Interleukin-10 Released by CD4⁺CD25⁺ Natural Regulatory T Cells Improves Microvascular Endothelial Function Through Inhibition of NADPH Oxidase Activity in Hypertensive Mice

Modar Kassan, Maria Galan, Megan Partyka, Mohamed Trebak, Khalid Matrougui

Objective—We previously demonstrated that a reduced number of CD4⁺CD25⁻regulatory T cells (Tregs) was associated with microvascular dysfunction in hypertension. However, the underlying mechanism by which Tregs regulate vascular endothelial function remains unknown.

Methods and Results—Control and interleukin (IL)-10⁻/⁻ knockout mice were infused with angiotensin II (400 ng/kg/min) for 2 weeks (hypertensive [HT] and HT-IL-10⁻/⁻). Endothelium-dependent relaxation (EDR) in response to acetylcholine was significantly reduced in mesenteric resistance artery (MRA) from HT and HT-IL-10⁻/⁻ compared with control and IL-10⁻/⁻ mice. Importantly, the incubation of MRA from HT mice with the conditioned media of cultured Tregs, isolated from control mice, reduced NADPH oxidase activity and improved EDR, whereas no effect was observed in MRA from control mice incubated with the same media. These effects were reversed when MRAs were preincubated with IL-10 antibody or IL-10 receptor antagonist, whereas incubation with transforming growth factor-β receptor antagonist had no effect. The transfer of cultured Tregs, isolated from control mice, into HT-IL-10⁻/⁻ mice reduced systolic blood pressure (SBP) and NADPH oxidase activity and improved EDR in MRA compared with untreated HT-IL-10⁻/⁻ mice. In vivo treatment of HT mice with IL-10 (1000 ng/mouse) significantly reduced SBP and NADPH oxidase activity and improved EDR in MRA compared with untreated HT mice. The transfer of cultured Tregs, isolated from IL-10⁻/⁻ mice, into HT mice did not reduce SBP or NADPH oxidase activity or improve EDR. The incubation of MRA from HT mice with apocynin improved EDR, whereas NADPH oxidase substrate attenuated EDR in MRA from control mice, which was reversed with exogenous IL-10.

Conclusion—These data demonstrate that IL-10 released from Tregs attenuates NADPH oxidase activity, which is a critical process in the improvement of microvascular endothelial function in hypertension, suggesting that Tregs/IL-10 could be a therapeutic target for treatment of vasculopathy in hypertension. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: hypertension □ nitric oxide synthase □ vascular biology □ vasodilation

Impaired microvascular endothelial-dependent relaxation (EDR) is an important risk factor for vasculopathy and myocardial infarction in cardiovascular diseases, such as hypertension. We and other investigators have demonstrated endothelial dysfunction in mesenteric resistance arteries in hypertensive (HT) animal models. In a previous study, we reported that impaired EDR in coronary arterioles was associated with the induction of inflammation and a decrease in Treg number and interleukin (IL)-10 content in angiotensin II (Ang II)–induced hypertension. The transfer of Tregs, isolated from control mice, to HT mice improved EDR in coronary arterioles. In the present study, we delineated the mechanism of Treg-mediated improvement in EDR, and we provide direct evidence for the role of Tregs in the regulation of microvascular function.

It has been established that hypertension induced by Ang II infusion increases the expression and activity of NADPH oxidase and increases the subsequent generation of reactive oxygen species responsible for impairment of endothelial function. IL-10 is a potent antiinflammatory cytokine that plays a pivotal role in the regulation of immune and inflammatory responses. Indirect evidence indicates that IL-10 has a protective effect on endothelial function in diabetes and hypertension.

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Thus, in this study, we demonstrated that treatment with Tregs improves microvascular endothelial function in HT mice by a paracrine effect that involves the release of IL-10. We also delineated the mechanism of IL-10-mediated improvement in endothelial function, which involves alteration in NADPH oxidase activity.

