Article

Different Characters of Spleen OX-62 Positive Dendritic Cells between Fischer and Lewis Rats

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The phenotype, DNA-binding activities of NF-κB, cytokine production, endocytosis and stimulatory capacity of spleen OX-62-positive dendritc cells (SDCs) from Fischer rats were compared with those from Lewis rats. Results showed that the expressions of CD11b, MHC-II, CD8, CD45RA, CD54 and CD86 on SDCs were significantly higher in Fischer than those in Lewis rats. The levels of IL-2, IL-4, IL-10 and IFN-γ in SDCs from Fischer rats were distinctly higher than those from Lewis. Both stimulatory capacity and DNA-binding activities of NF-κB in SDCs were all lower in Fischer than those in Lewis rats. These differences may partly contribute to rat strain-specificity in susceptibility to chronic inflammatory stimuli. *Cellular & Molecular Immunology*. 2006;3(2):145-150.

Key Words: SDC, NF-κB, endocytosis, stimulatory capacity

Introduction

Lewis and Fischer rats, two inbred rat strains, exhibit different responses to inflammatory stimuli of carrageenan, streptococcal cell walls and lipopolysaccharide (LPS) (1-3). Some researchers attributed the strain-specific differences in inflammatory and disease susceptibility to environmental, genetic, neuroendocrinal factors (4, 5). However, as is known, dendritic cells (DCs) are usually regarded as a potential antigen-presenting cell (APC) involved in T cell activation (6). They also directly or indirectly affect B cell function, antibody synthesis and isotype switch (7, 8). Furthermore, some studies find that rat spleen DCs show heterogeneity in population (9). Spleen is one of the most important immune organs, in which DCs play a key role in the regulation of the immune reactions (10). So we postulated that DCs were involved in the differences in susceptibility to inflammatory stimuli between rat strains. In the present study, we compared surface markers, DNA-binding activities of NF-κB, cytokines, endocytosis and stimulatory capacity of OX-62 positive DCs (SDCs) derived from Fischer and Lewis rats and tried to determine that the susceptibility to inflammatory stimuli is related to SDC.

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Materials and Methods

Animals

Lewis and Fischer rats (SPF) of 6-8 weeks were purchased from Shanghai SLAC Laboratory Animal, Co. Ltd., China. They were housed in a temperature and humidity controlled room with a 12 h light: dark cycle. Food and water were available and rats were maintained in our animal facilities for at least 1 week before being used in the following experiments.

Reagents

Collagenase D, Cell Counting Kit-8 (CCK-8), mitomycin C, phytohaemagglutinin (PHA), lipopolysaccharide (LPS), fetal calf serum (FCS), fluroscein isothiocyanate (FITC) conjugated dextran were all purchased from Sigma Chemical Co. (Louis, Mo, USA). RPMI 1640 was purchased from HyClone Laboratories (Logan, UT, USA). Rat Fc Block, Cytofix/Cytoperm Plus (with GolgiStop), R-phycoerythrin (PE) conjugated monoclonal antibodies of IgG2a, IgG2b, I-Ad, CD8α, CD11b, CD40, CD45R, CD80, CD86, IL-2, IL-4, IL-10, IL-12, IFN-γ and TNF-α were bought from BD Biosciences, Pharmingen. Anti-rat OX-62 antibody labeled microbeads, rat pan T cell microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Both PHA and LPS were dissolved in phosphate-buffered saline (PBS) to the final concentration of 5 μg/ml.

SDCs preparation

Rats were euthanatized by cervical dislocation and then kept

in 75% ethanol for 10 min. The spleens were teased apart and digested with 2 mg/ml collagenase D for 30 min at 37°C in the presence of 10 μM EDTA during the last 5 min (11). The cells were passed through a steel mash and the erythocytes were deleted by ACK solution (0.83% NH_4Cl, 0.1% $KHCO_3$ and 0.004% EDTA，pH 7.2-7.5). Then cells were passed through a filter, collected in a 50 ml tube, washed and counted. After supernatant removed completely, 20 μl of anti-rat OX-62 antibody labeled microbeads were added, and cells were resuspended in 80 μl of buffer per 10^7 cells at 4°C for 15 min. The OX-62 positive cells were then richened by immunomagnetic bead selection with MACS Column, then resuspended in RPMI 1640 media (10 % FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercapto-ethanol, 100 U/ml penicillin and 0.1 mg/ml streptomycin). The purity of SDCs obtained in this manner was up to 90% through the stain of PE-CD11c antibodies and analysis by flow cytometry. The viability was determined by trypan blue dye exclusion and was routinely more than 95%.

