ng/ml, eBioscience, USA), the primers (5'-ACA CCA CCC ACC ACC GCC ACT-3' and 5'-TCG GAT GAT GCC ACA GAT GAA GC-3') were used to measure the expression of Foxp3 mRNA, which were highly expressed in CD4⁺CD25⁺ T cells but not in CD4⁺CD25⁻ T cells in our work; The intracellular Foxp3 staining was performed with the APC-anti-human Foxp3 staining set (eBioscience USA) according to the manufacturer's instructions [14].

Co-culture of Tregs and T-effector

CD4⁺CD25⁻ (Tcon) and CD4⁺CD25⁺ T cells (Treg) (10⁴ 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 (50ng/ml) in 96-well plates (Nunc Germany). All cells were cultured in a final volume of 200 μ L in the presence of irradiated 10⁴ T cell-depleted accessory cells (APC) per well. After 48 hours, [³H] thymidine (1 μ Ci/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- [³H]-thymidine uptake (cpm) of co-culture of Treg with Tcon divided by cpm of Tcon alone) $\times$ 100%. Triplicate wells were used in all suppression experiments [15].

Co-culture experiments

HUVECs which have grown to 90% confluence on 6-well plates (1 $\times$ 10⁶ cells/well), were washed three times with PBS and cultured in serum-free medium for 24h to get synchronization. Then non-adherent cells were removed and culture medium was changed. First, HUVECs were cultured with different concentrations of ox-LDL (0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L). And then, HUVECs were cultured with ox-LDL (50 mg/L) for 0h, 3h, 6h, 12h, 24h. Last, HUVECs were cultured without T cells (ox-LDL) or with Tregs (CD25⁺) for 48h in the presence of anti-CD3 mAbs (50 ng/ml), and then with ox-LDL (50 mg/L) for an additional 6h, while as a control group (control), HUVECs were cultured without anything but serum-free MI99 medium for 6h. To explore whether there was a threshold effect for Tregs on the expression of KLF-2, eNOS and PAI-1 in mRNA levels in HUVECs, HUVECs (1 $\times$ 10⁶ cells/well) were cultured without or with various concentration of anti-CD3 mAbs pre-treated Tregs (1 $\times$ 10⁵, 2.5 $\times$ 10⁵, 5 $\times$ 10⁵ cells/well, respectively) in the presence of ox-LDL for 6h. After the incubation period, floating T cells were aspirated, HUVECs were harvested for further experiments.

Real-time RT-PCR for detection of Pro-inflammatory adhesion molecules, KLF-2, eNOS and PAI-1

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'); ICAM-1 (5'-GAG ATC ACC ATG GAG CCA AT-3' and 5'-GGG CCT CAC ACT TCA CTG TC-3'); E-selectin (5'-GGACAC AGC AAA TCC CAG TT-3' and 5'-CAC ACC TCA CCA AAC CCT TC-3'); KLF-2 (5'-GCA CGC ACACAG GTG AGA AG-3' and 5'-ACC AGT CAC AGT TTG GGA GGG-3'); eNOS (5'-CCA GCT AGC CAA AGT CAC CAT C-3' and 5'-GTC TCG GAG CCA TAC AGG ATT-3'); PAI-1 (5'-GGC TGA CTT CAC GAG TCT TTCA-3' and 5'-TTC ACT TTC TGC AGC GCC T-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 $2^{-\Delta\Delta C_t}$.

Western blot for detection of KLF-2, eNOS and PAI-1

After incubation with oxLDL, the serum containing floating T cells were discarded. The remnant HUVECs were washed with pre-cooled PBS, centrifuged at 4°C 3000 rpm for 5 min, lysed in ice-cold buffer containing 10mM Hepes (pH 7.4), 10mM KCl, 1.5mM MgCl₂, 0.1mM sodium EDTA, 0.1mM sodium EGTA, 1.0mM DTT, 1.0mM PMSF, and 1 mg/ml protease inhibitor aprotinin for 30 min and centrifuged at 4°C 2000 rpm for 10 min. The protein concentration in cellular supernatants was determined by the Lowry assay. Equal amounts of protein (20 μ g) were separated on 9% SDS-polyacrylamide (SDS-PAGE) gels and electrophoretically transferred to nitrocellulose (NC) membrane and blocked 2 hours (5% 'degrease milk powder' in Tween /PBS buffer; vacillating bed; and room temperature), and after incubation with first and second antibodies, were detected by ECL (Pierce Biotech). Bands were normalized to β -actin and expressed as a percent of control. Antibodies recognizing KLF-2, eNOS, and PAI-1 were from Abcam Cambridge, BD Biosciences, Santa Cruz Biotechnology, respectively.

