

Neutrophil Chemotaxis and Local Expression of Interleukin-10 in the Tolerance of Endotoxin-Induced Uveitis

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PURPOSE. To study the mechanism of lipopolysaccharide (LPS) tolerance in a rat model of footpad injection endotoxin-induced uveitis (EIU).

METHODS. EIU was produced by footpad injection of 1 mg/kg LPS in male Sprague-Dawley rats. Four experiments were undertaken in this study. First, on days 3, 7, 14, 28, 56, and 84 after LPS injection, the iris-ciliary body (ICB) was isolated. LPS tolerance-associated gene expression in the ICB was determined by quantitative polymerase chain reaction. Second, the distribution of IL-10-producing cells in frozen sections of ocular tissues was analyzed by fluorescence and confocal microscopy. Third, peripheral blood neutrophil chemotaxis was determined using a fluorescent in vitro migration assay. Fourth, for in vivo neutrophil chemotaxis assay, neutrophils isolated from EIU-tolerant or control rats were transfused into green fluorescence protein (GFP) rats injected with LPS 18 hours earlier. Six hours after transfusion, the percentage of GFP-negative neutrophils in the aqueous humor was determined by flow cytometry.

RESULTS. *IL-10* gene expression in ICB was significantly upregulated for at least 1 month. Immunohistochemical examination indicated that dendritic cells in the ICB produced IL-10. Peripheral blood neutrophil chemotaxis in EIU-tolerant rats was inhibited significantly in vitro and in vivo. IL-10 enhanced the reduction of neutrophil chemotaxis in EIU-tolerant rats in vitro.

CONCLUSIONS. The results suggest that continuous high expression of IL-10 in the eye and the reduction of peripheral blood neutrophil chemotaxis play significant roles in the mechanism of LPS tolerance in a rat model of footpad injection EIU. (*Invest Ophthalmol Vis Sci.* 2008;49:5450-5457) DOI:10.1167/iovs.08-1878

Footpad injection of bacterial lipopolysaccharide (LPS) causes pronounced anterior uveitis in susceptible species and strains.¹ This animal model of endotoxin-induced uveitis (EIU) serves as a useful paradigm of human anterior uveitis, such as acute anterior uveitis. Previously, many investigators have attempted to determine the exact mechanism of the onset

of EIU and to find an anti-inflammatory pathway in the pathogenesis of EIU.²⁻⁷

Pre-exposure to LPS induces reduced sensitivity of the biological system to a second LPS challenge, a phenomenon referred to as LPS tolerance or endotoxin tolerance. In the EIU model, repeated footpad injection of LPS also results in a state of LPS tolerance, and the animals do not develop uveitis.^{1,8} Although the ocular effects of LPS are well documented, few investigations have focused on this phenomenon, and the mechanism for the development of tolerance in EIU is still unclear.

LPS is a potent chemotactic component for polynuclear leukocyte neutrophils. Breakdown of the blood-aqueous barrier can be detected 4 hours after footpad injection of LPS, and a predominant neutrophil infiltration of the anterior segment is maximal at 18 to 24 hours.^{1,9-11} After the second challenge with LPS, neutrophil accumulation in the aqueous humor was inhibited dramatically by approximately 100%.¹² Because unilateral intraocular injection of LPS produced local tolerance that was not observed in the contralateral control eyes, some local changes in the ocular tissue had been considered as a main cause of the development of EIU tolerance.

Interestingly, Chang et al.¹³ recently reported that endotoxin tolerance might occur in peripheral blood neutrophils and monocytes in patients with acute anterior uveitis, suggesting that systemic factors may be involved in the development of EIU tolerance. In addition, though the inhibition of neutrophil migration into the inflamed ocular tissue is the clinical feature in EIU tolerance, little is known about neutrophil chemotaxis in EIU tolerance. Consequently, to understand the mechanism of EIU tolerance in the footpad injection model, we reproduced the LPS-tolerant rat EIU model and focused on the chemotaxis function of the peripheral blood neutrophils in EIU tolerance and on local expression of several potential LPS-tolerant factors.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (6 weeks old; weight range, 150-200 g) were obtained from a local animal supplier (Japan SLC, Hamamatsu, Japan). For the transfusion experiments, a male green fluorescence protein (GFP) transgenic Sprague-Dawley rat strain (SD-Tg [CAG-EGFP]; Japan SLC) was used as the transfusion recipient of neutrophils. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EIU Induction

The EIU model was induced as reported previously.⁷ Briefly, each rat received one injection of 1 mg/kg LPS (*Salmonella minnesota*; Sigma, St. Louis, MO) in 1 mL/kg sterile pathogen-free saline in one rear footpad. Control rats received the same volume of pathogen-free saline in one rear footpad.

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Second Challenge with LPS

LPS or saline-pretreated rats received a second challenge of 1 mg/kg LPS in one rear footpad on day 3, 7, 14, 28, 56, or 84 after the first injection.

Number of Infiltrating Cells and Protein Concentration in Aqueous Humor

Twenty-four hours after the second challenge with LPS, the rats were humanely killed, and the aqueous humor was collected immediately from both eyes by anterior chamber puncture. The cells were counted with a hemocytometer, and the total protein concentration in the aqueous humor samples was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).⁷

Collection of Iris-Ciliary Body

For gene expression analysis, the iris-ciliary body (ICB) of the rats was collected on days 3, 7, 14, 28, 56, and 84 after the first LPS or saline injection. Samples were homogenized with the use of a homogenizer (TissueLyser; Qiagen, Valencia, CA) in RLT buffer reagent (Qiagen) and were centrifuged to remove cellular debris.

Total RNA Isolation and cDNA Synthesis

Total RNA from the samples described previously was isolated (EZ1 RNA Tissue Mini Kit; Qiagen) according to the manufacturer's protocols. Isolation and a quality check of isolated RNA were performed as described previously.¹⁴ cDNA was synthesized from total RNA (High Capacity kit; Applied Biosystems, Inc. [ABI], Foster City, CA), according to the manufacturer's instructions.

