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OX62+OX6+OX35+ rat dendritic cells are unable to prime CD4+ T cells for an effective immune response following acute burn injury

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ABSTRACT

Co-stimulatory molecules expressed on Dendritic Cells (DCs) function to coordinate an efficient immune response by T cells in the peripheral lymph nodes. We hypothesized that CD4+ T cell-mediated immune suppression following burn injury may be related to dysfunctional DCs residing in gut associated lymphoid tissues (GALT), such as Mesenteric Lymph Nodes (MLN). Therefore, we studied co-stimulatory molecules expressed on burn rat MLN DCs as an index of functional DCs that would mount an effective normal CD4+ T cell immune response. In a rat model of 30% Total Body Surface Area (TBSA) scald burn, OX62+OX6+OX35+ DCs and CD4+ T cells were isolated from MLN of day 3 post-burn and sham control rats. DCs were tested for their expression of co-stimulatory molecules, and prime CD4+ T cell (DC:CD4+T cell co-culture assays) to determine an effector immune response such as CD4+ T cell proliferation. The surface receptor expressions of MLN DCs co-stimulatory molecules, i.e., MHC-II, CD40, CD80 (B7-1), and CD86 (B7-2) were determined by Flow cytometry (quantitatively) and confocal microscopy (qualitatively). Tritiated thymidine and CFDA-SE determined CD4+ T cell proliferation following co-incubation with DCs. Cytokine milieu of MLN (IL-12 and IL-10) was assessed by mRNA determination by RT-PCR. The results showed down-regulated expressions of co-stimulatory markers (CD80, CD86, CD40 and MHC-II) of MLN DCs obtained from burn-injured rats, as well as lack of ability of these burn-induced DCs to stimulate CD4+ T cell proliferation in co-culture assays, as compared to the sham rats. Moreover, anti-CD40 stimulation of affected burn MLN DCs did not reverse this alteration. Furthermore, a marked up-regulation of mRNA IL-10 and down-regulation of mRNA IL-12 in burn MLN as compared to sham animals was also observed. To surmise, the data indicated that dysfunctional OX62+OX6+OX35+ rat MLN DCs may contribute to CD4+ T-cell-mediated immune suppression observed following acute burn injury.

1. Introduction

Our previous studies have focused on gut-associated lymphoid tissues (GALT), precisely; the roles of Antigen-Presenting Cells (APC) and CD4 + T cells in immunosuppression following acute burn-injury [1,2]. We showed that naïve rat MLN CD4 + T cells exhibit markedly reduced IL-2 production and proliferation when stimulated in the presence of MLN APCs from burn-injured rats [2]. In the current study, we expanded our previous observations seen in rat burn MLN APCs to MLN DCs that are recognized to be unique in effectively activating naïve CD4 + T cells. The involvement of co-stimulatory molecules expressed on APCs such as DCs, which are responsible for T cell suppression in burn and septic injuries continues to be a subject of extensive studies in several laboratories [3–7]. Recent studies have emphasized the importance of DCs as exclusive antigen presenters and activators of naïve T cells in the draining lymph nodes [8,9]. Immature DCs with decreased expression of MHC-II, CD80/86, CD40, and their altered interactions with CD28, CTLA-4, and CD40L on the T cells, can adversely affect naïve T cell activation and their responses [10–12]. Studies have also shown that activated CD4 + T cells originating from MLN recirculate back to MLN, where they proliferate; the proliferation of CD4 + cells in the MLN was found to be greater than that of CD8 + cells [13]. Such altered interactions between DC and the T cells has been proposed to lead to an anergy-like state in T cells and/or active T cell suppression [14]. We hypothesized that disturbances in DC functions in burn-injury conditions could also contribute to impaired CD4 + T-cell mediated immunity. In the current study we limited our experiments to DCs and CD4 + T cells derived from MLN as they have been known to drain lymph, not only from intestinal wall, but also from burn-scalded skin, thus becoming a central place for initiating a competent immune response or suppressed immune response following acute burn injury.

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2. Materials and methods

2.1. Animal model

Male Sprague Dawley rats (250–300 g) were housed and used in compliance with the regulation of the Animal Care Facility of the Chicago State University. This rat model has been previously described as a suitable model demonstrating the effector immune response associated with burn injury [1,2]. Rats were anesthetized with sodium pentobarbital (40–50 mg/kg, intraperitoneally), their dorsal body surface hair shaved off, and placed in an appropriately sized template device such that the shaved area of the skin on the animals' back was exposed. Adequacy of anesthesia was tested by the absence of withdrawal response to toe pinching. The template device was then lowered into a hot water bath (95–97 °C) to immerse the exposed skin area in hot water for 8 s. With this technique, full thickness third degree burns comprising 30% of the total body surface was obtained. Sham rats were subjected to identical anesthesia and other treatments, except that they were immersed in 37 °C water. The animals were dried immediately, and given fluid resuscitation with 0.9% saline (3 cc/body surface area) to maintain urine output. Untreated and un-operated rats were used as controls.

2.2. Rat-specific antibodies

All rat specific antibodies, i.e., OX6 (MHC-II), OX35 (CD4 +), OX52 (pan T cell), OX8 (T cells and NK cells), OX12 (Ig L chain), and OX33 (pan-B cells), CD40, CD86 (B7-2), CD80 (B7-1) and anti-CD3 were obtained from BioLegend, San Diego, CA.

