Oxidized Low-density Lipoprotein-induced Proinflammatory Cytokine Response in Macrophages are Suppressed by CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells Through Downregulating Toll Like Receptor 2-mediated Activation of NF- κ B

Ming Li¹, Jing Lin¹, Zhixiao Wang, Shaolin He, Xuming Ma and Dazhu Li

Department of Cardiology, Institute of Cardiovascular Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, ¹These authors contributed equally to this work

Key Words

CD4⁺CD25⁺Tregs • Macrophage • Proinflammation • TLR • NF- κ B

Abstract

CD4⁺CD25⁺ regulatory T cells (Tregs) exert a suppressive activity on atherosclerosis but the underlying mechanism remains unclear. Here, we investigated whether and how Tregs affect oxLDL-induced proinflammatory response in macrophages. Tregs were isolated by magnetic cell sorting-column and analyzed by flow cytometry. Macrophages were cultured with or without Tregs in the presence of oxLDL for 48 hours to induce proinflammatory response. Our data showed that with oxLDL challenge, the Treg-modulated macrophages have decreased NO production and iNOS expression, decreased HLA-DR and CD86 expression, and down-regulated proinflammatory cytokine/chemokine production. Tregs can inhibit the pro-inflammatory properties of macrophages and steer macrophage differentiation toward an anti-inflammatory cytokine producing phenotype. Mechanistic studies reveal that Treg-mediated suppression of the monocyte response to oxLDL was reflected by a reduction in the up-regulation of NF- κ B activity accompanied by a

decreased expression of TLR2 but not TLR4 at the transcriptional level. These results suggest that CD4⁺CD25⁺Foxp3⁺ regulatory T cells may exert its suppressive functions on pro-inflammatory properties of OxLDL induced-macrophages partly through TLR2–NF- κ B signaling pathway.

Copyright © 2010 S. Karger AG, Basel

Introduction

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 [1-2]. Effector T cells recognize modified auto-antigens such as ox-LDL and heat shock proteins (i.e. HSP-60) that are presented by antigen-presenting cells such as macrophages or dendritic cells [3]. The accumulation of inflammatory cells within the arterial wall leads to local production of chemokines, interleukins and proteases that enhance the influx of monocytes and lymphocytes, thereby promoting the progression of atherosclerotic lesions [2]. The role of the

immune system in atherosclerosis has received considerable interest in recent years; however, sufficient knowledge to justify the immunomodulatory mechanisms has not yet been obtained.

In recent years, a novel subtype of T cell, called the regulatory T cells (Tregs), have been shown to play a critical role in the development of atherosclerosis. Natural Treg cells, characterized by the expression of CD4, CD25 and the transcriptional factor Foxp3 (forkhead-winged-helix transcription factor), have the capacity to the maintenance of immunological tolerance against self and non-self antigens and prevent the development of various immunoinflammatory diseases [4-7]. Several preliminary studies have shown this type of regulatory T cell inhibited the development and progression of atherosclerosis.

Tregs on the adaptive immune system and on CD4⁺ T cells in particular have been well documented [1, 2]. But their effects on innate immune are less well known. The macrophage, one of the principal effector cell of innate immunity, play a critical role in both innate and adaptive immunity through their ability to recognize pathogens and/or "danger signals" via Toll-like receptors (TLRs) and other pattern-recognition receptors; through their effector mechanisms, including phagocytosis, nitric oxide production, and killing of bacteria; and through their ability to produce a wide array of cytokines and chemokines. Recently, a family of TLRs has been identified as a key recognition component of innate-immunity [8, 9]. TLRs are originally identified as receptors that activate host defenses in response to microbial-derived ligands such as Gram-negative bacterial lipopolysaccharide (LPS) [10], they are now increasingly documented to respond to endogenous modified self, such as modified and/or oxidized low-density lipoprotein (OxLDL). There are growing evidences showing the contribution of the TLR-signaling pathway to initiation and progression of atherosclerosis [11, 12]. In the advanced atherosclerotic lesions in human, TLR1, TLR2, and TLR4 were detected in not only infiltrating macrophages but also vascular cells such as endothelial cells and vascular smooth muscle cells, and they might contribute to plaque activation [13, 14].