Gene Expression Analyses in ICB

Quantitative reverse transcriptase-polymerase chain reaction (PCR) using double-strand cDNA was performed for the selected 12 genes (*iNOS*, *TNF- α* , *IL-1 β* , *IL-6*, *IL-10*, *TGF- β* , *SHIP*, *SOCS-1*, *MyD88*, *TLR2*, *TLR4*, and *TLR9*). Gene selection was based on previous investigations related to macrophage LPS tolerance.^{15–26} Quantitative PCR reactions were performed in a mixture containing 50 ng/well cDNA samples, PCR mix (TaqMan Universal PCR Master Mix; ABI), and a set of oligonucleotides (TaqMan Gene Expression assays; ABI). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a calibrator in relative quantification calculations. Gene-specific PCR products were measured continuously by a sequence detection system (PRISM 7900HT; ABI) according to the manufacturer's protocol. Each sample was run in duplicate. Duplicate data from one RNA sample were averaged for each gene. For each sample, the results of gene expression were normalized relative to its *GAPDH*. The mean normalized gene expression of ICB in the day-matched saline-treated rats was expressed as an arbitrary value of 1.0, and the samples were plotted relative to that value.^{27,28}

Quantification of IL-10 Level in Aqueous Humor

Aqueous humor was collected from LPS-treated or control rats on days 1, 3, 7, and 14 after the first injection. The aqueous humor from 24 eyes of 12 rats in each group were pooled in one tube (total volume, 500 μ L) and condensed up to 50 μ L (Microcon 10; Millipore, Billerica, MA).²⁹ This sample then was assessed with a commercially available ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Histology and Immunohistochemistry

Freshly enucleated eyes were embedded in optical cutting temperature compound, snap frozen, and stored at -20°C . Sections 10- μ m thick were blocked. For double staining, the first goat anti-rat IL-10 primary polyclonal antibody (R&D Systems) was revealed by incubation with a secondary anti-goat antibody conjugated with FITC. Tissues were then incubated in the second mouse anti-rat primary monoclonal antibody

(dendritic cell marker: anti-OX62 [GeneTex, San Antonio, TX]; macrophage marker: anti-CD163 [Acris Antibodies, Herford Germany]) followed by incubation with secondary anti-goat antibody conjugated with rhodamine. Between incubation, samples were thoroughly washed. Sections were observed using confocal laser scanning microscopy.

Neutrophil Isolation

Neutrophil isolation was performed as described.³⁰ Briefly, peripheral blood was obtained by cardiac puncture and heparinized (10 U/mL). Collected rat blood was mixed with 3% dextran solution and allowed to settle for 30 minutes at room temperature. The supernatant was decanted and centrifuged. Pellets were suspended in HBSS, layered (Ficoll-Paque PLUS; GE Healthcare, Buckinghamshire, UK), and centrifuged at 18°C (500g for 25 minutes). The pellet was resuspended in ammonium chloride-based lysis reagent (PharmLyse; BD Biosciences, San Jose, CA) at 4°C to lyse the remaining erythrocytes. The cells were 96.3% pure neutrophils, as determined by flow cytometry (fluorescence-activated cell sorter [FACS]) light-scatter patterns.

In Vitro Neutrophil Migration Assay

We chose neutrophils obtained from rats on day 7 after the first LPS or saline injections as experimental samples. Neutrophils isolated from three identically treated rats were pooled as one sample and suspended in HBSS buffer at 10^7 cells/mL. The cells were incubated with 5 mM calcein AM for 15 minutes at 37°C and then washed and resuspended at 1×10^6 cells/mL in RPMI-1640 containing 5% fetal bovine serum (FBS), 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mM glutamine. The cells were incubated for 90 minutes under standard conditions using inserts (Falcon HTS 3 μ m Fluoroblok; BD Biosciences) with the multiwell 24-well companion plate (BD Biosciences). Chemoattractant solutions (1, 10, or 100 nM human IL-8; R&D Systems) in supplemented RPMI without FBS were placed in the 24-well plate. Two hundred fifty microliters of cell suspension was placed in the inserts (Fluoroblok; BD Biosciences). Fluorescence readings at 0, 30, 60, and 90 minutes were taken (ARVO MX; PerkinElmer, Waltham, MA) using excitation and emission wavelengths of 485 and 535 nm. The neutrophil migration ability in the LPS-treated group was evaluated by calculating the ratio of the fluorescence value in LPS-treated rats to that of control rats. This experiment was repeated five times, and the average ratio was calculated.

In Vivo Neutrophil Migration Assay

GFP rats (recipient rats) received one injection in one rear footpad of 200 μ g LPS. Eighteen hours later, they were transfused through the tail vein with 3×10^7 cells of purified neutrophils isolated from three wild-type rats (donor rats) on day 7 after the first LPS or saline injection. Six hours after transfusion of the neutrophils, peripheral blood was obtained from recipient rats through cardiac puncture of anesthetized rats and were immediately anticoagulated with EDTA. At the same time, aqueous humor was collected as described. For lysing red blood cells and fixation, lysing solution (Optilyse B; Beckman Coulter, Fullerton, CA) was used. Samples were applied for FACS analysis (FACSCalibur; BD Biosciences). In total, 50,000 events were collected and analyzed for blood preparation and 10,000 events were collected and analyzed for aqueous humor preparation. On the basis of forward and side scattering, the neutrophils were gated. The percentages of GFP-negative neutrophils (donor cells) in the aqueous humor and peripheral blood of EIU-induced recipient GFP rats were calculated.^{31–33}

Statistical Analysis

Data are expressed as mean \pm SEM. Statistical significance was analyzed by paired Student's *t*-test for migration assay and by unpaired Student's *t*-test for the other experiments. $P < 0.05$ was considered significant.

