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High mobility group box 1 protein suppresses T cell-mediated immunity via CD11c^{low}CD45RB^{high} dendritic cell differentiation

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ABSTRACT

Aim: High mobility group box 1 protein (HMGB1) has been identified as a late proinflammatory cytokine and plays a key role in immune regulation. However, it is not yet clear whether HMGB1 can induce the activation and differentiation of dendritic cell (DC) subsets and subsequently modulate immune function of T cells. This study was performed to investigate the effect of HMGB1 on the differentiation of splenic DCs and its influence on T cell-mediated immunity in terms of DC subsets CD11c^{low}CD45RB^{high} DCs and CD11c^{high}CD45RB^{low} DCs in male BALB/c mice spleens *in vitro*.

Results: MACS microbeads were used to isolate splenic DCs, CD11c^{low}CD45RB^{high} DCs, CD11c^{high}CD45RB^{low} DCs and CD4^{+} T cells. The percentage of CD11c^{low}CD45RB^{high} DCs was significantly increased after treatment with HMGB1 compared to their counterparts (CD11c^{high}CD45RB^{low} DCs). It was found that unlike the gradually increasing interleukin (IL)-12 secretion of CD11c^{high}CD45RB^{low} DCs induced by HMGB1, CD11c^{low}CD45RB^{high} DCs showed a obvious dose-dependent response between IL-10 production and HMGB1 stimulation. In order to verify whether the alteration of CD4^{+} T cells was mainly associated with the differentiation of splenic DCs mediated by HMGB1 to CD11c^{low}CD45RB^{high} DCs, anti-IL-12 receptor (IL-12R) or anti-IL-10R monoclonal antibody was used to inhibit the effect of CD11c^{high}CD45RB^{low} DCs or CD11c^{low}CD45RB^{high} DCs in CD4^{+} T cells mixed lymphocyte reaction culture. After treatment with anti-IL-12R or anti-IL-10 monoclonal antibody in CD4^{+} T cells + CD11c^{high}CD45RB^{low} DCs or CD11c^{low}CD45RB^{high} DCs mixed lymphocyte reaction, the induction of these DCs on T cells was inhibited dramatically.

Conclusion: These data demonstrated that HMGB1 might induce the differentiation of splenic DCs to CD11c^{low}CD45RB^{high} DCs followed by shifting of Th1 to Th2 with enhancement of T lymphocyte immune function *in vitro*. Also, the effect of HMGB1 on T cell differentiation to Th2 was not associated with the inhibition of IL-12 production in CD11c^{high}CD45RB^{low} DCs.

1. Introduction

High mobility group box 1 protein (HMGB1) is a nonhistone, DNA-binding protein, which plays a critical role in regulating gene transcription [1]. Recently, HMGB1 has been identified as a late proinflammatory cytokine, and when it is extracellularly released may result in inflammatory response and endotoxin like lethality [2]. HMGB1, which is found as a late mediator appearing after endotoxin challenge [3], has triggered a series of research on its extrachromosomal activity and function as it is also released by activated macrophages [4–6], necrotic tissue [7,8], dendritic cells (DCs) [9], or natural killer cells [10]. Extracellular HMGB1 has been shown to be able to provoke inflammation, to regulate the migration of monocytes [11], and to contribute to DC maturation via the receptor for advanced glycation end products (RAGE) as well as induction of immune responses *in vitro* [9,12–14].

HMGB1 acts as the second messenger and effector throughout all the process of DC differentiation and regulates important DC functions, suggesting that its release at sites of cell injury or damage might play a role in the initiation and/or perpetuation of an immune response. Our recent studies found that an excessive formation of HMGB1 significantly suppressed T cell proliferation during postburn days 1–7, and concomitantly expression levels of interleukin (IL)-2 in T cell supernatant and IL-2Rα on T cell surface were decreased to certain extent. It was also noted that T cells polarized to Th2 cells after HMGB1 stimulation *in vivo* [15]. The above findings indicate that there is a marked immune suppression of T cells induced by HMGB1 [16–18]. However, it is not clear whether HMGB1 can induce the differentiation of DC subsets and subsequently modulate T cell-mediated immunity. The present

Abbreviations: HMGB1, high mobility group box 1 protein; DC, dendritic cell; IL-4/10/12, interleukin-4/10/12; IFN-γ, interferon-γ; MLR, mixed lymphocyte reaction.