Analysis of SDC surface marker expression

Cell surface markers were studied by flow cytometry using antibodies suggested in the literature to reflect DC population at different states of maturation. The following PE-labeled monoclonal antibodies were used: OX-42 (anti-CD11b), OX-6 (anti-MHC-II), and OX-19 (anti-CD5), OX-8 (anti-CD8), OX-33 (anti-CD45RA), TLD-4C9 (anti-CD54), 3H5 (anti-CD80), 24F (anti-CD86). After SDCs were treated with 5 μg/ml LPS for 12 h, cells were incubated with primary mAbs for 30 min at 4°C and then washed. Fluorescence was measured using a FACS Calibur flow cytometry (Becton Dickinson) and data analysis was performed using the Cell Quest Software (Becton Dickinson, San Diego, CA).

Analysis of intracellular cytokines

Cytokine production was measured by flow cytometry. SDCs were cultured in the presence of 5 μg/ml LPS and GolgiStop for 12 h and followed by treatment with Fc Block for 15 min at 4°C. Then the cells were washed, fixed and permeabilized with Cytofix/Cytoperm solution. SDCs were incubated with PE-labeled monoclonal antibodies of IL-2, IL-4, IL-10, IFN-γ and TNF-α for 30 min. Then SDCs were washed twice with wash buffer and once with FACSstaining buffer (PBS, containing 1% BSA and 0.09% NaN_3) before analysis on the FACScan. For each experiment, the cells were stained with isotype control antibodies to establish background staining and to set the quadrants before calculating the percentage of positive cells, and 10,000 cells total events were collected.

Evaluation of NF-κB activation

SDCs were cultured in the presence of 5 μg/ml LPS for 12 h. NF-κB was detected in nuclear protein extracts by an electrophoretic mobility shift assay (EMSA).

Nuclear extracts were prepared by Nuclear Extract Kit. Cells were harvested and rinsed with cold PBS, scraped, collected by centrifugation, then resuspended in 300 μl lysis buffer (50 mM KCl, 25 mM HEPES, 0.5% IGEPAL CA630, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), transferred to a 1.5 ml eppendorf tube and kept on ice for 4 min. Nuclei were collected by centrifugation (10,000 rpm, 10 min at 4°C) and washed with 300 μl washing buffer (50 mM KCl, 25 mM HEPES, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 uM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Nuclei were pelleted (10,000 rpm, 10 min at 4°C) and resuspended in 30-100 μl extraction buffer (500 mM KCl, 25 mM HEPES, 10% glycerol, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 20 min. The suspension was centrifuged (14,000 rpm, 2 min at 4°C) and the supernatant was obtained for EMSA. Protein concentration was measured using the Bradford assay (Bradford, 1976) and adjusted to 1 μg/ml in extraction buffer.

EMSA was performed as the following. EMSA reactions consisted of 12 μl of 2 × gel shift reaction buffer (12% glycerol, 24 mM HEPES, 8 mM Tris-HCl, 2 mM EDTA, 1 mM dithiothreitol), 1 μl of bovine serum albumin (3 μg/μl), 2 μl of poly(dI-dC) (0.5 μg/μl), and 20,000-50,000 cpm γ^{32}P-DNA probe (Promega, Madison, Wisconsin, USA). Lastly, 3 μg of nuclear extract and a sufficient volume of extractin buffer were added to give 5 μl total volume. Samples were incubated on ice for 20 min, loaded on a 4% polyacrylamide gel and electrophoresed at 200 V for 1-1.5 h at 4°C. Gels were transferred to 3 MM Whatman paper, dried under vacuum at 80 for 1 h, and exposed overnight to either a phosphorimager screen or Kodak film.

Analysis of SDC endocytosis

The ability of SDCs to endocytose FITC-labeled dextran was determined by fluorescent cell counting. SDCs were incubated in the presence of 5 μg/ml LPS for 12 h and then cultured with 1 μg/ml FITC labeled dextran at 37°C for 0, 1.5, 3.0, 4.5, 6.0 h, respectively. Three parallel wells were set in each time point. In the indicated time, SDCs were collected and washed by cold PBS containing 1% FCS and 0.1% NaN_3 for four times. The cells were fixed in 1% polyformaldehyde and analyzed on a FACS Calibur flow cytometry (Becton Dickinson).