Because macrophages are essential partners in innate and acquired immunity and as such play a variety of roles in atherosclerotic plaque development and its clinical sequelae [3], so understanding the mechanisms behind the homeostatic control of macrophage function is, therefore, of fundamental importance. Thus, this study was designed to evaluate the effects of Tregs on Ox-LDL-activated macrophages and its possible mechanisms

from TLRs-dependent pathway.

Materials and Methods

Animals

All C57BL/6 background mice were obtained from Peking University. They were housed in the specific pathogen-free conditions in our animal facility and administered food and water ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and is 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 lipidemic 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 5000 rpm/min for 22 hours, using type 50 rotor. They were dialyzed against 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 [15].

Cell isolation and purification

For cells isolation, groups of 8- to 12-week-old male mice were used for all experiments. Murine peritoneal macrophages were harvested after intraperitoneal injection (5 ml per mouse) of phosphate-buffered saline (PBS). After centrifugation at 1000 × g for 5 minutes, the cells were resuspended into complete RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml of penicillin/streptomycin. Then adjusted to the concentration of 1×10⁶/ml and used for further experiments.

Splenocytes were prepared from murine spleens by Ficoll density gradient. CD4⁺ T cells were isolated from total splenocytes by negative selection using LD column (Miltenyi Biotech, Bergisch Gladbach, Germany). Purified CD4⁺ T cells were incubated with anti-mouse CD25 magnetic beads (Miltenyi Biotech), and separated into CD4⁺CD25⁺ and CD4⁺CD25⁻ fractions by positive selection using MS column (Miltenyi Biotech). The positively selected CD25⁺ cell fractions were separated again over an MS column to achieve higher purities. Purities of CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells were >95% and >90%, respectively, as assessed by using fluorescence-activated cell sorting (FACS) (Becton-Dickinson, Oxnard, NJ). To confirm the identity of these Tregs, 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.

Coculture experiments

Murine peritoneal macrophages were seeded in 6-well plates ($3\text{--}4 \times 10^6/\text{well}$), then non-adherent cells were removed and the culture medium were changed first after 8 hours incubation at 37°C . For coculture experiment, peritoneal macrophages were cultured without T cells (no T), with $\text{CD4}^+\text{CD25}^+$ T cells (CD25^+), or $\text{CD4}^+\text{CD25}^-$ T cells (CD25^-) for 40 hours in the presence of anti-CD3 antibody (50 ng/ml, eBioscience, California, USA) [16], then T cells were depleted by using CD2-microbeads (Miltenyi Biotec), and the purified macrophages were stimulated with oxLDL (50 $\mu\text{g}/\text{ml}$). After 48 hours, lipid-loaden macrophages were harvested and supernatants were collected for further experiments.

Phenotypic Analysis

After the incubation period, macrophages from different groups were incubated with human leukocyte antigen DR (HLA DR)-PE antibody solution and CD86-PE antibody solution (eBioscience, USA) at 4°C for 0.5 h, respectively. Then we washed them two times with PBS which contains 2% bovine serum albumin (BSA) and 0.1% NaN_3 . For isotype control, PE-conjugated human anti-IgG₁ antibodies (eBioscience, USA) were used. Cells were resuspended in 500 μL PBS with 1% paraformal-dehyde and immediately processed using a FACS LSR α (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).

Measurement of NO production

The amount of nitrite produced by the mouse macrophage was indicated by the amount of accumulation of NO_2^- that was measured in the above cell culture supernatant from different groups using the Greiss reagent, as described previously [17]. Briefly, equal volumes of culture supernatant and Greiss reagent (100 μL) were mixed for 10 min at room temperature. Absorbance at 540 nm was measured with a Labsystems Multiscan Ascent assay plate reader. A solution of NO_2^- was used to construct a standard curve.

ELISA assay for cytokines

The peritoneal macrophages were collected and coculture experiments were performed as described in the previous section. Supernatants were collected after 48 hours exposure to oxLDL, and kept frozen at -80°C until the cytokine levels (TNF- α , MCP-I, MMP-9, IL-10, and TGF- β) were determined by ELISA assays according to the manufacturer's instructions. Each sample was tested for each cytokine in triplicate.