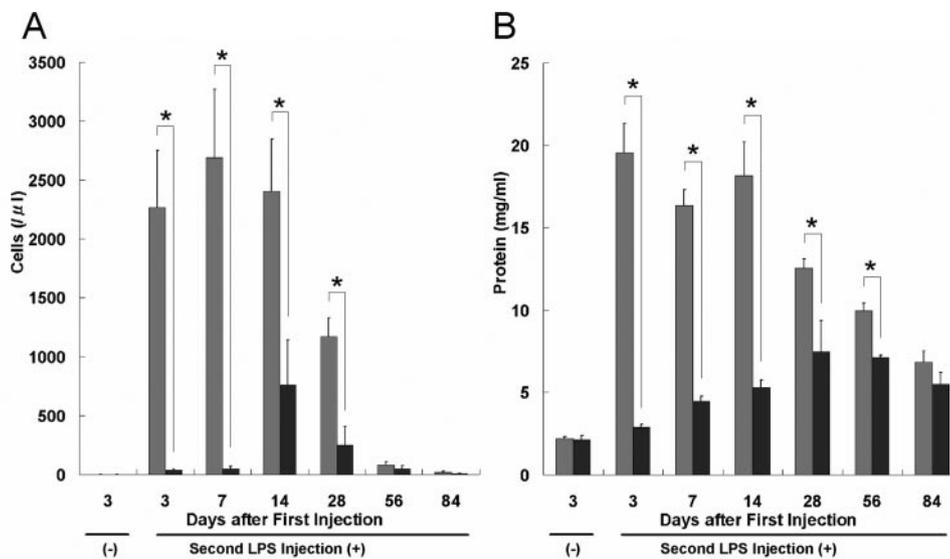


FIGURE 1. Effects of tolerance on (A) cellular infiltration and (B) protein concentration in the aqueous humor of an LPS-pretreated rat (black bar) and a saline control-pretreated rat (gray bar). Cell numbers and protein concentrations are expressed as mean \pm the SEM of three rats. Significant differences: * $P < 0.05$ by the unpaired t -test.

RESULTS

Induction of EIU Tolerance

Twenty-four hours after the second challenge with LPS, on days 3, 7, 14, and 28 after the first injection of LPS or saline, there were significantly fewer infiltrating cells in the aqueous humor of the LPS-pretreated rats than in the control rats (LPS-pretreated rats, 40 ± 6 , 48 ± 22 , 761 ± 384 , and 249 ± 160 cells/ μ L, respectively; control rats, 2267 ± 484 , 2689 ± 580 , 2407 ± 435 , and 1170 ± 159 cells/ μ L, respectively; $P < 0.05$; Fig. 1A). No significant differences were observed on days 56 and 84 between the two groups. Without a second challenge, no infiltrating cells were observed in the aqueous humor on day 3 in either group.

Regarding protein concentration in the aqueous humor, the second challenge with LPS induced a significant increase in the protein concentration in control rats compared with LPS-pretreated rats up to day 56 (control rats: 19.6 ± 3.1 , 16.2 ± 1.7 , 18.1 ± 3.6 , 12.5 ± 1.0 , 10.0 ± 0.8 , and 6.9 ± 1.1 mg/mL, respectively; LPS-pretreated rats: 2.9 ± 0.3 , 4.5 ± 0.5 , 5.3 ± 0.8 , 7.5 ± 3.3 , 7.1 ± 0.3 , and 5.5 ± 1.2 mg/mL, respectively; $P < 0.05$; Fig. 1B).

Because older rats were less sensitive to LPS than younger rats, we could not report that EIU tolerance developed definitively on day 56. However, EIU tolerance did continue up to day 28 in our EIU model. The data at each point were obtained from three rats, and the mean \pm SEM was calculated.

Gene Expression Changes in ICB throughout the Duration of EIU Tolerance

On day 3 after the first injection, the expression of most genes analyzed in this experiment, except for *TNF- α* , *iNOS*, and *SOCS-1*, were upregulated in EIU-tolerant rats compared with control rats. Interestingly, only *IL-10* showed a high level of gene expression up to day 28 throughout the observation period (7.9-, 5.4-, 2.8-, and 3.0-fold higher than control rats; $P < 0.05$; Fig. 2).

The expression of other genes decreased to levels comparable to those of control rats on day 7 and thereafter. The expression of each gene checked in this experiment was constant in control rats throughout the observation period. Data at each point were obtained from three rats, and the mean \pm the SEM was calculated.

IL-10 Protein Level in Aqueous Humor

This part of the experiment was performed twice independently, and 192 rats were included in the examination. ELISA analysis showed a high level of IL-10 protein in the aqueous humor on days 1, 3, and 7 after the first LPS injection (mean, 46.7 [first experiment, 35.5; second experiment, 57.86], mean, 14.7 [first experiment, 17.6; second experiment, 11.6], and mean, 2.7 [first experiment, 3.3; second experiment, 2.2] pg/mL, respectively). However, on day 14 after the first LPS injection, IL-10 protein was undetectable (<1.0 pg/mL). We did not detect IL-10 protein at any point in the control rats.

Immune Cells That Produced IL-10

Histologic examination indicated that dendritic cells and macrophages remained in the ICB of an EIU-tolerant rat on day 7 after the first LPS injection. There were neither neutrophils nor lymphocytes (data not shown). These findings were consistent with previous findings.⁹

The results of double immunohistochemical staining demonstrated that there were a number of double-positive cells for OX62 and IL-10; however, few cells were double positive for CD163 and IL-10 (Fig. 3). This suggested that dendritic cells played a role in maintaining a high level of IL-10 in the eye of EIU-tolerant rats. Figure 3 shows representative data in five independent examinations.

In Vitro Neutrophil Chemotaxis Function in EIU-Tolerant Rats

Because there was an obvious difference between days 7 and 14 in the number of cells infiltrating the aqueous humor in EIU-tolerant rats after the second challenge with LPS (Fig. 1A), we used neutrophils collected on day 7 after the first LPS or saline injection in the in vitro and in vivo experiments of neutrophil chemotaxis.

