Original Article Protection by LPS-induced inhibitory CD11b⁺ cells on corneal allograft

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Received December 31, 2014; Accepted February 28, 2015; Epub March 15, 2015; Published March 30, 2015

Abstract: Objective: It is widely reported that CD11b⁺Gr1+ myeloid-derived suppressor cells can cause allograft tolerance in mice and human, however, little is known on the therapy role in chronic transplantation rejection. In this paper, their role in corneal transplantation was studied for the first time. Method: Inhibitory CD11b⁺ cells were obtained by murine LPS-induced septic model. Phenotype, endocytosis, antigen presenting ability, and T cell suppression assays were performed by flow cytometry analysis. The suppressive ability *in vivo* was analyzed by targeting allogeneic corneal transplantation. Results: LPS was intraperitoneally injected into C57BL/6 mice, the percentage of CD11b⁺ Gr1+ cells was increased in mice spleen, blood, and bone marrow, respectively. Compared with control mice, Ly6C, TLR2, and MHC-11 expression were higher in LPS treated mice. CD11b⁺ Gr1+ cells could inhibit allogenic corneal reaction *in vivo* after adoptive transfer, in consistent with an observation of inhibition effect on the antigen presenting cells (APCs) and CD4+ T cells proliferation *in vitro*. Conclusion: CD11b⁺ cells induced by LPS could function as inhibitory APCs, suppress CD4+ T cells proliferation and improve corneal allograft survival. Predictly, its application for cells transfer therapy in clinic in the further.

Keywords: CD11b⁺, corneal transplantation, LPS

Introduction

T cell-dependent immune response plays a relatively important role in allograft rejection, because the ultimate fate of allografts was partially determined by the balance between the effector and suppressor T cells. Acute corneal allograft rejection is closely connected with T-helper (Th) 1 and Th2 differentiation, and often correlates with INF-y expression in allograft, and INF-y production upon CD4+ T cells re-stimulation with alloantigen [1-3]. Actually, graft rejection is mainly mediated by Th1 cells, even more aggressively if Th1 and Th2 synergistic reaction happens [4, 5]. Recently, regulatory T cells (Tregs), including CD4+, CD25+, and Foxp3+, were reported to be associated with graft tolerance induction in mice and human [6-9]. Besides, tolerogenic dendritic cells (tDCs) and immature dendritic cells (iDCs) were proved to prevent alloreactive T cells in transplantation [10, 11].

As a heterogeneous population of cells that expand during cancer, inflammation, infection

and transplantation, myeloid-derived suppressor cells (MDSCs) have a remarkable ability to regulate adaptive immune and innate immune responses [12-16]. Consisting of myeloid progenitor cells and immature myeloid cells, MD-SCs can propagate continuously in pathological conditions and results in the up-regulation of expression of immune suppressive factors, such as arginase 1 (ARG1) and inducible nitric oxide synthase (iNOS), as well as the increase production of nitric oxide (NO) and reactive oxygen species (ROS) [17, 18]. MDSCs lack the expression of cell-surface markers that are specifically expressed by monocytes, macrophages or dendritic cells (DCs), but they are characterized by the co-expression of myeloid-cells lineage differentiation antigen GR1 and CD11b in mice [19, 20]. Based on the new insights into the MDSCs suppress mechanism provided with Treg induction, cell-cell contact inhibition, and antigen-specific T-cell suppression [21-24], the hypothesis that adoptive cellular therapy with MDSCs may offer the opportunity to inhibit immune responses in transplantation is proposed. Until very recently, a feasible method for Treg cells expansion to promote corneal allograft survival has been established [25], and the aims of this study include: (i), defining LPSinduced *in vivo*; (ii) determining the expression, the function, and phenotype of resulted; (iii) testing the possibility of adoptive transfer with CD11b⁺ cells in corneal transplantation.

Material and methods

Mice and materials

The 6-8 week old male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Institute of Zoology, Chinese Academy of Sciences, and used as donors and recipients, respectively. LPS (*Escherichia coli*, Serotype 055:B5) was dissolved in 0.9% saline. 2.5 mg/kg LPS was given intraperitoneally to C57BL/6 mice once a day for 7 days. This dose and time was chosen because preliminary tests showed that it led to a lower mortality rate about 30% in C57 mice.