Materials and Methods

General Protocol

All experiments were performed according to the American Guidelines for the Ethical Care of Animals and were approved by Tulane University Health Sciences Center Animal Care and Use Committee. One hundred forty C57BL/6J mice (8-week-old male) and 15 IL-10−/− mice (8-week-old male) were purchased from the Jackson Laboratory (Bar Harbor, ME), housed in groups of 5, and maintained at a temperature of 23°C with a 12-hour light-dark cycle. Mice were fed on a solid standard diet (Na+ content 0.4%) and water. Seventy control and 5 IL-10−/− mice were euthanized to isolate Tregs and perform experiments on EDR and NADPH oxidase activity. Animals were divided into 2 main groups, control and IL-10−/−. The control group was divided into 4 subgroups: (1) mice were infused with Ang II (400 ng·kg−1·min−1) using subcutaneous miniosmotic pumps for 2 weeks (HT, n=35); (2) mice received the same dose of Ang II with IL-10 (1000 ng/mouse) for 2 weeks using subcutaneous miniosmotic pumps (HT-IL-10, n=10); (3) mice received the same dose of Ang II using subcutaneous miniosmotic pumps and were injected with Tregs, isolated from IL-10−/− mice (3 injections per week for 2 weeks, HT+Tregs, n=10); and (4) control group (n=15). The IL-10−/− group was divided into 3 subgroups: (1) mice infused with Ang II (400 ng·kg−1·min−1) using subcutaneous miniosmotic pumps for 2 weeks (HT-IL-10−/−, n=5); (2) mice received the same dose of Ang II with Tregs, isolated from control mice (3 injections per week for 2 weeks, HT+Treg-IL-10−/−, n=5); and (3) IL-10−/− without treatment (n=5).

At the end of treatment, animals were anesthetized with isoflurane, and mesenteric resistance arteries were rapidly harvested, placed in PSS solution (composition in mmol/L: NaCl 118; KCl 4.7; CaCl2 2.5; KH2PO4 1.2; MgSO4·7H2O 1.2; NaHCO3 25; glucose 11), pH 7.4, and processed appropriately for further study.

Tregs and CD4+ T-Cell Isolation

Tregs and CD4+ T cells were isolated from cell suspension of spleen as previously described (Supplemental Figure II, available online at http://atvb.ahajournals.org).1 Isolated Tregs and CD4+ T cells were then cultured for 24 hours in wells precoated with anti-CD3 (clone 2C11, R&D Systems) in RPMI 1640 with 10% fetal bovine serum, 100 μg/mL streptomycin (all from Gibco and Invitrogen) 50 μg of 2-mercaptoethanol (Sigma-Aldrich), and 10 ng/mL human recombinant IL-2 (R&D Systems).

Endothelial Cell Culture

Primary cultured microvascular endothelial cells were used as previously described.12 Cells were stimulated with (1) NADPH oxidase substrate (NADPH, 100 μmol/L) with and without IL-10 (5 ng/mL); (2) NADPH in the presence of p38 mitogen-activated protein (MAP) kinase inhibitor (2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one, 10 μmol/L, Calbiochem) and JAK 1 inhibitor (2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benzo[h]-imidaz[4,5-f]isquinolin-7-one, Pyridone 6, P6, DBI, 10 μmol/L, Calbiochem), added 30 minutes before NADPH oxidase adduction with or without IL-10.

Vascular Reactivity

In Vitro Experiments

Mesenteric resistance arteries were isolated, carefully cleaned of fat and connective tissue, and cut into rings (2 mm in length). Mesenteric resistance arteries (100 to 120 μm in diameter) from control and HT mice were mounted in a small-vessel dual-chamber myograph for measurement of isometric tension. Two steel wires (25 μm) were introduced through the lumen of the mesenteric resistance artery (MRA) and mounted according to the method described by Mulvany and Halpern.13 After a 30-minute equilibration period in PSS solution bubbled with carbogen at 37°C and pH 7.4, MAs were stretched to their optimal lumen diameter for active tension development. Internal diameter (100 to 120 μm) of all microvessels was set to a pretension corresponding to an intraluminal pressure of 80 mm Hg. After a second 30-minute equilibration period, the vessels were exposed to phenylephrine (PE) (10−4 mol/L), and the presence of functional endothelium was assessed by the ability of acetylcholine (ACh) (10−6 mol/L) to induce relaxation. After precontraction with PE (10−4 mol/L) and steady maximal contraction (the lumen diameter and the contraction in response to PE were similar in all groups of mice), cumulative concentration response curves were obtained for ACh (1×10−8 to 3×10−5 mol/L).