siRNA experiments

HUVEC were transfected with two KLF2 siRNAs synthesized by Qiagen (Valencia, CA). The sense and antisense strands of human KLF2 siRNA were: (a) 5'-CCAAGA GUU CGC ATC UGA ATT-3' (sense) and 5'-UUC AGA UGC GAA CUC UUG GTG-3' (anti-sense) and (b) 5'-GCG GCA AGA CCU ACA CCA ATT-3' (sense) and 5'-UUG GUG UAG GUC UUG CCG CAG-3' (antisense). Negative control siRNA were 5'-UUC UCC GAA CGU GUC ACG UTT-3' (sense) and 5'-ACG UGA CAC GUU CGG AGA ATT-3' (antisense). HUVEC were plated 16 h prior to transfection at a density of 30000 cells/cm² in HUVEC growth medium without antibiotics. At the time of transfection with siRNA, the cells were 85-95% confluent. Individual siRNAs (KLF2 siRNA a and b, 0.6 nmol each, or negative control siRNA,

Fig. 1. Effects of Tregs on the expression of CAMs of HUVECs. HUVECs were cultured without T cells (ox-LDL) or with Tregs (CD25⁺) for 48h in the presence of anti-CD3 mAbs (50 ng/ml), and then with ox-LDL (50 mg/L) for an additional 6h, while as a control group (control), HUVECs were cultured without anything but serum-free M199 medium for 6h. Total RNA from HUVECs were collected and VCAM-1, ICAM-1 and E-selectin mRNA were analyzed by Real-Time PCR. GAPDH was used as endogenous control. Data are presented as mean±SEM of triplicate wells, and are representative of at least three independent experiments. (* is indicated for V.S. control group; # is indicated for CD25⁺ group V.S. ox-LDL group. *: p<0.05, **: p<0.01, ***: p<0.001; #: p<0.05, ##: p<0.01, ###: p<0.001).

Fig. 2. ox-LDL modulate the expression of KLF2, eNOS and PAI-1 in HUVECs at a time dependent manner. HUVECs were cultured with ox-LDL (50 mg/L) for 0h, 3h, 6h, 12h, 24h. A: Total RNA from HUVECs were collected and KLF2, eNOS, PAI-1 mRNA were analyzed by Real-Time PCR. GAPDH was used as endogenous control. Data are presented as mean±SEM of triplicate wells, and are representative of at least three independent experiments. (*and§ is indicated for V.S. 0 hour group. *: p<0.05, **: p<0.01, ***: p<0.001; §: p>0.05) B: KLF2, eNOS and PAI-1 protein level were analyzed by Western-blotting. These blots are representative of n=3 independent experiments. Quantitative data for the Western blot studies is shown graphically. (*and§ is indicated for V.S. 0 hour group. *: p<0.05, **: p<0.01, ***: p<0.001; §: p>0.05).

1.2 nmol) were mixed with Oligofectamine (8 ul) and Opti-MEM (Invitrogen) in a final volume of 200 ul and incubated at room temperature for 15 min for the lipid-siRNA complex to form. Cells were washed once, and 800 ul of Opti-MEM was added. 200 ul of the Oligofectamine-siRNA complex solution was then added to the cells. The cells were then incubated for 6 h at 37°C. HUVEC growth medium was then added, and cells were incubated for an additional 18 h. Tregs and or ox-LDL were added as described above, and total RNA was isolated 6 h later.

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 (with or without HUVECs). After 48 hours of co-culture, the top compartments (inserts) were removed, and the HUVECs in the lower well were stimulated with ox-LDL (40 ug/ml) for 24h. After the incubation period, HUVECs were collected for further experiments.

Data analysis

All data are expressed as means of duplicate samples from three to five 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. A value of P<0.05 was considered significant. All statistical analyses were performed with SPSS software (version 11.0, SPSS Inc., Chicago, USA).

Results

Isolation of CD4⁺CD25⁺ Foxp3⁺ T cells

We sorted CD4⁺CD25⁺ T cells from PBMCs from normal volunteers. Consistent with our previous studies [15], the purity of CD4⁺CD25⁺ T cells was > 93%, the vast majority of sorted CD4⁺CD25⁺ T cells activated by anti-CD3 mAbs were Foxp3⁺ and they may also displayed functional Treg cells characteristics.