First, we fixed the IL-8 concentration at 100 nM. Neutrophils in EIU-tolerant rats also showed significantly low migration ability at all assay time points (30, 60, and 90 minutes) (% of control rats: $49\% \pm 14\%$, $58\% \pm 9\%$, $50\% \pm 11\%$, respectively; $P = 0.0239$, 0.0040 , 0.0049 , respectively; Fig. 4A)

We then fixed the incubation time as 90 minutes. Neutrophils in EIU-tolerant rats and control rats migrated across the filter toward IL-8 in a dose-dependent fashion. Maximum neutrophil migration occurred at 100 nM in both groups (data not shown). The migration ability toward IL-8 significantly de-

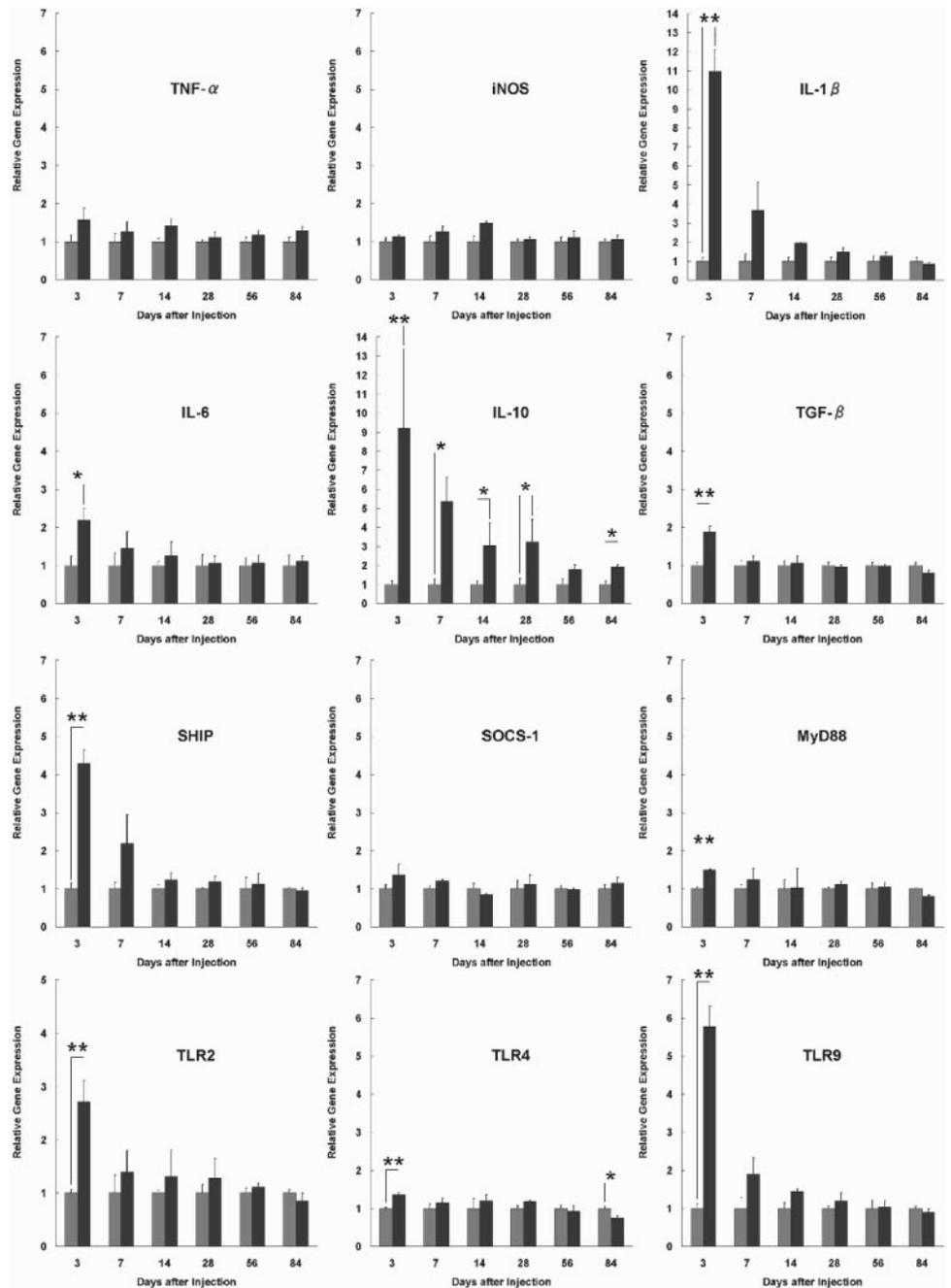


FIGURE 2. Relative gene expression ratios of the LPS tolerance-related proteins in the ICB of an LPS-pretreated rat (black bar) and a saline-pretreated control rat (gray bar). Data are expressed as mean \pm SEM of three rats. Statistical analysis was performed using the unpaired *t*-test to compare the tolerant rats and the control rats. Significant differences: **P* < 0.05 and ***P* < 0.01 by the unpaired *t*-test.

creased in neutrophils of EIU-tolerant rats at all doses of IL-8 (0, 4, 20, and 100 nM [control rats, 44% \pm 8%, 42% \pm 13%, 42% \pm 13%, and 50% \pm 11%, respectively; *P* = 0.0039, 0.0155, 0.0157, 0.0049, respectively; Fig. 4B]). This experiment was repeated five times, and the average ratio was calculated. Data are expressed as mean \pm SEM.

In Vivo Neutrophil Chemotaxis Function in EIU-Tolerant Rats

We evaluated the in vivo chemotactic function of neutrophils in EIU-tolerant rats. Using GFP rats as recipients, we distinguished transfused GFP-negative neutrophils from recipient GFP-positive neutrophils by FACS analysis. The percentages of GFP-negative neutrophils in the aqueous humor and peripheral blood of EIU-induced recipient GFP rats were calculated by

FACS analysis. This experiment was performed three times. Figure 5 shows representative data.

The percentage of GFP-negative neutrophils in the peripheral blood of the EIU-tolerant donor group was almost the same as that of the control donor groups (% of control, 260.7% \pm 157.3%; *P* = 0.24). However, the percentage of GFP-negative neutrophils in the aqueous humor was significantly lower in the EIU-tolerant group (control, 26.2% \pm 21.8%; *P* = 0.048), indicating that in vivo neutrophil migration decreased in EIU-tolerant rats compared with control rats.