After a wash period, rings were incubated for 30 minutes with the conditioned media of Tregs or of CD4+ T cells or with complete media alone, and then dose-response curves in response to ACh (1×10−8 to 3×10−5 mol/L) were performed after precontraction of mesenteric resistance arteries rings with 10−4 mol/L PE. In other experiments, Treg conditioned media and MRAs were pretreated with an antibody against IL-10 (5 μg/mL), IL-10 receptor antagonist (5 μg/mL), and transforming growth factor-β (TGFB) receptor antagonist (SB 431542, 10 μmol/L) respectively.

To evaluate the direct effect of IL-10, MRAs with and without endothelium from HT and control groups were used. After a stabilization period in PSS solution, MRAs were precontracted with PE, and a dose-response curve for relaxation in response to exogenous IL-10 (5, 10, and 60 ng) was obtained.

To determine the role of NADPH oxidase in the impaired EDR in HT mice, MRAs were incubated with 2 types of NADPH oxidase inhibitors (apocynin, 100 μmol/L; gp91 ds-tat, 3 μmol/L) for 30 minutes, and then EDR responses to ACh were studied after precontraction with PE. The apocynin concentration used in the present study makes the inhibitor less specific for NADPH.14 Therefore, we used another NADPH inhibitor (gp91 ds-tat) to strengthen our results. To determine the effect of IL-10 on NADPH oxidase activity, EDR responses to ACh were determined in the presence or absence of the substrate of NADPH oxidase (100 μmol/L) with or without IL-10 (5 μmol/L).

In Vivo Experiments

Another series of experiments was carried out using MRA from mice infused with Ang II with or without Treg transfer or IL-10 infusion (HT, HT-IL-10−/−, HT+Tregs, and HT-IL-10−/−+Tregs). Mice were trained for tail cuff measurements 1 week before starting the experiments. Systolic blood pressure (SBP) was measured in conscious mice using the CODA tail-cuff blood pressure system (Kent Scientific Torrington). Systolic arterial blood pressure measurements were performed at the same time (between 9 AM and 11 AM) to avoid the influence of the circadian cycle, and the value of SBP was obtained by estimating the average reading of 8 measurements for a single trial. The tail cuff procedure accurately measures SBP but underestimates diastolic blood pressure. Also, changes in subtle behavioral differences between groups, strains, or individual animals can introduce experimental variability to the measurement.

At the end of treatment period, mice were anesthetized with isoflurane, and the MRAs were cleaned and cut into rings to study reactivity, NADPH oxidase activity, and endothelial nitric oxide synthase (eNOS) phosphorylation levels. After a stabilization period in PSS solution, EDR in response to ACh (1×10−8 to 3×10−5 mol/L) was determined in PE-precontracted rings.

NADPH Oxidase Activity

Superoxide anion levels generated by NADPH oxidase activity were measured in lysates of MRA using lucigenin chemiluminescence. Briefly, lysates were prepared in a sucrose buffer containing...
50 mmol/L KH₂PO₄, 1 mmol/L EGTA, and 150 mmol/L sucrose, pH 7.0, in a TissueRuptor (Bullet Blender, Next Advance, Inc). The lysates supernatants were collected and stored at −80°C. To measure NADPH oxidase activity, a volume of 100 μL of each lysate was used in a total volume of 1 mL of PBS buffer containing lucigenin (5 μmol/L) and NADPH (100 μmol/L) and then preheated at 37°C. Blank samples were prepared using 100 μL of sucrose buffer. Lucigenin activity was measured every 30 seconds for a 10-minute period in a luminometer (Turner Biosystems 20/20 single-tube luminometer). Superoxide anions generated by NADPH oxidase were also measured in mouse endothelial cells. The area under the curve was used to quantify chemiluminescence results. Data are expressed as relative light units normalized to protein content (μg of protein). As a negative control for NADPH oxidase activity, we used cells incubated with apocynin (100 μmol/L) before the addition of NADPH oxidase substrate.

In other experiments, mice were infused with Ang II for 2 weeks. Then, isolated MRAs were incubated with IL-10 (10 pg/mL) for 15, 30, and 60 minutes, and NADPH oxidase activity was then determined.