Effects of Tregs on the expression of pro-inflammatory adhesion molecules in HUVECs impaired by ox-LDL

The activation of endothelial cells (ECs), characterized by induced expression of pro-inflammatory

Fig. 3. ox-LDL modulate the expression of KLF2, eNOS and PAI-1 in HUVECs at a dose dependent manner. HUVECs were cultured with different concentrations of ox-LDL (0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L). A: Total RNA from HUVECs were collected and KLF2, eNOS, PAI-1 mRNA were analyzed by Real-Time PCR. GAPDH was used as endogenous control. Data are presented as mean $\pm$ SEM of triplicate wells, and are representative of at least three independent experiments. (* is indicated for V.S. 0 mg/L group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). B: KLF2, eNOS and PAI-1 protein level were analyzed by Western-blotting. These blots are representative of $n = 3$ independent experiments. Quantitative data for the Western blot studies is shown graphically. (* is indicated for V.S. 0 mg/L group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, is an early step of atherosclerosis and often evoked by various atherogenic risk factors, including increased level of inflammatory cytokines, dyslipidemia, hyperinsulinemia, and insulin-resistant (type 2) diabetes [16]. These proteins mediate early leukocyte attachment and rolling on the endothelial surface. Subsequent events such as firm adhesion and transmigration across the endothelial lining then set the stage for a developing inflammatory response within tissues [16]. Given the importance of these adhesion molecules in inflammation, we investigated the effects of Tregs on the expression of adhesion molecules in HUVECs impaired by ox-LDL. As shown in Fig. 1, HUVECs exposed to ox-LDL have a significant increased expression of VCAM-1, ICAM-1 and E-selectin (all $P < 0.001$) compared with that from control group; Tregs treated HUVECs significantly decreased VCAM-1 ($P < 0.01$), ICAM-1 ($P < 0.05$) and E-selectin ($P < 0.001$) expression compared with that from ox-LDL group. These results imply that Tregs may exert their protective effects against activation of HUVECs impaired by ox-LDL in part through decreasing the expression of pro-inflammatory adhesion molecules.

Ox-LDL mediated inhibition of KLF-2, eNOS expression but promotion of PAI-1 expression in HUVECs

Previous research has shown that KLF-2 inhibits endothelial activation by many proinflammatory stimuli,

including IL-1 β , TNF- α , lipopolysaccharide (LPS), and thrombin. But whether KLF-2 can be regulated by ox-LDL is not clear. Here, we investigated the influence of ox-LDL at different concentration on KLF-2 and its direct target gene eNOS and PAI-1 mRNA and protein levels in control cells for various time periods. As shown in Fig. 2, the time course of KLF2 and eNOS suppression by ox-LDL at a dose of 50 mg/L was assessed by measuring KLF2 levels in HUVEC at different time points. The first detectable decrease of KLF2 and eNOS was observed at 3 h, and they got the lowest levels at 6 h, and then KLF-2 and eNOS levels start to rebound and last had no different past 24 h when compared with that in HUVECs cultured alone. These data indicate that KLF2 and eNOS levels reached through at 6 h in response to ox-LDL treatment and returned to baseline level past 24 h, suggesting the changes in KLF2 and eNOS expression is a rapid transient response, while the levels of PAI-1 in the parallel experiments started to rise at 3h, and it peaked at 6h when compared with that in HUVECs cultured alone.

To further investigate the effects of ox-LDL on KLF-2 expression, we cultured HUVECs with ox-LDL at different concentration. As shown in Fig. 3, the expression of KLF-2 and eNOS were strongly decreased when HUVECs exposed to ox-LDL at the concentration of 50 mg/L, while the levels of PAI-1 were strongly increased in the parallel experiments. These data suggested that incubation of HUVECs with ox-LDL