All animal care and procedures were approved by the Institutional Animal Care and Use Committee, and mice were used in accordance with the Association for research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Monoclonal antibodies for FACS

Anti-CD4-APC, anti-CD11b-APC, anti-Gr1-Per-CP5.5, anti-Ly6C-PE, anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, anti-CD273-PE, anti-CD275-PE, anti-TLR2-PE, anti-TLR4-PE, anti-MHC-II-PE, 7-amino-actino-mycin D (7-AAD)-PerCP5.5, Annexin-V-FITC, and isotype control antibodies were obtained for FACS analysis (eBioscience, San Diego, CA, USA).

Preparation of CD11b+ and CD4+ T cells

CD11b⁺ and CD4+ T cells were isolated by magnetic separation using CD11b⁺ positive isolation kit and CD4+ T negative isolation kit (Mitenyi biotec, Germany), a great purity of the sorted cells was detected by flow cytometry as expected (> 90%).

Endocytosis and antigen presenting ability (APA) of CD11b⁺ cells by FACS analysis

Isolated CD11b⁺ cells (1×10⁵/well) were re-suspended in complete medium and incubated at 37°C with 5% of CO₂. FITC-conjugated OVA (OVA-FITC) was added at a final concentration of 1 mg/mL or a same ratio of CD11b⁺ and

CD4+ T cells was instead. The cells were washed four times with cold phosphate buffered saline (PBS), and 5% bowin AB serum, and then analyzed by FACScalibur (Becton Dickinson) using FlowJo 7.6 software (Tree Star).

CD11b⁺ cells suppression assays

Suppression assays were performed in 96-well plates in triplicate. Fresh isolated naïve CD4+ T cells (1×10^5 /well) were incubated with mouse anti-CD3/CD28 mAb and added with indicated ratios of CD11b+ cells for 72 h. The T cells proliferation was determined by CFSE dilution profile. The suppression percentage was calculated using the following formula: suppression% = (T cells without MDSCs - T cells with MDSCs)/(T cells without MDSCs) ×100%.

Orthotropic corneal transplantation

Standard protocol for murine orthotropic corneal transplantation was used according to a previous description [26]. A 2 mm diameter of donor corneas was excised from BALB/c mice and sutured onto the recipient graft, a 2 mm of excising site in the C57BL/6 mice central corneal. All grafts were evaluated using slit-lamp biomicroscopy at weekly intervals. Rejected graft was defined when opaque occurred, in which condition that the iris details could not be clearly recognized with the standardized opacity grading (range of 0-5) scheme.

Measurement for CD11b⁺ cell function in vivo

Spleen CD11b⁺ cell obtained from LPS-induced mice $(1 \times 10^6/\text{mouse})$ was transferred to allograft recipients at the time of surgery. Allograft survival rate in each group (n = 10) was monitored up to 8 weeks.

Statistical analysis

Software SPSS13.0 was used for Student's t test. Allograft survival data was generated as Kaplan-Meier survival curves, and log-rank analysis was conducted for the comparisons between groups. Data are presented as mean \pm SD. A value of *P* < 0.05 was considered to be statistically significant.

Results

CD11b⁺ Gr1⁺ cells expansion by LPS-induced

To investigate the immune-suppressive cells expression in LPS-indued mice, Two third of whole models were survived permanent, and



Figure 1. MDSCs expression on day 8 after LPS induced. A. Flow cytometry analysis for CD11b⁺Gr1+ cell intensity. B. CD11b⁺Gr1+ cell increase in spleen, blood and bone marrow by LPS, respectively. Data are mean \pm SD of triple determinations. Significant difference between the LPS and PBS mice (control): #P < 0.001.