Western Blot Analysis
The phosphorylation and expression of eNOS, p38 MAP kinase, and JAK-1 were determined using specific antibodies (1:1000 dilution, Cell Signaling Technology, Inc) as previously described. 12,16

Real-Time Polymerase Chain Reaction
IL-10 receptor mRNA levels were determined in MRA from control and IL-10−/− infused or not infused with Ang II and untreated or treated with Tregs isolated from control mice for 2 weeks. Total RNA was obtained using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer’s recommendations. A total of 1 μg of DNase I-treated RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with random hexamers in a 20-μL reaction. Polymerase chain reaction was performed in duplicate for each sample using 1 μL of cDNA as a template, 1X TaqMan Universal PCR Master Mix (Applied Biosystems), and 10X of TaqMan Gene Expression Assays (Applied Biosystems) in a 20-μL reaction. Assays-on-Demand (Applied Biosystems) of TaqMan fluorescent real-time polymerase chain reaction primers and probes were used for IL-10 ra (Mm00434151_m1) and 18S rRNA (Hs99999901_s1), which was used as endogenous control to normalize results. Quantitative reverse transcription–polymerase chain reaction was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Relative mRNA levels were determined using the 2−ΔΔCt method. Results are expressed as the relative expression of mRNA in treated mice compared with untreated mice.

Drugs
PE hydrochloride, ACh chloride, apocynin, NADPH, IL-10, and Ang II were obtained from Sigma-Aldrich. The gp91 ds-tat was purchased from Anaspec, and SB 431542 was purchased from Tocris Bioscience. Stock solutions of drugs were prepared in ultrapure water and stored at −20°C, and appropriate dilutions were made on the day of the experiment.

Statistical Analysis
Data are expressed as mean±SEM. Concentration-response curves were analyzed using GraphPad Prism 4.0 software. The responses to ACh and IL-10 are expressed as percentages of PE contraction. Statistical calculations for significant differences were performed using the Student t test and 2-way ANOVA to analyze dose response in multiple groups. Significance was accepted at P<0.05.

Results
To determine whether Tregs directly regulate resistance artery endothelial function in hypertension, we first examined...
EDR in MRA in response to dose response of ACh in control and HT mice. EDR in MRA was significantly attenuated in HT mice compared with control (Figure 1A to 1C). We observed that incubation of MRA from HT mice in the conditioned media from Tregs significantly improved EDR (Figure 1A), and no effect on the response to ACh was observed when MRAs were incubated with complete media alone (Figure 1B). Furthermore, the specificity of the effect of Tregs on endothelial function was determined by incubating MRA from HT mice with conditioned media from Tregs alone or in combination with transforming growth factor-β receptor antagonist (SB 431542). The results revealed no effect on EDR (Figure 1C).

To elucidate whether IL-10 released by Tregs is an important factor in the improvement in endothelium-dependent MRA relaxation, we examined the effect of IL-10 antibody (5 μg/mL) and IL-10 receptor antagonist (5 μg/mL). The results of these experiments revealed that the IL-10 antibody and the IL-10 receptor antagonist completely blocked the improvement in EDR of MRA incubated with conditioned media from Tregs (Figure 2A and 2B). Figure 2C illustrates an increase in NADPH oxidase activity in MRA from HT mice, incubated with or without conditioned media from Tregs alone, in combination with transforming growth factor-β receptor antagonist (SB 431542). The effect was reversed when MRAs were incubated with the IL-10 antibody or IL-10 receptor antagonist.

To determine whether TGF-β is involved in EDR in MRA, we incubated arteries with the TGF-β receptor antagonist (SB 431542) for 1 hour, and the results revealed no effect on MRA relaxation (Figure 2D), even at high concentration of the TGF-β receptor inhibitor (data not shown).