Fig. 4. Tregs induces resistance to suppression of KLF2, eNOS but promotion of PAI-1 in HUVECs stimulated by ox-LDL. Co-culture system was set up as described in Fig. legend 1. A: Total RNA from HUVECs were collected and KLF2, eNOS, PAI-1 mRNA were analyzed by Real-Time PCR. GAPDH was used as endogenous control. Data are presented as mean±SEM of triplicate wells, and are representative of at least three independent experiments. (* is indicated for V.S. 0 mg/L group; # is indicated for CD25+ group V.S. ox-LDL group. *: p<0.05, **: p<0.01, ***: p<0.001; #:p<0.05, ##: p<0.01, ###: p<0.001). B: KLF2, eNOS and PAI-1 protein level were analyzed by Western-blotting. These blots are representative of n=3 independent experiments. Quantitative data for the Western blot studies is shown graphically. (* is indicated for V.S. 0 mg/L group. *: p<0.05, **: p<0.01, ***: p<0.001) C: HUVECs (1×10⁶ cells/well) were cultured without or with various concentration of anti-CD3 mAbs pre-treated Tregs (1×10⁵, 2.5×10⁵, 5×10⁵ cells/well, respectively) in the presence of ox-LDL for 6 h, then HUVECs KLF-2 and its target gene expression were measured by Real-Time PCR. GAPDH was used as endogenous control. Data are presented as mean±SEM of triplicate wells, and are representative of at least three independent experiments. (* is indicated for V.S. control group; # is indicated for CD25+ group V.S. ox-LDL group. *: p<0.05, **: p<0.01, ***: p<0.001; #:p<0.05, ##: p<0.01, ###: p<0.001). D: To further demonstrate the direct up-regulation of KLF2 by Tregs in HUVECs impaired by ox-LDL, KLF2 knockdown experiments were performed. HUVECs were transfected with KLF-2 siRNA or control siRNA before treatment with ox-LDL and/or anti-CD3 mAbs activated Tregs, while as a control group, HUVECs were cultured without anything but serum-free MI99 medium. Gene expressions were determined by RT-PCR. (* is indicated for HUVECs + ox-LDL + KLF-2 siRNA V.S. HUVECs + control siRNA; # is indicated for HUVECs + ox-LDL + KLF-2 siRNA + Tregs V.S. HUVECs + ox-LDL + control siRNA + Tregs. *: p<0.05, **: p<0.01, ***: p<0.001; #:p<0.05, ##: p<0.01, ###: p<0.001).

resulted in a dose-related regulation of KLF-2.

Tregs induces resistance to suppression of KLF-2, eNOS but promotion of PAI-1 in HUVECs stimulated by ox-LDL

We have recently shown that Tregs may exert their protective effects against atherogenesis in part through inducing an immune-inhibitory phenotype of ECs. Here, we study whether Tregs could regulate the expression of KLF-2 and its target genes like eNOS or PAI-1 in HUVECs. HUVECs were cultured without T cells (ox-LDL) or with Tregs (CD25⁺) for 48h in the presence of

anti-CD3 mAbs (50 ng/ml), and then with ox-LDL (50 mg/L) for an additional 6h, while as a control group (control), HUVECs were cultured without anything but serum-free MI99 medium for 6h. The mRNA levels of KLF-2, eNOS and PAI-1 of the HUVECs were determined by Real Time PCR. HUVECs exposed to ox-LDL have a significant decreased expression of KLF-2 and eNOS but increased expression of PAI-1 compared with that in control system. Importantly, the expression of KLF-2, eNOS and PAI-1 were significantly reversed in the Tregs treated HUVECs (Fig. 4 A).

Fig. 5. Tregs mediated various expression of KLF-2, eNOS and PAI-1 in HUVECs requires cell contact as well as soluble factors. HUVECs were cultured without T cells (ox-LDL), with Tregs in the presence of anti-CD3 mAbs in either a co-culture (CC) or a transwell system (TW). After 6 h of culture, the inserts were removed, and HUVECs in the lower well were stimulated with ox-LDL, while as a control group (control), HUVECs were cultured without anything but serum-free M199 medium. Total RNA from HUVECs were collected and KLF2, eNOS, PAI-1 mRNA were analyzed by Real-Time PCR. GAPDH was used as endogenous control. Data are presented as mean±SEM of triplicate wells, and are representative of at least three independent experiments. (* is indicated for Treg group V.S. CC group; # is indicated for TW group V.S. TW + HUVECs group. *: p<0.05, **: p<0.01, ***: p<0.001; #: p<0.05, ##: p<0.01, ###: p<0.001).

The protein levels of the KLF-2, eNOS and PAI-1 were also determined by Western Blot analysis. As shown in Fig. 3 B, coincidence with the mRNA activity data above, KLF-2 and eNOS protein expression were suppressed in ox-LDL system, but were reversed in CD25⁺ system relative to control system, while the PAI-1 protein expression has opposite phenomena, respectively (Fig. 4 B).