Effect of IL-10 on Neutrophil Migration

We fixed the assay time at 90 minutes and the concentration of IL-8 at 100 nM. Isolated neutrophils were incubated in various concentrations (0, 0.1, 1, and 10 ng/mL) of recombinant rat IL-10 (PeproTech EC, London, UK) for 30 minutes before the

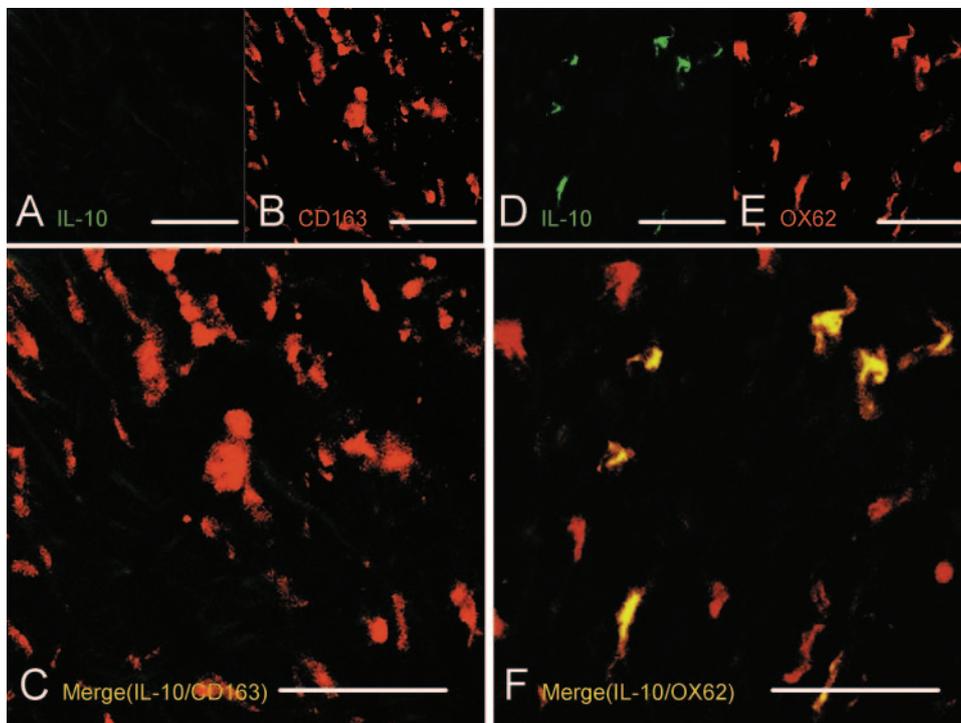


FIGURE 3. Double staining of IL-10 with OX62 or CD163 in the ICB of the EIU-tolerant rat. Colocalization of IL-10 with CD163 or OX62 was examined by double immunofluorescence staining. No IL-10-positive cells were observed where there were many macrophages. (A) IL-10 (green). (B) CD163 (red). (C) IL-10/CD163 merge. (D) IL-10 (green). (E) OX62 (red). (F) IL-10/OX62 merge. Scale bars, 50 μ m.

assay was performed. Neutrophil chemotactic function in EIU-tolerant rats significantly decreased in the presence of IL-10 in a dose-dependent fashion (neutrophil chemotaxis without IL-10: 0.1, 1, and 10 ng/mL; $91\% \pm 30\%$, $62\% \pm 12\%$, and $48\% \pm 14\%$, respectively; $P = 0.0117$ at 1 ng/mL and $P = 0.0066$ at 10 ng/mL; Fig. 6B). In contrast, the presence of IL-10 increased neutrophil chemotactic function in control rats (neutrophil chemotaxis without IL-10: 0.1, 1, and 10 ng/mL; $162\% \pm 38\%$, $161\% \pm 22\%$, and $130\% \pm 32\%$, respectively; $P = 0.0236$ at 1 ng/mL; Fig. 6A). This experiment was repeated five times, and the average ratio was calculated. Data are expressed as the mean \pm the SEM.

DISCUSSION

This study demonstrated four important findings in the development of EIU tolerance. First, the expression level of IL-10, which is a potent inhibitor of cytokine production, increased

throughout the duration of EIU tolerance. Second, the immune cells that produced IL-10 were dendritic cells, not macrophages. Third, the neutrophil chemotactic function decreased significantly in EIU-tolerant rats in vivo and in vitro. Fourth, the presence of IL-10 dramatically suppressed neutrophil chemotactic function in EIU-tolerant rats in vitro, though it significantly activated neutrophil chemotactic function in control rats.

LPS binds to LPS-binding protein (LBP), and this complex is delivered to the cell surface receptor CD14 before it is transferred to the transmembrane signaling receptor toll-like receptor 4 and its accessory protein MD2.³⁴ Because CD14 is expressed abundantly on mature monocytes and macrophages,³⁵⁻³⁷ it is believed that monocytes and macrophages play pivotal roles in the LPS response and LPS tolerance. LPS stimulation activates several intracellular signaling pathways, which in turn activate a variety of transcription factors that coordinate the induction of many genes encoding inflammatory mediators and anti-inflammatory cytokines. In

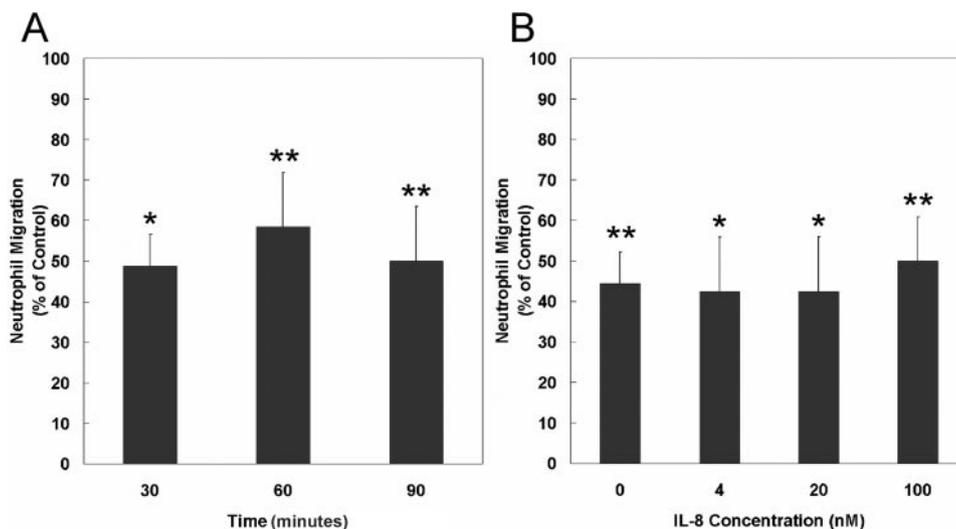


FIGURE 4. Change in the migration of neutrophils in LPS-tolerant rats toward human IL-8 stimulus (A) over time and (B) with concentration. The mean ratio of fluorescence of the LPS-pretreated rats to that of the controls is expressed as a percentage. Significant differences: * $P < 0.05$ and ** $P < 0.01$ by the paired *t*-test.