Mixed lymphocyte reaction (MLR)

The ability of SDCs to stimulate an allogeneic MLR was determined as the following. SDCs, as stimulant cells, were cultured with 5 μg/ml LPS for 12 h and treated with 25 μg/ml mitomycin C. Allogeneic T cells, as responder cells, were obtained from spleen by rat pan T cell microBeads. The reactions were carried out in round-bottomed 96-well plates to ensure efficient DC/T cell contact. SDCs were cocultured with T cells at a ratio of 0, 1:16, 1:8, 1:4, 1:2; 1:1 (SDCs/T cells) for 5 days in a CO_2 incubator. Three parallel wells were set in each ratio of SDC/T cell. T cell proliferation was measured by Cell Counting Kit-8 using a microplate reader.

Statistical analysis

Results were presented as the mean ± SD. Statistical

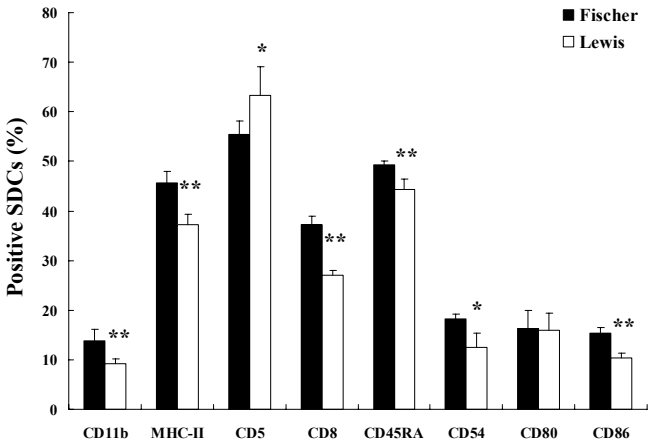

Figure 1. Comparison of the phenotype of SDCs between Fisher and Lewis rats. The surface expressions of different molecules on purified SDCs were analyzed by flow cytometry. The levels of CD11b, MHC-II, CD8, CD45RA, CD54 and CD86 molecules on SDCs from Fischer rats were higher than those from Lewis while CD5 was lower. Data represent the mean ± SD from three independent experiments. *$p < 0.05$, ** $p < 0.01$ *vs* Lewis rats.

significance between groups was analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple comparisons tests. A p value of < 0.05 was considered significant, and $p < 0.01$ was considered very significant.

Results

Comparison of the phenotype of SDCs

Our results showed that CD11b, MHC-II, CD8, CD45RA, CD54 and CD86 expressions on SDCs were notable higher in Fischer rats than those in Lewis rats excepting CD5. There was no obvious difference in CD80 expression from both rats (Figure 1).

Comparison of NF-κB DNA-binding activity of SDCs

DNA-binding activities of NF-κB in SDCs (5 μg/ml LPS) were lower in Fischer rats than those in Lewis rats and all had statistical differences. Furthermore, in the control (no LPS stimulation), NF-κB DNA-binding activities of SDCs were notably lower than those treated with LPS (Figure 2).

Comparison of the levels of intracellular cytokines of SDCs

The levels of IL-2, IL-4, IL-10 and IFN-γ in SDCs were significantly higher in Fischer rats than those in Lewis rats, while the level of TNF-α was lower (Figure 3).

Comparison of the endocytosis of SDCs

There were slight changes in endocytosis of SDCs from Fischer rats compared to Lewis, but no statistical difference was observed (Figure 4).

Comparison of the stimulatory capacities of SDCs

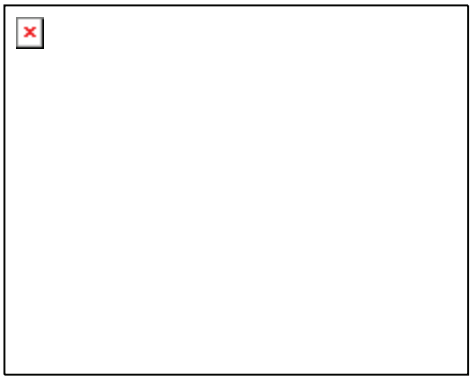

Figure 2. Comparison of DNA-binding activity of NF-κB in SDCs between Fischer and Lewis rats. NF-κB DNA-binding activity was analyzed by EMSA. FP: free probe; PC: positive control; BC: blank control.

The stimulatory capacities of SDCs were lower in Fischer rats than those in Lewis rats and all had statistical differences (Figure 5).