To explore whether there was a threshold effect for Tregs mediated various expression of KLF-2, eNOS and PAI-1 in mRNA levels in HUVECs, HUVECs (1×10^6 cells/well) were cultured without or with various concentration of anti-CD3 mAbs pre-treated Tregs (1×10^5 , 2.5×10^5 , 5×10^5 cells/well, respectively) in the presence of ox-LDL for 6 h, then HUVECs KLF-2 and its target gene expression were measured. A dose-dependent effect on HUVECs KLF-2 and its target gene expression was noted in HUVECs (Fig. 4C).

We further performed stable siRNA-mediated knockdown of KLF2 to demonstrate the direct up-regulation of KLF2 by Tregs in HUVECs. Tregs-induced KLF-2 mRNA levels were significantly reduced by the KLF-2 siRNA by about 77.5% as compared to mRNA levels in cells transduced with control siRNA. Moreover, The effects of tregs on inducing resistance to suppression of eNOS but promotion of PAI-1 in HUVECs impaired by ox-LDL were significantly inhibited by knocking down KLF-2 mRNA (all $P < 0.001$, Fig. 4D).

Tregs regulating the expression of KLF-2, eNOS and PAI-1 in HUVECs requires cell contact as well as soluble factors

To investigate whether modulation of KLF-2 and its target gene expression in HUVECs by Tregs was

depended on cell contact or soluble factors, HUVECs were cultured without T cells (ox-LDL), with Tregs in the presence of anti-CD3 mAbs in either a co-culture (CC) or a transwell system (TW). After 6 h of culture, the inserts were removed, and HUVECs in the lower well were stimulated with ox-LDL, while as a control group (control), HUVECs were cultured without anything but serum-free M199 medium. By disrupting physical contact between HUVECs and Tregs (TW) the increasing of KLF-2 and eNOS and suppression of PAI-1 was significantly reversed compared with the co-culture system (Fig. 5). This partial reversal could be due to the requirement of cell contact between Tregs and HUVECs, or due to insufficient co-stimulation of Tregs in the absence of HUVECs. We therefore restored cell contact in the upper well by adding HUVEC to the Treg population in the insert (TW + HUVECs). Interestingly, under these conditions modulation of KLF-2 and its target gene expression was completely restored to the levels seen in co-cultures. This finding indicates that upon HUVECs-Treg interaction soluble factors are produced that reduce the ability of HUVECs in the lower well to respond to ox-LDL, even once the inserts containing the Tregs are removed.

Discussion

Atherosclerosis starts with an innate immune response involving the recruitment and activation of monocytes/macrophages that respond to an excessive accumulation of modified lipids within the arterial wall, followed by an adaptive immune response involving antigen-specific T lymphocytes [16, 17]. The historical

focus of immunological 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 the atherogenic initiation, changing their quiescence into activated phenotypes to support every phase of the inflammatory process [18].

Although the Regulatory T-cell immunity and its relevance to atherosclerosis is now clearly established, its role in vascular inflammation at the endothelial level remains to be elucidated. In the present study, we have examined the interactions of Tregs with ox-LDL impaired HUVECs and found a critical role of Tregs in exerting endothelial atheroprotective effects via the KLF2 transcription Factor.

Kruppel-like factors (KLFs) are a subclass of the zincfinger family of transcription factors characterized by the DNA binding domain containing the conserved sequence CX₂CX₃FX₅LX₂HX₃H (X is any amino acid; underlined cysteine and histidine residues coordinate zinc) [19]. KLFs typically bind to GC-rich or CACCC sequences in the promoter region of target genes to regulate transcriptional activity. Most information on the function of KLFs in endothelial cell biology and pathobiology has come from studies of KLF2. With respect to the vasculature, KLF2 is expressed exclusively in the endothelium. KLF-2 inhibits endothelial activation by many proinflammatory stimuli, including IL-1 β , TNF- α , lipopolysaccharide (LPS), and thrombin, which are important in the pathogenesis of atherosclerosis [9]. Overexpression of KLF2 in endothelial cells induces anti-thrombotic factors like eNOS, which inhibit blood coagulation and platelet aggregation. In contrast, KLF2 inhibits expression of a key regulator of the plasminogen activator system (PA) system named PAI-1, which controls the formation and activity of plasmin modulating thrombosis [20]. Oxidative stress, especially ox-LDL has been shown to contribute to endothelial dysfunction by inhibiting eNOS and increasing PAI-1 [21, 22]. In this study we show that in Fig. 1-4, ox-LDL treated HUVECs decreased eNOS but increased PAI-1 expression accompanied with significant downregulating of KLF-2 at a dose and time dependent manner, which suggesting that ox-LDL may exert its pro-thrombotic function in part through inhibiting KLF-2 expression. Studies by Huddleson JP et al. reveal that TNF- α inhibits KLF-2 via NF- κ B and HDAC4, which cooperatively inhibit MEF-2, an essential transcriptional activator of KLF-2 [23]. Whether similar mechanisms are operative in ox-LDL-mediated suppression of KLF2 in HUVECs