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study was performed in order to elucidate the potential effect of HMGB1 on the differentiation of splenic DCs to IL-10 producing $CD11c^{low}CD45RB^{high}$ DCs, which induced suppression of T lymphocyte immune function and shifting of helper T cell Th1 to Th2 *in vitro*.

2. Materials and methods

2.1. Mice

Male BALB/c mice were purchased from Laboratory Animal Center of Chinese Academy of Medical Sciences, Beijing. They were 6–8 weeks old, weighing 20 ± 1 g. All animals were housed in separate cages in a temperature-controlled room with 12 h light and 12 h darkness to acclimatize for at least 7 days before being used. All experimental manipulations were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Chinese PLA General Hospital (The Permit Number 'SYXK2002-006' for this study), Beijing, China.

2.2. Reagents and kits

Recombinant HMGB1 was purchased from Sigma, St. Louis, MO. Polymyxin B-agarose (Sigma, St. Louis, MO) was used for removing endotoxin from recombinant human HMGB1. Collagenase D from *Clostridium histolyticum* was purchased from Sigma, St. Louis, MO. Ficoll-Papue was purchased from Axis-shield Co., Norway. RPMI 1640, fetal calf serum (FCS), glutamine, penicillin, streptomycin, and HEPES were purchased from TianRunShanda Biotech Co. Ltd., Beijing, China. Polymyxin B was added to the cell culture medium at 10 μg/ml to neutralize the activity of endotoxin. The following monoclonal antibodies (mAbs) were used in mixed lymphocyte reaction (MLR): clone 1B1.3a (rat IgG1), a blocking mAb reactive with mouse IL-10 receptor (IL-10R), an appropriate immunoglobulin isotype control was clone G155–178 (BD Pharmingen, San Diego, CA). Clone 114 (rat IgG2), a blocking mAb reactive with mouse IL-12 receptor (IL-12R), an appropriate immunoglobulin isotype control was clone G155–178 (BD Pharmingen, San Diego, CA). Biotinylated mouse dendritic cell enrichment cocktail was obtained from BD Biosciences, Mountain View, CA comprising the following biotin-conjugated mAbs: anti-mouse CD2 (LFA-2), clone RM2-5; anti-mouse CD3e (CD3 epsilon chain), clone 145-2C11; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse CD49b (Integrin alpha2 chain), clone HMα2; anti-mouse CD147 (Basigin), clone RL73; anti-mouse Ly-6G and Ly-6C (Gr-1), and clone RB6-8C5; anti-mouse TER-119/erythroid cells, clone TER-119. The biotinylated mouse dendritic cell enrichment cocktail contains mAbs that recognize antigens expressed on peripheral erythrocytes and leukocytes that are not DCs. Concanavalin A (Con A), thiazole blue (MTT) and TritonX-100 were purchased from Sigma, St. Louis, MO.

2.3. Purification of splenic DCs, $CD11c^{high}CD45RB^{low}$ DCs and $CD11c^{low}CD45RB^{high}$ DCs

A single-cell suspension was prepared from the murine spleens under aseptic condition with the following method. Isolated spleen was placed in a 6 cm petri-dish with sufficient collagenase D solution to completely cover the bottom of the dish (5 ml/spleen). The spleen was then injected with 500 μl of collagenase D solution per spleen using a 1 ml syringe and a 25G needle, then the tissue was cut into small pieces by using a pair of sharp scissors. The splenic tissue was incubated in collagenase D solution for 30 min at 37 °C, then the entire substance was gently passed through a 70 μm cell strainer using a plunger. Splenic DCs were negatively selected from splenocytes using mouse dendritic cells enrichment Set-DM. Plasmacytoid dendritic cells (pDCs) were deleted from spleen DCs through positive selection by anti-mPDCA-1 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). First, $mPDCA\text{-}1^+$ PDCs are magnetically labeled with Anti-mPDCA-1 MicroBeads. Then the cell suspension is loaded onto a Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled $mPDCA\text{-}1^+$ PDCs are retained on the column. The unlabeled cells run through and this cell fraction is depleted of $mPDCA\text{-}1^+$ PDCs.