MDSCs	LPS		PBS	
	Positive Cells Percentage	Mean Fluorescence Intensity (MFI)	Positive Cells Percentage	Mean Fluorescence Intensity (MFI)
Ly6C	98.6 ± 1.16***	1105 ± 176***	5.6 ± 1.32	5.6 ± 1.32
CD40	18.2± 2.29	805.4 ± 50.7	12.7 ± 4.82	765 ± 278.6
CD80	54.0 ± 2.40	54.0 ± 2.40	49.0 ± 4.48	49.0 ± 4.48
CD86	39.3 ± 7.23	550.2 ± 135.1	41.6 ± 4.22)	552 ± 35.3
CD273	10.6 ± 1.73	1040 ± 172	14.3 ± 1.41	1072 ± 142
CD275	2.0± 0.30	514 ± 21.3	2.73 ± 0.55	503 ± 21.7
TLR2	66.9 ± 2.77***	366 ± 26.2	15.8 ± 3.2	157 ± 17.1
TLR4	10.0 ± 2.60	319 ± 76.0	12.9± 4.12	348 ± 55.2
MHC-II	62.3 ± 1.54	1194± 107*	62.0 ± 1.91	929 ± 135

Table 1. Positive CD11b⁺ Gr1⁺ cells percentage in LPS and PBS mice (control)

Note: Data are presented as mean \pm SD of triple independent experiments. Significant difference between the LPS and PBS mice: *: P < 0.05; **: P < 0.02; **: P < 0.02; **: P < 0.01.

then the expression of CD11b⁺ Gr1+ cells in LPS-induced model at day 8 was subsequently analyzed. As shown in **Figure 1**, the number of CD11b⁺ Gr1+ cells in spleen, blood and bone marrow was increased. The CD11b⁺ Gr1+ cell number in spleen was increased by 2.9% in sham mice, and 15.8% in LPS-induced mice, respectively. Similarly, an increase from 5.4% to 29.4% in bone marrow, and from 0.3% to 9.3% in blood was observed in LPS-induced mice. These results suggested that CD11b⁺ Gr1+ cells were increased in spleen, blood and marrow in sepsis mice.

Phenotypic profile of CD11b⁺ Gr1+ cells

To examine CD11b⁺ Gr1+ cells phenotype alteration of LPS-induced mice, various cell surface markers expressions were detected by flow cytometry analysis. Although the expression level for co-stimulation molecule CD80/CD86, CD40, immune-suppressive molecule PD-1L/PD-1, and Toll-like receptor 4 (TLR4) in CD11b⁺ Gr1+ cells from LPS-induced was similar to that in sham, a significantly higher expression of Ly6C, TLR2, and MHC-11 were observed in LPS-induced mice (**Table 1**). These results suggest that CD11b⁺ Gr1+ cells tend to elicit the immune response to endotoxin infection.

Functional analysis of CD11b⁺ Gr1+ cells by LPS

To determine CD11b $^+$ Gr1+ cells role on T lymphocytes activities, including antigen phago-



Figure 2. Phagocytic capacity, antigen presenting ability (APA) and suppressive function of CD11b+ cells. A. CD11b⁺ cells with (gray line) or without OVA-FITC (black line) after 6 h. B. CD11b+ cells with OVA-FITC and CD4+ T cells after 12 h (gray line) and 24 h (black line). C. CD11b⁺ cells from bone marrow of LPS (c-MDSCs, white histogram) and PBS mice (bm-MDSCs, gray histogram), as well as the spleen of LPS-induced mice (sp-MDSCs, black histogram). D. CD4+ T cells (1×10⁵/well) cultured with decreasing ratios of CD11b⁺ cells from spleen of LPS-induced mice or bone marrow of PBS mice in the presence of anti-CD3/CD28 beads, and E. CD4+ T cells proliferation. Data are mean \pm SD of triple determinations. Significant difference obtained by Wilcoxon rank sum test between the LPS and PBS mice: **P* < 0.05, ***P* < 0.02.