need more experiments.

Contrary to the suppression of KLF2 by proinflammatory cytokines, expression of KLF2 in endothelial cells is highly upregulated by prolonged laminarflow shear stress and statins. Consequences of flow-induced KLF2 expression in endothelial cells include activated expression of eNOS and repressed expression of angiotensin converting enzyme, endothelin-1, and adrenomedullin, all of which are involved in the control of vascular tone in response to flow [9]. KLF2 therefore has flow-dependent, atheroprotective functions in endothelial cells. The atheroprotective activity of KLF2 was demonstrated by its induction in response to statins [24, 25], which protect against atherosclerosis [26-28]. Importantly, upregulation of KLF2 is required for many of the transcriptional effects of statins in endothelial cells, thus KLF2 might mediate their atheroprotective effects [24]. Consistent with these findings, importantly, here we demonstrated that the mRNA and protein levels of KLF2 in human umbilical vein endothelial cells activated with ox-LDL increased in the presence of tregs. Ox-LDL inhibited KLF2 expression was reversed by addition of tregs. Furthermore, Treg-mediated KLF2 expression was followed by the regulation of several of its downstream transcriptional targets including increase eNOS and decrease PAI-1. Most importantly, suppressive effect of Tregs on HUVECs activated by ox-LDL was significantly inhibited by knocking down KLF-2 mRNA (Fig. 4D). Together, these observations support a critical role for KLF2 as a "molecular switch," regulating many of the known endothelial atheroprotective transcriptional responses characterized to date for tregs.

We have recently shown that Tregs may exert their protective effects against atherogenesis in part through inducing an immune-inhibitory phenotype of ECs involving a CTLA-4-dependent cell-to-cell contact manner and also requiring soluble factors, by mainly IL-10 and TGF- β . To further investigate the mechanisms of Tregs-mediated KLF2 expression in HUVECs, transwell experiments were used. By disrupting physical contact between HUVECs and Tregs (TW), the KLF-2 and its target gene were only partly reversed suggesting that cell-to-cell contact was required in Treg-mediated KLF2 expression. Hence, these experiments demonstrated that KLF2 expression induced by Tregs involved direct contact dependent interactions with HUVECs. However, the fact that KLF-2 and eNOS levels in TW group still markedly reduced and PAI-1 markedly increased compared with that in CC group,

indicating that soluble factors may play a role in KLF2 expression mediated by Tregs. This finding suggests that Treg-mediated KLF2 up-regulation in HUVECs may involve a novel pathway that depends on both cell-to-cell contact and soluble factors.

In summary, here we provide evidence for a previously uncharacterized role of human CD4⁺CD25⁺Foxp3⁺Tregs, namely their ability to induce the expression of anti inflammatory factor KLF2 in activated ECs involving cell-to-cell contact and also requiring soluble factors. The identification of KLF2 as a

major target of Tregs, raises the possibility that Tregs could promote a global athero-protective endothelial phenotype that is largely dependent on KLF2 up-regulation.

Acknowledgements

This work was supported by National Natural Science Foundation to Dr. Dazhu Li (NO. 30670855).