Discussion

Fischer and Lewis rats have been characterized by their different reactivity to a variety of pro-inflammatory stimuli (3, 12). Numerous studies have compared a number of biochemical (13), physiological (14), and behavioral endpoints (5) of these inbred strain rats. Previous researches also found that PS-PG-induced IBD model could be built in Lewis rats, but not in Fischer. Our results showed that CD11b, MHC-II, CD8, CD45RA, CD54 and CD86 expressions on

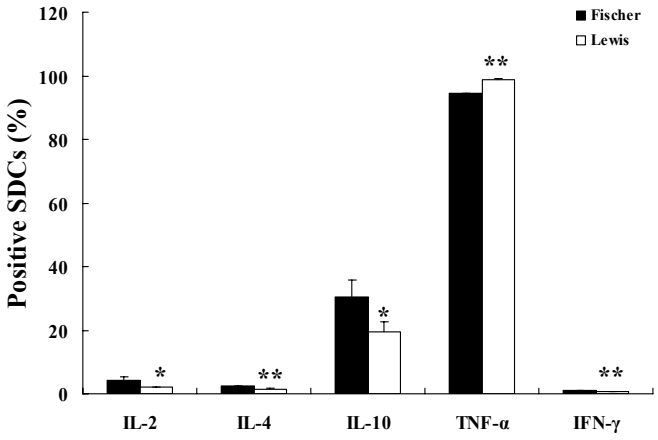

Figure 3. Comparison of cytokine expression in SDCs between Fisher and Lewis rats. After 12 h culture with 5 μg/ml LPS, SDCs were stained with R-PE labeled monoclonal IL-2, IL-4, IL-10, IFN-γ and TNF-α antibodies. Fluorescence was analyzed by flow cytometry. Data represent the mean ± SD from three independent experiments. *$p < 0.05$, ** $p < 0.01$ *vs* Lewis rats.

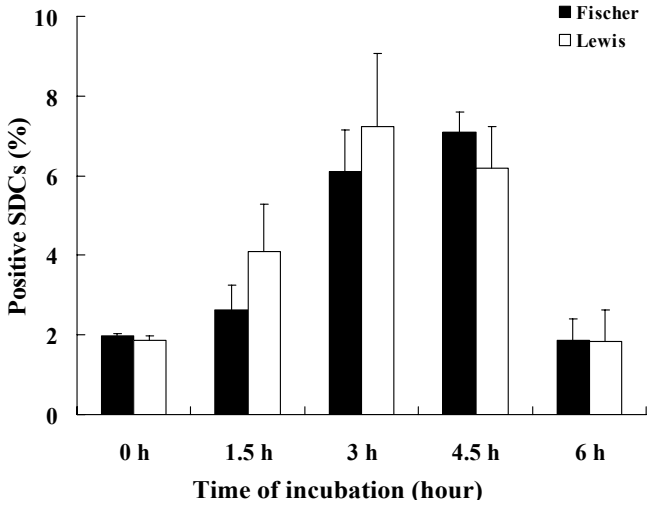

Figure 4. Comparison of the endocytosis of SDCs between Fisher and Lewis rats. SDCs were purified and cultured with FITC-dextran in the medium for 0, 1.5, 3, 4.5 and 6 h respectively. The dextran uptake was analyzed by flow cytometry. The results showed there was no statistical difference between these two rat strains.

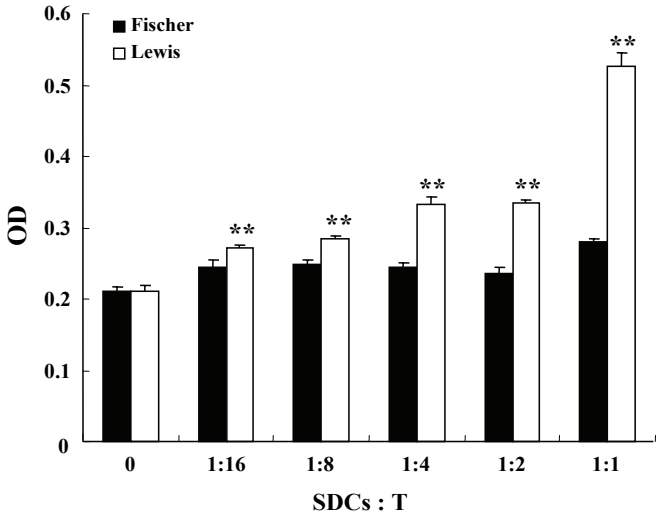

Figure 5. Comparison of the stimulatory capacity of SDCs between Fisher and Lewis rats. SDCs were used as stimulators, while allogeneic T cells, also obtained from the spleens, were served as responders. SDCs were added in graded doses to T cells at various ratios. Then the proliferation of T cells was measured by CCK assay. Data represent the mean ± SD of five parallel wells from three independent experiments. *$p < 0.05$, **$p < 0.01$ *vs* Lewis rats.