In addition, we also determined whether IL-10 itself could be a relaxing factor. Figure 3A and 3B illustrates that IL-10 can act as a very weak relaxing factor, producing 20% of maximum relaxation in control mice at very high doses, with no effect on tension in HT mice.
Incubation of isolated MRA from HT mice with IL-10 reduced NADPH oxidase activity within 30 minutes of incubation (Figure 2E). To further delineate the mechanism by which IL-10 improves microvascular endothelial function in hypertension, we first incubated MRA from HT mice with apocynin and gp 91 ds-tat (NADPH oxidase inhibitors). Our results revealed that the inhibition of NADPH oxidase activity significantly improved EDR in MRA from HT mice (Figure 3C). We also examined the relationship between NADPH oxidase activity and EDR. In these experiments, the incubation of MRA from control mice with NADPH oxidase substrate produced a significant attenuation in the EDR in response to ACh (Figure 3D). Importantly, the effect of NADPH oxidase substrate was reversed when MRAs were incubated with or without IL-10 (Figure 3E). The inhibitory effect of IL-10 was reduced with the p38 MAP kinase inhibitor but not with the JAK-1 inhibitor (Figure 3E). Western blot analysis revealed that IL-10 increased p38 MAP kinase and JAK-1 phosphorylation (Figure 3F).

To further elucidate the mechanism of the effect of Tregs/IL-10 on the regulation of microvascular endothelial function, control mice were infused with Ang II with and without exogenous IL-10 for 2 weeks. IL-10 treatment significantly reduced SBP in mice infused with Ang II (Figure 4A). Figure 4B and 4C shows the improvement of EDR in MRA from HT mice infused with IL-10 compared with HT mice. The inhibition of eNOS significantly reduced EDR in MRA from both groups (Figure 4C). These results were associated with an increase in eNOS phosphorylation and a decrease in NADPH oxidase activity in HT mice with IL-10 compared with HT without IL-10 treatment (Figure 4D and 4E). We did not observe any change in total eNOS expression (Figure 4E).

In another series of experiments, isolated Tregs from control mice, when transferred into HT mice, significantly reduced SBP and NADPH oxidase activity and improved the EDR in MRA associated with enhanced eNOS phosphorylation and IL-10 receptor expression (Figure 5A to 5E).
The importance of IL-10 in the regulation of microvascular EDR was also observed with Tregs isolated from IL-10/H11002 mice and then transferred to HT mice. The experiments revealed no changes in SBP and EDR (Figure 6).

Supplemental Figure I is a schematic diagram summarizing how Tregs regulate microvascular endothelial function.

**Discussion**

The present study identifies CD4+/CD25+ regulatory T cells (Tregs) and its cytokine IL-10 as novel targets for the treatment of microvascular endothelial dysfunction in hypertension. We demonstrated that IL-10 released by Tregs reduces NADPH oxidase activity, increases eNOS activity, and subsequently improves EDR in resistance arteries from HT mice. We also ascertained that HT mice treated with IL-10 significantly reduced SBP and NADPH oxidase activity and improved EDR in resistance arteries. Importantly, the transfer of Tregs, isolated from control mice, into HT IL-10/H11002 mice reduced SBP and NADPH oxidase activity and improved EDR in resistance arteries, whereas the transfer of Tregs isolated from IL-10/H11002 knockout mice had no effect in HT mice. These results suggest that Treg-IL-10 plays an important role in the regulation of microvascular endothelial function.

Hypertension, with its cardiovascular complications, is the leading cause of morbidity and mortality in the world. The connection between hypertension and impaired microvascular endothelial function is well established. Endothelial dysfunction is associated with an imbalance between nitric oxide bioavailability and reactive oxygen species production. One source of oxidative stress in the vascular system is NADPH oxidase. The superoxide anions that are excessively generated interact with NO and form peroxynitrite, which reduces NO bioavailability, resulting in impaired eNOS-dependent relaxation. It has been reported that Ang II-induced hypertension enhances the generation of superoxide anions by increasing NADPH oxidase activity, which causes vascular endothelial dysfunction. Tregs play an important role in the immune system, maintaining immunologic unresponsiveness to self-antigens and suppressing excessive immune responses deleterious to the host. Recent studies illustrated a relationship between Tregs and vascular wall function in cardiovascular diseases. Additionally, an association between the immune system and hypertension has been reported. We recently demonstrated that an increase in apoptotic Tregs is responsible for the induction of vascular inflammation and impaired EDR in coronary arterioles in hypertension. The transfer of Tregs, isolated from control mice, into HT mice reduces arterial blood pressure and improves coronary arteriolar endothelial function. However, the underlying molecular mechanisms of how Tregs improve microvascular endothelial function remain largely unknown.