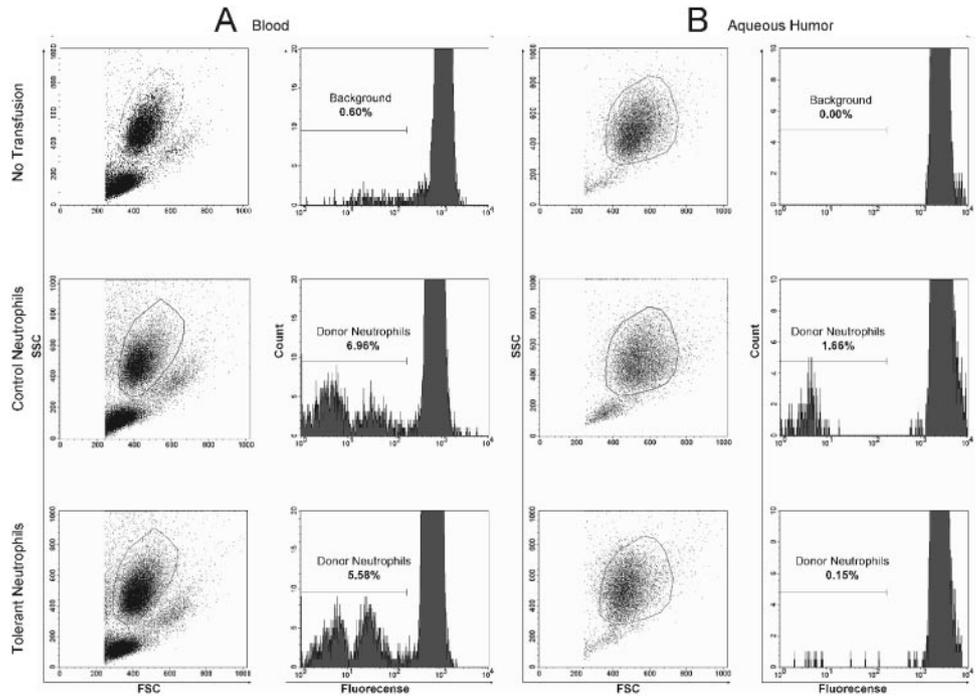


FIGURE 5. Flow cytometric identification of peripheral blood and aqueous humor neutrophils of nontransfused, control-neutrophil transfused, and LPS-tolerant neutrophil-transfused rats (*upper column*: no transfusion; *middle column*: transfusion of control neutrophil; *lower column*: transfusion of tolerant neutrophils). (**A, B, left column**) Gating of neutrophils was performed based on forward and side scattering. (**A, B, right column**) FACS histogram shows GFP fluorescence (*x-axis*) of cells, and donor cells are identified as GFP-negative cells. The percentages of GFP-negative cells are shown in the histogram.

LPS tolerance, the production of these cytokines by monocytes and macrophages is low to absent.

EIU could have been induced by systemic treatment and local injection of LPS. Unilateral intraocular injection of LPS produced local tolerance, but this was not observed in the contralateral control eyes. These previous observations suggested that resident macrophages in the eye bind circulating LPS-LBP complexes and induce an inflammatory reaction (EIU).⁹ When resident ocular macrophages become LPS tolerant, a second challenge with LPS cannot induce a subsequent ocular inflammation (EIU tolerance). In addition to this scenario, the results of the present study suggested that local IL-10 plays an important role as an anti-inflammatory agent in the induction of EIU tolerance. To the best of our knowledge, this is the first report that the expression of *IL-10* mRNA in the eye increases significantly throughout the duration of EIU tolerance.

Recent studies have indicated that there are multiple cellular mechanisms for downregulating TLR activation.³⁸ Although we could not investigate all potential inhibitory agents and

sites targeted by endogenous inhibitors in the TLR signaling pathway, no significant changes occurred in representative factors, such as *SHIP*, *SOCS-1*, and *MyD88*, in the eyes of EIU-tolerant rats. *TLR* expression also was not downregulated. Transforming growth factor- β , which is thought to be a key immunosuppressive agent in the aqueous humor, was not upregulated in EIU tolerance. Among the genes we investigated, only *IL-10* seemed to be involved in this event.

Previously, LPS tolerance for IL-10 production was observed in murine and human monocytes/macrophages *in vivo* and *ex vivo*.³⁹⁻⁴³ On the other hand, several researchers have recently reported that LPS induced IL-10 production in dendritic cells.^{44,45} The immunohistochemical examination in this study demonstrated that dendritic cells, not macrophages, produced IL-10 in the ICB of the EIU-tolerant rat. Therefore, we could speculate that dendritic cells also play some role in the development of EIU tolerance. This must be further investigated.

Given that CD14 also was expressed at low levels in the neutrophils,⁴⁶ it is reasonable that neutrophils are affected by an LPS challenge. However, though many investigators have

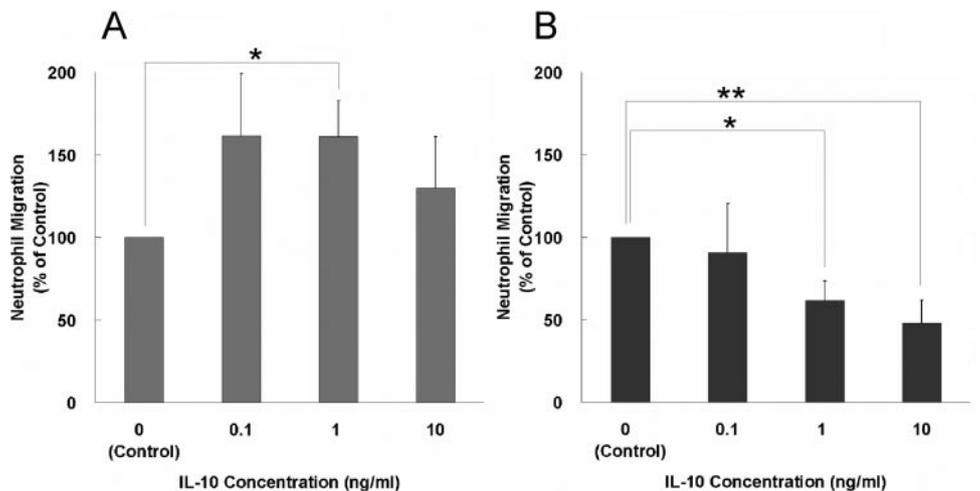


FIGURE 6. Change in the migration of neutrophils in tolerant and control rats after incubation of IL-10. (**A**) Neutrophils from control rats. (**B**) Neutrophils from EIU-tolerant rats. Isolated neutrophils were incubated for 30 minutes in various concentrations of recombinant rat IL-10. The mean ratio of fluorescence of neutrophils with IL-10 to that without IL-10 is shown as a percentage. Significant differences: * $P < 0.05$ and ** $P < 0.01$ by the paired *t*-test.