RT-PCR and real-time PCR

Total RNA from macrophages was isolated using Trizol reagent (Invitrogen) according to manufacturer's instruction. PCR was performed after cDNA synthesis using Platinum PCR SuperMix (Invitrogen, Carlsbad, CA) and primer pairs specific for inducible NO synthase (iNOS) and GAPDH. Real-time PCR was performed on GAPDH, TLR2 and TLR4. Quantitative real-time PCR was carried out with ABI PRISM 7900 Sequence Detector system (Applied Biosystem, Foster City, CA). GAPDH was used as endogenous control. PCR reaction mixture

contained the SYBR Green I (Takara Biotechnology), 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 $2^{-\Delta\Delta\text{Ct}}$. The sequences of primers for PCR were used: GAPDH (5'- CCA TCA CCA TCT TCC AGG AGC AGC GAG-3' and 5'- CAC AGT CTT CTG GGT GGC AGT GAT-3'), iNOS (5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3' and 5'- GGC TGT CAG AGC CTC GTG GCT TTG G-3'), TLR2 (5'- CCAGAC ACT GGG GGT AAC ATC -3' and 5'-CGG ATC GAC TTT AGA CTT TGG G-3'), TLR4 (5'-GCC TTT CAG GGA ATT AAG CTC C-3' and 5'- AGA TCA ACC GAT GGA CGT GGACGT GATA -3').

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed by a nonradioactive method (Pierce, USA) as described previously. After 48 hours incubation with oxLDL, nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, USA) according to the manufacturer's instructions, and EMSA was performed with a LightShift chemiluminescent EMSA kit (Pierce, USA). For EMSA, the binding reactions were performed for 20 minutes in 10 mmol/L Tris-HCl (pH 7.5), 50mmol/L KCl, 5 mmol/L MgCl_2 , 1 mmol/L dithiothreitol, 50 ng/ μL poly (dI-dC), 0.05% NP-40, 2.5% glycerol, biotin 3-end-labeled double-stranded oligonucleotide, and nuclear protein extract (30 $\mu\text{g}/\text{well}$). Samples were electrophoresed on a native polyacrylamide gel and then transferred to a nylon membrane. The biotin end-labeled DNA was detected by chemiluminescence. The double-stranded NF κB oligonucleotide where the 3' end of the probe was labeled with biotin contained: 5'-AGT TGA GGG GAC TTT CCC AGC C-3'.

Statistical analyses

Results are shown as mean $\pm$ SEM of at least three independent experiments. 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 SPSS software (version 11.0, SPSS Inc., Chicago, IL).

Results

Effects of Tregs on the expression of immune phenotypic markers of ox-LDL activated macrophage

Murine peritoneal macrophages co-cultured without T cells (no T), with Tregs (CD25^+), or with $\text{CD4}^+\text{CD25}^+$ T cells (CD25^+) for 48h in the presence of anti-CD3 mAbs, and then with ox-LDL for an additional 24h. The expression of CD86 and HLA DR in macrophages was determined by Flow cytometry. ox-LDL activated macrophages displayed a activating phenotype by high expression of CD86 and HLA DR, Treg treated macrophages (CD25^+) significantly decreased CD86 and

Fig. 1. Effects of Tregs on HLA DR and CD86 expression in ox-LDL activated macrophage. Macrophages co-cultured without T cells (no T), with Tregs (CD25⁺), or CD4⁺CD25⁻ T cells (CD25⁻) for 48h in the presence of anti-CD3 mAbs , and then with ox-LDL for an additional 24h. The expression of HLA DR and CD86 in macrophages was determined by Flow cytometry. Figures are representative of at least three independent experiments.

Fig. 2. Effects of Tregs on oxLDL-induced nitric oxide (NO) production in macrophages. Murine peritoneal macrophages were cultured as described as above. Equal volumes of cell supernatants were mixed with Greiss reagent for 10 min, and the absorbance at 540λ was measured. A solution of NO²⁻ was used to construct a standard curve. The results are reported as means ±SEM of three independent experiments. Data are presented as mean ±SEM of triplicate wells, and are representative of at least three independent experiments. (# is indicated for CD25⁺ vs no T; * is indicated for CD25⁺ vs CD25⁻. ###: p<0.001; **: p<0.01.).