Figure 3. Protection of corneal allograft survival by inhibitory $CD11b^+$ cells. Graft survival with $CD11b^+$ cells, and PBS treatment after 5-week post-transplantation by Kaplan-Meier survival analysis. Data are mean ± SD. statistic difference between the $CD11b^+$ cells treatment and the PBS control: P =0.02.

cyte, antigen presenting ability (APA), and suppression function of T cells, coculture of isolated CD11b⁺ cells with OVA, or with OVA and CD4+ T cells was conducted. Co-culture of CD11b⁺ cells with CD4+ T cells and OVA showed that over 98% CD11b⁺ cells pulsed soluble Ag

OVA after 6 h, while 67% and 72% CD4+ T cells after 12 h and 24 h, respectively (**Figure 2A**, **2B**). To determine the suppressive ability of CD11b⁺ cells, CD4+ T cells were stimulated by anti-CD3/28 antibody in decreasing ratios of CD11b⁺ cells. A 52% inhibition of CD4+ T cells proliferation was achieved at CD11b⁺/T ratio of 1:2. At the ratio of 1:8, a 30% inhibition was observed in LPS-induced mice while no inhibitive effect was found in sham mice (**Figure 2D**). These findings suggested that CD11b⁺ cells may be functioned as immune-suppressive antigen presenting cells (APCs).

Protection of corneal allograft survival by inhibitory CD11b cells

To study the suppressive properties of inhibitory CD11b⁺ cells *in vivo*, CD11b⁺ cells (1×10⁶/ mouse) extracted from LPS-induced mice was transferred into syngeneic C57BL/6 mice receiving BALB/c cornea allografts without any immunosuppression drug treatment. The CD-11b treatment group showed improvement on allograft survival rate compared with control group (P = 0.02) (**Figure 3**). The mean survival time of CD11b treatment group was 21.4 days compared 10.9 days of control group. The results indicated that inhibitory CD11b⁺ cells adoptive transfer had immune-suppressive function to corneal alloreaction *in vivo*.

Discussion

The results here showed that CD11b⁺Gr1+ cells were markedly increased in the spleen, blood and bone marrow after LPS induced, and highly suppressive for activated CD4+ T cells. Although Ly6C was subsequently highly expressed, the level of co-stimulatory molecules CD80/86 and positive immune regulatory molecules PD-1/PD-1L was still low. Similar results in experimental autoimmune encephalomyelitis (EAE), tumor bearing, organ transplant and inflammatory bowel disease (IBD) were reported [27-29]. The high expression of TLR2 on sepsis MDSCs indicated a different phenotype between endotoxin infection and non-endotoxin model. According to Samia J. Khoury et al, the CD11b+ cells were divided into Ly6C^{high}. and Ly6C^{int} repress immature mononuclear and neutrophils cells [28]. In this study, Ly6C^{high} and Ly6C^{int} constitute 30% and 45% of CD11b+ splencytes, respectively.

It is proved that MDSCs can suppress the T cells by different mechanisms in vitro, including inhibitive factors production (iNOS, ARG1, IDO, and HO-1), Treg cells induction, and cell-cell contact inhibition [30]. The LPS-MDSCs were found more efficient than normal BM-MDSCs in antigen processing, despite that the 50% normal BM-MDSCs were conjunct with CD4+ T cells and OVA. Similar results were obtained in T cells suppressive experiment, indicating that LPS-MDSCs had a greater inhibition effect than normal MDSCs, even at low cell ratio. Considering the performance of MDSCs in co-stimulatory molecules expression, antigen processing ability and T cells suppressive function, this population could be defined as regulatory APCs.

Attentions on MDSCs transfer and transplantation have already been paid. In different organ transplants models, it is concluded that MDSCs transfer therapy can induce a long-term survival of allograft in kidney and skin, with the protection against experimental graft versus host disease (GVHD), but no prevention effect of MDSCs on allograft rejection in wide-type was reported [29, 31, 32]. However, our observations for prevention were obtained here, which could be explained clearly of it's immune suppressive role in organ transplantation [29, 33].

The MDSCs participation in tolerance induction or maintenance *in vivo* remains questionable. The role for adoptive transfer was directly evaluated in several transplants, the phenomenon that inhibitory CD11b⁺ shown suppressive alloreaction in experimental mode was demonstrated here. In summary, a reliable approach for MDSCs preparation by LPS is provided. The cells obtained show a characteristic phenotype of CD11b⁺ Gr1+ LyC6+ TLR2, with antigen processing function and immunosuppressive activity *in vitro*, suggesting that the MDSCs could be applied for transfer therapy in clinic.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No.81270975).

Disclosure of conflict of interest

None.

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