focused on monocytes and macrophages to investigate the mechanism of LPS tolerance, little is known about neutrophil function in LPS tolerance. Results of an in vitro experiment clearly showed that the ability of peripheral blood neutrophils to migrate in EIU-tolerant rats decreased by as much as 50% compared with that in normal rats. However, the results of an in vivo experiment showed that the ability decreased by one-fourth in EIU-tolerant rats. One reason for the significant difference between the in vitro and in vivo results might be the high expression of IL-10 in the ocular tissue of EIU-tolerant rats.

The most interesting finding in the present study was that IL-10 dramatically reduced the migratory ability of peripheral blood neutrophils in EIU-tolerant rats in vitro but significantly increased it in control rats. Results of an ELISA experiment showed that the protein level of IL-10 in the aqueous humor dramatically increased 24 hours after EIU induction. This high level of IL-10 enhanced the decrease of migratory ability in the donor neutrophils from the EIU-tolerant rats, resulting in few donor neutrophils that could invade the host inflamed eye.

The same phenomenon might occur in EIU-tolerant rats in our footpad model. The protein level of IL-10 in the aqueous humor increased up to 7 days after the first LPS injection, when neutrophil infiltration into the eye by a second LPS challenge was almost completely shut down. On day 14 after the first LPS injection, the protein level of IL-10 in the aqueous humor decreased to an undetectable level, and more than 500 neutrophils infiltrated the eye as a result of the second LPS challenge (Fig. 1A), suggesting that the protein level of IL-10 in the aqueous humor has some correlation with the number of neutrophils infiltrating the aqueous humor by the second LPS challenge in EIU-tolerant rats. PCR results clearly showed that gene expression of *IL-10* mRNA in the ICB sample was upregulated continuously throughout the period of EIU tolerance. Therefore, we speculated that the protein level of IL-10 in EIU-tolerant eyes somehow increased for a long period and regulated neutrophil infiltration into the eye by the second LPS challenge.

Because older rats are less sensitive to LPS than younger rats, one can speculate that similar mechanisms might be involved in the reduced capacity of aged rats to develop EIU. Interestingly, though we could not detect protein expression of IL-10 in the aqueous humor of 6-week-old and 18-week-old rats, we detected significantly high expression levels of IL-10 (309.8 pg/mL) in the aqueous humor of aged (1-year-old) rats. On the other hand, there was no difference between aged and young rats in neutrophil chemotactic function (data not shown). These results suggested that IL-10 may also play a role in the lower sensitivity to LPS in aged rats, but the mechanism was somehow different from that for LPS tolerance. Further investigation is needed for an understanding of the reduced capacity of the aged rat to develop EIU.

In summary, we investigated the mechanism of LPS tolerance in a footpad injection EIU model. Although few investigations have focused on the mechanism of the development of EIU tolerance, recent studies have suggested that macrophages play a central role in LPS tolerance. In addition to previous findings, this study revealed two other players in the development of EIU tolerance, circulating neutrophils and dendritic cells in ICB. The former decreased their chemotactic ability in EIU-tolerant rats. The latter produced IL-10 in EIU-tolerant rats and IL-10 modulated neutrophil chemotactic function. Understanding how dendritic cells infiltrate the eye of the EIU-tolerant rat and secrete IL-10 and how LPS and IL-10 synergistically suppress neutrophil migration will facilitate the development of new therapeutic strategies in ocular inflammatory disease, especially neutrophil dominant inflammation, such as acute anterior uveitis and Behçet disease.