References

- Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z: Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med* 2006;12:178-180.
- Yang KP, Li D, Luo M, Hu Y: Generation of HSP60 specific regulatory T cell and effect on atherosclerosis. *Cell Immunol* 2006;243:90-95.
- Lin J, Li M, Wang Z, He S, Ma X, Li D: The role of CD4⁺CD25⁺regulatory T cells in macrophage-derived foam cells formation. *J Lipid Res* 2010;51:1208-1217.
- Mor A, Planer D, Luboshits G, Afek A, Metzger S, Chajek-Shaul T, Keren G, George J: Role of naturally occurring CD4⁺CD25⁺regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007;27:893-900.
- Sakaguchi S, Yamaguchi T, Nomura T, Ono M: Regulatory T cells and immune tolerance. *Cell* 2008;133:775-787.
- Quillard T, Devallière J, Coupel S, Charreau B: Inflammation dysregulates Notch signaling in endothelial cells: implication of Notch2 and Notch4 to endothelial dysfunction. *Biochem Pharmacol* 2010;80:2032-2041.
- Pober JS: Endothelial activation: intracellular signaling pathways. *Arthritis Res* 2002;4:S109-116.
- Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Elderkamp YW, Meijers JC, Voorberg J, Pannekoek H, Horrevoets AJ: KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. *Blood* 2006;107:4354-4363.
- McConnell BB, Yang VW: Mammalian Krüppel-like factors in health and diseases. *Physiol Rev* 2010;90:1337-1381.
- He S, Li M, Ma X, Lin J, Li D: CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells Protect the Proinflammatory Activation of Human Umbilical Vein Endothelial Cells. *Arterioscler Thromb Vasc Biol* 2010;30:2621-2630.
- Wagner AH, Gebauer M, Pollok-Kopp B, Hecker M: Cytokine-inducible CD40 expression in human endothelial cells is mediated by interferon regulatory factor-1. *Blood* 2002;99:520-525.
- Holven KB, Aukrust P, Holm T, Ose L, Nenseter MS: Folic acid treatment reduces chemokine release from peripheral blood mononuclear cells in hyperhomocysteinemic subjects. *Arterioscler Thromb Vasc Biol* 2002;22:699-703.
- Li M, Che J, Li YS, Feng YB, Zeng QT: Folic acid reduces chemokine MCP-1 release and expression in rats with hyperhomocysteinemia. *Cardiovasc Pathol* 2007;16:305-309.
- Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G: Ex vivo isolation and characterization of CD4⁺CD25⁺T cells with regulatory properties from human blood. *J Exp Med* 2001;193:1303-1310.
- Levings MK, Sangregorio R, Roncarolo MG: Human CD25⁺CD4⁺T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001;193:1295-1302.
- Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;76:301-314.
- Hansson GK: Inflammation, atherosclerosis and coronary artery disease. *N Engl J Med* 2005;352:1685-1695.
- Tedgui A, Mallat Z: Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 2006;86:515-581.
- Pober JS, Sessa WC: Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* 2007;7:803-815.

- 20 Bieker JJ: Isolation, genomic structure and expression of human erythroid Kruppel-like factor (EKLF). *DNA Cell Biol* 1996;15:347-352.
- 21 Lin Z, Kumar A, SenBanerjee S, Staniszewski K, Parmar K, Vaughan DE, Gimbrone MA Jr, Balasubramanian V, Garcia-Cardena G, Jain MK: Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function. *Circ Res* 2005;96:e48-e57.
- 22 Steinberg D, Witztum JL: Is the oxidative modification hypothesis relevant to human atherosclerosis? *Circulation* 2002;105:2107-2111.
- 23 Galle J, Hansen-Hagge T, Wanner C, Seibold S: Impact of oxidized low density lipoprotein on vascular cells. *Atherosclerosis* 2006;185:219-226.
- 24 Huddleson JP, Srinivasan S, Ahmad N, Lingrel JB: Fluid shear stress induces endothelial KLF2 gene expression through a defined promoter region. *Biol Chem* 2004;385:723-729.
- 25 Parmar KM, Nambudiri V, Dai G, Larman HB, Gimbrone MA Jr, Garcia-Cardena G: Statins exert endothelial atheroprotective effects via the KLF2 transcription factor. *J Biol Chem* 2005;280:26714-26719.
- 26 Sen-Banerjee S, Mir S, Lin Z, Hamik A, Atkins GB, Das H, Banerjee P, Kumar A, Jain MK: Kruppel-like factor 2 as a novel mediator of statin effects in endothelial cells. *Circulation* 2005;112:720-726.
- 27 Bellosta S, Ferri N, Bernini F, Paoletti R, Corsini A: Non-lipid-related effects of statins. *Ann Med* 2000;32:164-176.
- 28 Liao JK, Laufs U: Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* 2005;45:89-118.