Fig. 3. Effects of Tregs on oxLDL-induced iNOS expression in macrophages. Murine peritoneal macrophages were co-cultured without T cells (no T), with Tregs (CD25⁺), or CD4⁺CD25⁻ T cells (CD25⁻) in the presence of oxLDL(50 μg/ml) for 48 hours. Total RNA was collected and iNOS mRNA were analyzed by RT-PCR. GAPDH served as endogenous control. Figures are representative of at least three independent experiments. (# is indicated for CD25⁺ vs no T; * is indicated for CD25⁺ vs CD25⁻. ###: p<0.001; **: p<0.01.).

HLA DR expression compared with that in no T and in CD25⁺ (Fig. 1).

Tregs Inhibit NO production in oxLDL-stimulated macrophage

We initially examined the inhibitory effects of the Tregs on the production of the inflammatory mediators, NO. Therefore, we examined iNOS production in each of the three oxLDL-exposed macrophage populations. First, we measured the accumulation of NO²⁻ in culture supernatants by the Greiss assay. Tregs strongly inhibited oxLDL-induced NO production (noT: 41.2±5.1 μM CD25⁺: 12.5±7.4 μM and CD25⁻: 39.8±4.6 μM,

respectively.) (Fig. 2).

Tregs reduce iNOS expression in oxLDL-stimulated macrophage

RNA was isolated from the three populations of activated macrophage 4 h after stimulation and analyzed by RT-PCR. We examined iNOS mRNA in each of the three populations. Consistent with the protein activity data above, iNOS levels were reduced in Treg-treated cultures (0.89±0.3) compared with that in no T (3.52±0.3) and CD25⁻ cultures (3.16±0.7) (Fig. 3). This indicates that the reduced expression of iNOS due to Treg treatment was responsible for the inhibition of NO production.

Fig. 4. Tregs Inhibit the Proinflammatory Response of Macrophages to oxLDL. Macrophages (3.4×10^6 /well) were cultured alone (no T), with Tregs (CD25⁺, 5×10^5 /well), or CD4⁺CD25⁻ T cells (CD25⁻, 5×10^5 /well) for 40 hours in the presence of anti-CD3 antibody (50ng/ml), after which T cells were depleted and the different cultures were stimulated for 48 hours with oxLDL (50 μ g/ml). After the incubation period, cytokine/chemokine production was measured by ELISA (TNF- α , MCP-1, MMP-9, IL-10, and TGF- β). The average production of six independent experiments $\pm$ SEM of proinflammatory (A)

and antiinflammatory (B) cytokines/chemokines are shown for the three cultures. (# is indicated for CD25⁺ vs no T; * is indicated for CD25⁺ vs CD25⁻; + is indicated for CD25⁻ vs no T. #p<0.05; ##p<0.01; ###p<0.001; *p<0.05; **p<0.01; ***p<0.001; +p<0.05).

Tregs inhibit the proinflammatory response in oxLDL-induced macrophage

To determine if Tregs modulate the capacity of oxLDL-induced inflammatory response in macrophage, the levels of proinflammatory cytokines (TNF- α , MCP-1, MMP-9, TGF- β , and IL-10) were determined by ELISA assay. Our data show that, after coculture with Tregs, macrophages displayed a decrease in their capacity to produce proinflammatory cytokines/chemokines (TNF- α , MCP-1, and MMP-9) (Fig. 4A). In contrast, the production of the antiinflammatory cytokines TGF- β and IL-10 was enhanced (Fig. 4B) in Treg-treated cultures. As a control, CD4⁺CD25⁺ T-treated cultures displayed an increased production of proinflammatory and a less production of antiinflammatory cytokines/chemokines. Results collectively indicated rather than inhibiting proinflammatory response, Tregs could induce an antiinflammatory response in oxLDL-induced macrophage foam cell formation.

Effects of Tregs on NF- κ B expression in oxLDL-induced macrophage

NF- κ B is a ubiquitous transcription factor that regulates expression of proinflammatory and antiapoptotic genes and is thought to play an important role in driving the inflammatory response [18]. Therefore, we examined NF- κ B activity in macrophages cocultured with or without