SDCs were notable higher in Fischer rats than those in Lewis rats excepting CD5. As we have known, immature DCs have a low expression of costimulatory molecules, but are very efficient in antigen uptake and processing. With further maturation, they lose their endocytic capacity and acquire a full repertoire of costimulatory antigens and allostimulatory activity (15, 16). According to our results, higher levels of MHC-II and costimulatory molecules reflected more mature status of SDCs from Fischer rats than those from Lewis.

DNA-binding activity of NF-κB in SDCs from Fischer rats was notable low compared with that of Lewis. The nuclear transcription factor NF-kB is important in the expression of many genes which are involved in the control of apoptosis (cell suicide), development of B and T cells, anti-viral and bacterial responses, responses to multiple stresses, embryonic development and inflammatory responses. In most untrans- formed cells, NF-κB complexes are largely cytoplasmic and remain transcriptionally inactive until the cell is stimulated (17). Once the cell is activated, NF-κB will be liberated and accumulated in the nucleus where it activates the expression of specific genes involved in immunity, inflammation, and proliferation (18, 19). Recently, NOD2 (nucleotide-binding oligomerization domain), the first susceptibility gene linked with Crohn's disease, was highly restricted to monocytes, macrophages and DCs (20). It directly binds bacterial LPS and subsequently acts as an activator of NF-κB (21). NF-κB has more than two hundred biological effect and plays an important role in the inflammation (7). DNA-binding activity of NF-κB in SDCs from Fischer rats was deeply low and maybe reflected the blocking of NOD2 signaling pathway and impaired immune response.

NF-κB is a major regulator of cytokine production but there isn't a positive linear relationship of cytokine expression and DNA-binding activity of NF-κB (22). In previous reports, when exposure to MDP (muramyl dipeptide) derived from peptidoglycan, human DCs secrete IL-1β, IL-6, IL-12, and TNF (23-25). Our results showed that the levels of IL-2, IL-4, IL-10 and IFN-γ in SDCs from Fischer rats were notable higher than those from Lewis while the level of TNF-α was lower. IL-2 produced by T cells or DCs induces proliferation and activation of both $CD4^+$ and $CD8^+$ T cells (26-28). IL-4, an anti-inflammatory cytokine, downregulates both the innate and the adaptive immune responses (29). IL-10 is mostly secreted by DC and Th2 cells (30, 31), and inhibits the production of all pro-inflammatory cytokines (32). IFN-γ partly produced by DCs (33) has a strong role to regulate the immune response and enhance the expression of MHC-II while TNF-α is a pro-inflammatory cytokine (34). So we concluded that high level of TNF-α and low levels of IL-4 and IL-10 in SDCs from Lewis rats might partly explain their more susceptibility to PG-PS-induced IBD than Fischer.

Our results also showed that the stimulatory capacities of SDCs from Fischer rats were lower than those from Lewis. In view of cytokine production of SDCs from these two strains of rats, the higher levels of IL-4 and IL-10 (35) and the lower level of TNF-α in SDCs from Fischer rats may reflect both lower levels of Th1 and Th2 type immune responses. The level of IL-2 in SDCs is lower in Fischer rat than that in Lewis rat, which might have a slight influence on the proliferation of T cells. This seemed to be a paradox to the more mature of SDCs from Fischer rats. In fact, stimulatory

capacity of DCs stemmed not only from the high levels of MHC they express, but also from their capacity to produce peptide-MHC complexes from minute quantities of almost any antigen (36). Moreover, rat SDCs comprised two major subsets identified by CD4/SIRPα^+ and CD4/SIRPα^- expression. Further studies showed that the CD4/SIRPα^+ subset selectively expressed CD5 (37) and was more potent in inducing T cell proliferation than CD4/SIRPα^- SDCs. Our results showed that the expression of CD5 on SDCs from Fischer rats was lower than that in Lewis rats, which meant the amount of CD4/SIRPα^+ subset of SDCs from Fischer rats was less and the stimulatory capacity of them was lower than those from Lewis.

In conclusion, SDCs from Fischer and Lewis rats appeared notable different in maturation, DNA-binding activity of NF-κB, cytokine production and stimulatory capacity. These might partly explain rat strain-specific differences in susceptibility to chronic inflammatory stimuli.

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