References

- Rosenbaum JT, McDevitt HO, Guss RB. Endotoxin-induced uveitis in rats as a model for human disease. *Nature*. 1980;286:611-613.
- Koizumi K, Poulaki V, Doehmen S, et al. Contribution of TNF-alpha to leukocyte adhesion, vascular leakage, and apoptotic cell death in endotoxin-induced uveitis in vivo. *Invest Ophthalmol Vis Sci*. 2003;44:2184-2191.
- Pouvreau I, Zech JC, Thillaye-Goldenberg B. Effect of macrophage depletion by liposomes containing dichloromethylene-diphosphate on endotoxin-induced uveitis. *J Neuroimmunol*. 1998;86:171-181.
- Suzuma K, Mandai M, Kogishi J. Role of P-selectin in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 1997;38:1610-1618.
- Suzuma I, Mandai M, Suzuma K. Contribution of E-selectin to cellular infiltration during endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 1998;39:1620-1630.
- Becker MD, O'Rourke LM, Blackman WS. Reduced leukocyte migration, but normal rolling and arrest, in interleukin-8 receptor homologue knockout mice. *Invest Ophthalmol Vis Sci*. 2000;41:1812-1817.
- Ohta K, Nakayama K, Kurokawa T. Inhibitory effects of pyrrolidine dithiocarbamate on endotoxin-induced uveitis in Lewis rats. *Invest Ophthalmol Vis Sci*. 2002;43:744-750.
- Howes EL Jr, Goldyne ME, Perez HD. Lipopolysaccharide tolerance inhibits eye inflammation, I: reduced immune complex or lipopolysaccharide effects. *Arch Ophthalmol*. 1985;103:257-260.
- McMenamin PG, Crewe J. Endotoxin-induced uveitis: kinetics and phenotype of the inflammatory cell infiltrate and the response of the resident tissue macrophages and dendritic cells in the iris and ciliary body. *Invest Ophthalmol Vis Sci*. 1995;36:1949-1959.
- Yoshida M, Yoshimura N, Hangai M. Interleukin-1 alpha, interleukin-1 beta, and tumor necrosis factor gene expression in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 1994;35:1107-1113.
- de Vos AF, van Haren MA, Verhagen C. Kinetics of intraocular tumor necrosis factor and interleukin-6 in endotoxin-induced uveitis in the rat. *Invest Ophthalmol Vis Sci*. 1994;35:1100-1106.
- Bhattacharjee P, Parke A. The reduction of inflammatory responses in lipopolysaccharide-tolerant eyes. *Am J Pathol*. 1986;122:268-276.
- Chang JH, Hampartzoumian T, Everett B. Changes in Toll-like receptor (TLR)-2 and TLR4 expression and function but not polymorphisms are associated with acute anterior uveitis. *Invest Ophthalmol Vis Sci*. 2007;48:1711-1717.
- Hashida N, Ohguro N, Nakai K, et al. Microarray analysis of cytokine and chemokine gene expression after prednisolone treatment in murine experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci*. 2005;46:4224-4234.
- Cuschieri J, Billigren J, Maier RV. Endotoxin tolerance attenuates LPS-induced TLR4 mobilization to lipid rafts: a condition reversed by PKC activation. *J Leukoc Biol*. 2006;80:1289-1297.
- Sly LM, Rauh MJ, Kalesnikoff J. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity*. 2004;21:227-239.
- Rauh MJ, Ho V, Pereira C, et al. SHIP represses the generation of alternatively activated macrophages. *Immunity*. 2005;23:361-374.
- Nomura F, Akashi S, Sakao Y, et al. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol*. 2000;164:3476-3479.
- Hoekzema R, Murray PI, van Haren MA. Analysis of interleukin-6 in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 1991;32:88-95.
- Nakagawa R, Naka T, Tsutsui H, et al. SOCS-1 participates in negative regulation of LPS responses. *Immunity*. 2002;17:677-687.
- Alves-Rosa F, Vulcano M, Beigier-Bompadre M. Interleukin-1beta induces in vivo tolerance to lipopolysaccharide in mice. *Clin Exp Immunol*. 2002;128:221-228.
- Randow F, Syrbe U, Meisel C, et al. Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta. *J Exp Med*. 1995;181:1887-1892.

23. Smith JR, Hart PH, Coster DJ. Mice deficient in tumor necrosis factor receptors p55 and p75, interleukin-4, or inducible nitric oxide synthase are susceptible to endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 1998;39:658-661.
24. Liew FY, Xu D, Brint EK. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol.* 2005;5:446-458.
25. Ellaban E, Bolgos G, Remick D. Selective macrophage suppression during sepsis. *Cell Immunol.* 2004;231:103-111.
26. Kawai T, Adachi O, Ogawa T. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity.* 1999;11:115-122.
27. Lang R, Heeg K. Semiquantitative determination of human cytokine mRNA expression using TaqMan RT-PCR. *Inflammopharmacology.* 1998;6:297-309.
28. Thornton AM, Donovan EE, Piccirillo CA. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol.* 2004;172:6519-6523.
29. Milburn HJ, Poulter LW, Dilmec A. Corticosteroids restore the balance between locally produced Th1 and Th2 cytokines and immunoglobulin isotypes to normal in sarcoid lung. *Clin Exp Immunol.* 1997;108:105-113.
30. Duffy AJ, Nolan B, Sheth K. Inhibition of alveolar neutrophil immigration in endotoxemia is macrophage inflammatory protein 2 independent. *J Surg Res.* 2000;90:51-57.
31. Bellingan GJ, Caldwell H, Howie SE. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol.* 1996;157:2577-2585.
32. Lovas K, Knudsen E, Iversen PO. Sequestration patterns of transfused rat neutrophilic granulocytes under normal and inflammatory conditions. *Eur J Haematol.* 1996;56:221-229.
33. Cheretakis C, Leung R, Sun CX. Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model. *Blood.* 2006;108:2821-2826.
34. Bochud PY, Calandra T. Pathogenesis of sepsis: new concepts and implications for future treatment. *BMJ.* 2003;326:262-266.
35. Haziot A, Chen S, Ferrero E. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J Immunol.* 1988;141:547-552.
36. Setoguchi M, Higuchi Y, Yoshida S, et al. Insertional activation of N-myc by endogenous Moloney-like murine retrovirus sequences in macrophage cell lines derived from myeloma cell line-macrophage hybrids. *Mol Cell Biol.* 1989;9:4515-4522.
37. Wright SD, Ramos RA, Tobias PS. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* 1990;249:1431-1433.
38. Han J, Ulevitch RJ. Limiting inflammatory responses during activation of innate immunity. *Nat Immunol.* 2005;6:1198-1205.
39. Frankenberger M, Pechumer H, Ziegler-Heitbrock HW. Interleukin-10 is upregulated in LPS tolerance. *J Inflamm.* 1995;45:56-63.
40. Randow F, Syrbe U, Meisel C, et al. Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta. *J Exp Med.* 1995;181:1887-1892.
41. Wysocka M, Robertson S, Riemann H, et al. IL-12 suppression during experimental endotoxin tolerance: dendritic cell loss and macrophage hyporesponsiveness. *J Immunol.* 2001;166:7504-7513.
42. Sato S, Nomura F, Kawai T, et al. Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J Immunol.* 2000;165:7096-7101.
43. Karp CL, Wysocka M, Ma X, et al. Potent suppression of IL-12 production from monocytes and dendritic cells during endotoxin tolerance. *Eur J Immunol.* 1998;28:3128-3136.
44. Yanagawa Y, Onoe K. Enhanced IL-10 production by TLR4- and TLR2-primed dendritic cells upon TLR restimulation. *J Immunol.* 2007;178:6173-6180.
45. Samarasinghe R, Tailor P, Tamura T, Kaisho T, Akira S, Ozato K. Induction of an anti-inflammatory cytokine, IL-10, in dendritic cells after toll-like receptor signaling. *J Interferon Cytokine Res.* 2006; 26:893-900.
46. Ball ED, Graziano RF, Shen L. Monoclonal antibodies to novel myeloid antigens reveal human neutrophil heterogeneity. *Proc Natl Acad Sci U S A.* 1982;79:5374-5378.