The importance of Tregs in suppressing inappropriate inflammation has become increasingly clear in the past several years. The release of the antiinflammatory IL-10 by Tregs could be an important mechanism by which Tregs can reverse cardiovascular diseases such as atherosclerosis and vascular dysfunction in hypertension.

Our data provide evidence that Tregs release IL-10, which plays an important role in the improvement of microvascular
Nitric oxide synthase (eNOS), total (T) eNOS, and mRNA in mesenteric resistance artery (MRA) from IL-10 increase in oxidative stress,9,37 which is in part responsible for phosphorylation and subsequently EDR. Therefore, IL-10 most likely functions to rescue eNOS.

Previous study, the in vivo IL-10 level measured was 5 pg.5 (5 ng IL-10 induced 2% of maximum relaxation). The small vasorelaxing factor and whether it rescues eNOS phosphorylation and improved EDR. These in vivo studies in which control mice were infused with Ang II with and without IL-10. Thus, SBP and NADPH oxidase activity were significantly reduced in HT mice treated with IL-10. These responses were associated with an increase in eNOS phosphorylation and improved EDR. These in vivo data emphasize the pathophysiological relevance of IL-10 in the regulation of vascular endothelial function. Our studies are in agreement with previous studies reporting that IL-10 inhibits the expression of proinflammatory cytokines, including IL-6 and tumor necrosis factor-α, and the activation of transcription factors such as nuclear factor-κB, which are important mediators of vascular endothelial dysfunction.45–47 Previous in vitro studies using large arteries exposed subjected to Ang II with or without a high dose of IL-10 (300 nmol/L) for 24 hours prevented endothelial dysfunction.46 Our findings are supported by...
previous studies\textsuperscript{35,36} and indicate that IL-10 reduces oxidative stress and improves microvascular endothelial function in hypertension.

To extend our results, we transferred isolated Tregs from control mice into IL-10$^{−/−}$ mice infused with Ang II. The results of these studies showed a reduction in SBP and NADPH oxidase activity associated with a rescue of eNOS phosphorylation and EDR. Treg transfer into IL-10$^{−/−}$ mice infused with Ang II significantly reduced SBP and rescued EDR. We determined that there was an increased expression of IL-10 receptor, indicating that IL-10 receptor expression is regulated by IL-10. Thus, IL-10 receptor upregulation could explain the beneficial effect of Tregs in IL-10$^{−/−}$ mice. Interestingly, the transfer of Tregs from IL-10$^{−/−}$ mice into HT mice did not alter SBP or microvascular EDR, providing support for the role of IL-10 in the regulation of endothelial function.

In conclusion, the present study provides novel and significant insight into the basic mechanism of how Tregs control resistance artery endothelial function. We have demonstrated that Tregs release IL-10, which reduces NADPH oxidase activity by a p38 MAP kinase–dependent mechanism, rescues eNOS phosphorylation, and improves EDR in hypertension. Thus, Tregs/IL-10 are a potential target for treating microvascular complications in hypertension.

Disclosures

None.

References


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Figure I. In Ang II-induced hypertension, there is a decrease in Tregs number and IL-10 levels associated with increased NADPH oxidase activity and reduced eNOS phosphorylation. These events are responsible for impaired microvascular endothelium-dependent relaxation. Our results indicate that Tregs release IL-10, which binds to the IL-10 receptor, and then through intermediate signaling pathways rescue eNOS phosphorylation, inhibit NADPH oxidase activity and subsequently improve microvascular endothelium-dependent relaxation.
CD4+CD25+ regulatory T cells were isolated from mice spleen cell suspension using the CD4+CD25+ regulatory T Cell Isolation Kit “miltenyibiotec.com” (A) negative control without any antibody, (B) isolation of CD4+ using CD4+FITC antibody, (C) isolation of CD25+ using CD25+PE antibody, (D) CD4+CD25+ regulatory T cells isolation using CD4+FITC and CD25+PE antibodies.

**Figure II:** Isolation of CD4+CD25+ regulatory T cells by flow cytometry sorting.