Splenic $CD11c^{low}CD45RB^{high}$ DCs were then further positively selected from negative selection of cell fraction depleted of $mPDCA\text{-}1^+$ PDCs with biotinylated anti-mouse CD11c mAb (BD Biosciences, Mountain View, CA) plus IMag streptavidin Particles Plus-DM (BD Biosciences, Mountain View, CA). Therefore, splenic DCs were divided into two sections: $CD11c^{high}$ ($CD11c^{high}CD45RB^{low}$ DCs) and $CD11c^{low}$ DCs. Splenic $CD11c^{low}CD45RB^{high}$ DCs were then further positively selected from $CD11c^{low}$ DCs with biotinylated anti-mouse CD45RB mAb (BD Biosciences, Mountain View, CA) plus IMag streptavidin Particles Plus-DM (BD Biosciences, Mountain View, CA). The purity of $CD11c^{high}CD45RB^{low}$ DCs or $CD11c^{low}CD45RB^{high}$ DCs was higher than 85% as shown by fluorescence-activated cell sorting (FACS) analysis.

2.4. Purification of splenic $CD4^+$ T cells

The $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec GmbH, Germany) was an indirect magnetic labeling system for the isolation of untouched $CD4^+$ T cells from suspensions of murine spleen cells. Non-$CD4^+$ T cells, i.e. cytotoxic T cells, B cells, natural killer cells, DCs, macrophages, granulocytes and erythroid cells were indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD8a (Ly-2), CD45R (B220), DX5, CD11b (Mac-1) and Ter-119, and anti-biotin MicroBeads. Isolation of highly pure $CD4^+$ T cells was achieved by depletion of magnetically labeled cells.

2.5. Cell culture

Both DCs and DC subsets were cultured in RPMI 1640 + FCS (10%) approximately 24 h in the next step. Subsequently, the incubated cells were collected by gentle pipetting, washed with a 10 × excess volume of 1 × BD IMag™ buffer and centrifuged at 300*g* for 7 min. The supernatants were collected from each well for determination of IL-10 and IL-12 levels with enzyme-linked immunosorbent assay (ELISA) kits, strictly following the protocols provided by the manufacturer.

2.6. Mixed lymphocyte reaction (MLR) assay

$CD4^+$ T cells were suspended in RPMI 1640 culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and placed in 48-well round bottom plates for proliferation, reaching a final cell density of 10^6/ml with $CD11c^{low}CD45RB^{high}$ DCs in ratio of 1:50 and 1:100 ($CD11c^{low}CD45RB^{high}$ DCs to $CD4^+$ T cells). Cells were treated with 1 μg/ml anti-CD3 for 68 h at 37 °C in 5% CO_2/100% humidified air. Hundred microliters supernatant was decanted from each well, and 10 μl MTT (5 mg/ml) was added. After cultured for another 4 h, 100 μl Triton-Isop (10% TritonX-100, 50% Isopropanol, 0.01 mol/L HCl) was added into each well, the proliferation of $CD4^+$ T cells was measured by detection of optical density with a microplate reader (Spectra MR, Dynex, Chantilly, VA, USA) at the wavelength of 540 nm ($OD_{540\ nm}$). The whole procedure was repeated in quintuplicates.

After being stimulated for 24 h with 100 ng/ml HMGB1, DCs were separated from HMGB1 + DCs mixed culture, and subse-