Fig. 5. NF- κ B DNA binding activity in oxLDL-induced inflammatory response in macrophages. Coculture was set up as described in Figure legend 1. A, EMSA was performed with nuclear extracts from macrophages in different cultures to examine NF- κ B activity: lane 1, macrophages alone (no T); lane 2, macrophages coculture with Tregs (CD25⁺); lane 3, macrophages coculture with CD4⁺CD25⁻ T cells (CD25⁻); and lane 4, competition with a 100-fold excess of unlabeled probe was performed in macrophages alone cultures. Nuclear protein extracted from CD25⁻ and no T cultures (lane 1 and 3) showed strong binding activity for NF- κ B oligonucleotide probe compared with that from CD25⁺ (lane 2). Binding was specifically inhibited by excess unlabeled NF- κ B oligonucleotide (lane 4). Experiment was done in triplicate. B, DNA binding activity of NF- κ B in different cultures was shown in relative quantitative method. Quantitative analysis of EMSA with NF- κ B consensus oligonucleotide was performed in each animal group. Data were expressed as mean $\pm$ SEM of at least three independent experiments. (# is indicated for CD25⁺ vs no T; * is indicated for CD25⁺ vs CD25⁻; #p<0.05; *p<0.05).

Fig. 6. TLR expression in oxLDL-induced inflammatory response in macrophages. Macrophages were precultured without T cells (no T), with CD4⁺CD25⁻T cells (CD25⁻) or with Tregs (CD25⁺), they were exposed to oxLDL. After 48 hours of incubation, the expression levels of TLR2 and TLR4 were measured by real-time PCR. The mean expression $\pm$ SEM of three independent experiments is shown. (# is indicated for CD25⁺ vs no T; * is indicated for CD25⁺ vs CD25⁻; #: $p < 0.05$; *: $p < 0.05$).

T cells after oxLDL treatment. We performed EMSA with specific oligonucleotide probes for the NF- κ B binding-site regions, as shown in Fig. 5. The reduced proinflammatory response was reflected at the transcriptional level by a clear decreased NF- κ B up-regulation upon oxLDL stimulation from CD4⁺CD25⁺T treated macrophages (CD25⁺) (lane 2). In contrast, control macrophages (no T) or macrophages precultured with CD4⁺CD25⁺ T cells (CD25⁺) displayed an increase in NF- κ B activation upon oxLDL triggering (lane 1 and 3) ($P < 0.05$). The identity of the NF- κ B specific band was confirmed by competition analyses with unlabeled oligonucleotide (lane 4).

Effects of Tregs on TLRs expression in oxLDL-induced macrophage

To explore whether TLRs involved in Treg-modulating macrophages inflammatory response to oxLDL, TLR2 and TLR4 mRNA were measured by real-time PCR. Our data have shown that compared with untreated cultures (no T) and CD4⁺CD25⁺ T-treated cultures (CD25⁺), the expression of TLR4 mRNA was decreased, but without significant difference, indicating that the decreased proinflammatory cytokine response to oxLDL of Treg-modulated macrophages was not explained by a decreased expression of TLR4 after Treg contact. However, TLR2 mRNA expression had a clear decrease in CD4⁺CD25⁺ T-treated cultures (Fig. 6, $P < 0.05$).

Discussion

In the current study, we provide evidence that CD4⁺CD25⁺ regulatory T cells can inhibit CD86 and HLA DR expression and the pro-inflammatory properties of OxLDL induced-macrophages (decreased NO production and iNOS expression, down-regulated proinflammatory cytokine/chemokine production) and steer macrophage

differentiation toward an anti-inflammatory cytokine producing phenotype, this finding indicates that Treg modulating the polarization of macrophage a switch to an M2 phenotype; Moreover, Mechanistic studies reveal that the decreased pro-inflammatory cytokine response to oxLDL of Treg-modulated macrophages was reflected at the transcriptional level by a clear decreased NF- κ B up-regulation and TLR2 expression, indicating that CD4⁺CD25⁺Foxp3⁺ regulatory T cells may exert its suppressive functions on pro-inflammatory properties of OxLDL induced-macrophages partly through TLR2-NF- κ B signaling pathway.

Monocyte and macrophage phenotypic diversity is important in atherogenesis. Macrophages are an extremely heterogeneous lineage displaying a range of both pro- and antiinflammatory functions [19]. Two extremes in the spectrum of macrophage function are represented by the “classically” activated (or M1) and the alternative (or M2) phenotypes [20-23]. The M1 phenotype is typified by IFN- γ -primed macrophages and is characterized by increased MHC class II, IL-12, and inducible nitric oxide synthase (NOS2) expression. M2 macrophages are characterized by decreased expression of NOS2, MHC class II, and IL-12, but elevated expression of antiinflammatory cytokines such as IL-10. The M2 phenotype is promoted by Th2 cytokines such as IL-4 and IL-13. Generally, M2 macrophages are considered antiinflammatory cells, whereas M1 macrophages are proinflammatory [23, 24]. The signaling pathways that regulate these phenotypes in vivo are not well understood. Our data showed that with oxLDL challenge, the Treg-modulated macrophages display features of AAM subsets: These cells have decreased NO production and iNOS expression, decreased HLA-DR and CD86 expression, and down-regulated proinflammatory cytokine/chemokine production. Tregs can inhibit the pro-inflammatory properties of macrophages and steer macrophage differentiation toward an anti-inflammatory cytokine producing

phenotype compared with untreated macrophages, indicating that rather than inducing a general cytokine down-regulation in macrophages, Tregs shift the balance from a proinflammatory toward an antiinflammatory cytokine profile. A paper from M. M. Tiemessen's group reported similar findings in CD4⁺CD25⁺Foxp3⁺ regulatory T cells induce alternative activation of human monocytes/macrophages [16]. Taken together, this emerging evidence suggests that Treg modulating the polarization of macrophage a switch to an M2 phenotype may limit the innate immune response and be required for the resolution of inflammation.

Our study further demonstrated that Treg-mediated suppression of the monocyte response to oxLDL is accompanied by a reduction in the up-regulation of NF- κ B activity. NF- κ B activation is required for proinflammatory cytokine gene expression. The decreased NF- κ B activation may well be due to the increased levels of IL-10 and TGF- β in the Treg-monocyte culture, because it has been shown that both IL-10 and TGF- β can suppress NF- κ B activation [25]. Moreover, IL-10-induced suppression of NF- κ B activation is also found in tumor-associated macrophages, which have an alternatively activated phenotype [26]. Furthermore, blocking NF- κ B activation in classically activated macrophages results in a decreased proinflammatory response and reduced expression of costimulatory markers, whereas the phagocytic capacity remains intact and bacterial killing is enhanced [18]. More recent reports have shown NF- κ B has tissue-specific roles in inflammation; although NF- κ B activation in epithelial cells is required to drive the inflammatory response, NF- κ B activity in macrophages has an opposing role in the resolution of inflammation.

Macrophages are the predominant participants in innate immune responses in atherosclerosis via protein receptors. In particular, the members of the toll-like receptor (TLR) family play a critical role in the inflammatory components of atherosclerosis. So far 11 TLRs have been found in mammals and a great number of exogenous and endogenous ligands has been identified. Their activation by various ligands triggers a signaling cascade leading to initiate the activation of NF- κ B, resulting in the transcription of a variety of genes involved in the inflammatory and proliferative responses of cells critical to atherogenesis [13] and ultimately leading to the

synthesis and release of antimicrobial peptides and inflammatory cytokines that provide a critical link to adaptive immunity [8, 27].

Several reports have documented the expression of TLRs, in particular TLR4, TLR1, TLR2, and to a lesser extent TLR5 in both human plaques and murine models of atherosclerosis [14, 27], where they appear to be mainly localized to macrophages and endothelial cells. In these cells, both TLR2 and TLR4 frequently colocalized with NF- κ B [27], a transcription factor that induces TLR expression but also mediates the downstream signaling of TLR on ligand engagement. Similarly, the presence of NF- κ B p65 was also observed in TLR positive macrophages. This suggests that the TLR-NF- κ B pathway is activated in the lesion. Our data showed that Treg-mediated suppression of the monocyte response to oxLDL was reflected by a reduction in the up-regulation of NF- κ B activity accompanied by a decreased expression of TLR2 but not TLR4 at the transcriptional level, indicating that TLR2-NF- κ B signaling pathway may play critical role in CD4⁺CD25⁺Foxp3⁺ regulatory T cells modulated macrophage functions. This may be partly explained by that the motif pathogen-associated molecular patterns such as lipopolysaccharide (LPS) are recognized by TLR-4 [28], and those such as lipoteichoic acid (LTA), diacylated and triacylated lipopeptides are recognized mainly by TLR-2 [29]. Recent studies have shown that CD16⁺ monocytes express higher levels of TLR-2 and produce larger amounts of TNF α in response to the TLR-2-specific bacterial lipopeptide when compared with CD16 monocytes, suggesting the relevance of TLR-2 to CD16⁺ monocyte activation [30]. Interestingly, the majority of CD14⁺ CD16⁺ monocytes seem anti-inflammatory, as they produce the cytokine IL-10 in response to bacterial LPS. Conversely, the smaller CD14^{low}CD16⁺ population seems proinflammatory because it produces proinflammatory mediators in response to LPS and shows an increase in plasma levels during inflammatory conditions, including atherosclerosis [19].

Acknowledgements

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

References

- 1 Hansson GK: Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685-1695.
- 2 Libby P: Inflammation in atherosclerosis. *Nature* 2002;420:868-874.
- 3 Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993;362:801-809.
- 4 Sakaguchi S: Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345-352.
- 5 Lindley S, Davan CS, Bishop A, Roep BO, Peakman M, Tree TI: Defective suppressor function in CD4+CD25+T-cells from patients with type 1 diabetes. *Diabetes* 2005;54:92-99.
- 6 Cao D, Malmstrom V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C: Isolation and functional characterization of regulatory CD25 bright CD4+T cells from the target organ of patients with rheumatoid arthritis. *Eur J Immunol* 2003;33:215-223.
- 7 Cheng X, Chen Y, Xie JJ, Yao R, Yu X, Liao MY, Ding YJ, Tang TT, Liao YH, Cheng Y: Suppressive oligodeoxynucleotides inhibit atherosclerosis in apoE-/- mice through modulation of Th1/Th2 balance. *J Mol Cell Cardiol* 2008;45:168-175.
- 8 Medzhitov R, Preston-Hurlburt P, Janeway CA Jr: A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394-397.
- 9 Heine H, Lien E: Toll-like receptors and their function in innate and adaptive immunity. *Int Arch Allergy Immunol* 2003;130:180-192.
- 10 Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085-2088.
- 11 Bjorkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, Means T, Halmen K, Luster AD, Golenbock DT, Freeman MW: Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. *Nat Med* 2004;10:416-421.
- 12 Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M: Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci U S A* 2004;101:10679-10684.
- 13 Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ: Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* 2002;105:1158-1161.
- 14 Xu XH, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M: Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 2001;104:3103-3108.
- 15 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.
- 16 Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS: CD4+CD25+Foxp3+ regulation T cells induce alternative activation of human monocytes/macrophages. *Proc Natl Acad Sci U S A* 2007;104:19446-19451.
- 17 Stuehr DJ, Nathan CF: Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 1989;169:1543-1555.
- 18 Groesdonk HV, Schlottmann S, Richter F, Georgieff M, Senftleben U: *Escherichia coli* prevents phagocytosis-induced death of macrophages via classical NF-kappaB signaling, a link to T-cell activation. *Infect Immun* 2006;174:5989-6000.
- 19 Shimada K: Immune System and Atherosclerotic Disease Heterogeneity of Leukocyte Subsets Participating in the Pathogenesis of Atherosclerosis. *Circul J* 2009;73:994-1001.
- 20 Auffray C, Sieweke MH, Geissmann F: Blood monocytes: Development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* 2009;27:669-692.
- 21 Mosser DM, Edwards JP: Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958-969.
- 22 Libby P, Nahrendorf M, Pittet MJ, Swirski FK: Diversity of denizens of the atherosclerotic plaque: Not all monocytes are created equal. *Circulation* 2008;117:3168-3170.
- 23 Gordon S: Macrophage heterogeneity and tissue lipids. *J Clin Invest* 2007;117:89-93.
- 24 Taylor PR, Gordon S: Monocyte heterogeneity and innate immunity. *Immunity* 2003;19:2-4.
- 25 Lentsch B, Shanley TP, Sarma V, Ward PA: In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. *J Clin Invest* 1997;100:2443-2448.
- 26 Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van Damme J, Mantovani A: Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *Journal Immunol* 2000;164:762-767.
- 27 Akira A, Takeda K, Kaisho T: Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675-680.
- 28 Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, Mantovani A, Rothe M, Muzio M, Arditi M: Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J Biol Chem* 1999;274:7611-7614.
- 29 Schenk M, Belisle JT, Modlin RL: TLR2 Looks at Lipoproteins. *Immunity* 2009;31:847-849.
- 30 Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T, Ziegler-Heitbrock L: The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol* 2002;168:3536-3542.