2.3. Dendritic cell isolation

For DC separation, Anti-DC (OX62) Microbeads, rat (Miltenyi Biotech Inc., Auburn, CA) developed for the positive selection or depletion of OX62 + dendritic cells (DC) from lymphoid tissues was used as per instructions of the manufacturer. Lymphoid organs (MLN) were minced and digested in 2 mg/ml Collagenase D (Roche) in RPMI, 1% FCS for 30 min at 37 °C. EDTA at 10 nM was added during the last 5 min, and the cell suspension was pipetted several times and filtered. Cells were washed once in PBS/EDTA, 2 mM/1% FCS, and low-density cells were obtained after centrifugation on 14.5% Nycodenz gradient. When Nycodenz gradient was found not enough to remove dead cells, we employed Dead Cell Removal Kit (Miltenyi, Cat no. 130-090-101) to improve yield of live cells. To remove clumps that clogged the columns, cells were passed through 30 μm nylon mesh (Miltenyi). After centrifugation cells were re-suspended in 80 μl of buffer per 10^7 cells. Twenty microlitre of Anti-rat DC (OX62) Microbeads per 10^7 cells were added, mixed and incubated for 15 min at 6–12 °C. PE-conjugated Rat anti-OX62 monoclonal antibody was added at this time. Cells were re-suspended in 500 μl buffer for upto 10^8 total cells. Cell suspension was applied to MS columns in a magnetic environment. One millilitre of buffer was pipetted onto the columns. Cell mix was removed away from magnetic field and firmly flushed out fraction with magnetically labeled cells using plunger supplied with the columns. For flow cytometric and confocal image analysis, cells were stained with fluorescent antibodies for OX62, MHC Class II (OX6) and CD4 + (OX35) co-stimulatory markers expressed on DCs.

2.4. Enrichment of CD4 + T cells

CD4 + T cells were isolated using IMMULAN goat anti-mouse IgG-coated beads (Biotech Laboratories, Houston, TX). The Immulan (TM) T cells subpopulation kits are affinity chromatography based for the separation and selection of rat T cell subpopulations. Ninety nine percent of macrophages and B cells were removed from enriched CD4 + T cell populations. Purity of T cells subpopulations range (89–98%). Single cell suspension were prepared and incubated for 2 h to get rid of adherent monocytes. Fifteen millilitre of rabbit anti-rat IgG coated Immulan beads were then introduced onto columns. Cell suspensions were layered onto columns for 10 min. Effluent were then collected with 10 ml of RPMI media at a flow rate of 1 drop/s. Cells were labeled with mouse anti-rat CD8 Ab for 1 h. Labeled cells were then loaded again on columns containing goat anti-mouse IgG coated beads. The columns were washed with 10 ml medium at a flow rate of 1 drop/s. Effluent was the enriched CD4 + T cells. We were able to obtain 8–10 × 10^6 CD4 + T cells per rat MLN by this separation method.

2.5. CD4 + T cell:DC co-culture assays

CD4 + T cells and DCs at (10:1 ratio) were co-cultured in 96-well flat-bottomed microtiter plates (Falcon, Lincoln Park, NJ) for 72 h/37 °C/5% CO_2. Cells were activated with anti-CD3 (1 μg/ml) by immobilizing on the treated tissue culture 96-well plates that were coated with the antibodies (∼10 μg/ml) and incubated at 37 °C for 90 min, prior to cell cultures. Tritiated thymidine 1 μCi (37Bq) or CFDA-SE (Molecular probes, Life Technologies, Grand Island, NY) was added to each well in the last 16–18 h of culture. Cells were harvested at the end of culture period onto filtermats (Skatron, Sterling, VA) with a semi-automatic PHD cell harvester (Cambridge Technology Inc.). The counts per minute (cpm) of the filter membrane were measured in scintillation liquid on a Beckman LS 6500 liquid scintillation counter (Fullerton, CA).

2.6. Flow cytometry

FACS sorting core facilities of the Department of Biological Sciences Chicago State University was used to sort out DCs after labeling with fluorescent labels. Flow cytometry was also used for the determination of T cell type and activation cell markers labeling in the experiments. DCs were labeled with respective FITC/PE/APC-tagged rat-specific antibodies at 1–5 μg/ml per 10^6 cells. Isotype control antibodies and unstained cells were used as controls. Cells were fixed in 0.7 ml of 1% paraformaldehyde solution and acquired on a FACScan flow cytometer (Becton Dickinson).

2.7. Confocal microscopy and image analyses

MLN DCs and CD4 + T cells were labeled with appropriate FITC/APC or PE-labeled antibodies and an aliquot was cytospun onto microscopic slides. The slides were then analyzed with a Zeiss LSM 510 laser-scanning microscope. C-Apochromat 40 × 1.20 water immersion was used for viewing and image was acquired with Zeiss LSM 510; version 4.2 SPI software. One hundred cells labeled with a fluorescent dye uptake were counted for each specimen. The cell, which had taken respective fluorescent-antibody label was considered as positive. Moreover, DCs and CD4 + T cells were also differentiated from each other by their size and morphology and only those cells that fulfilled the criteria were counted as positive. Isotype control antibodies and unstained cells were used as controls.

2.8. Reverse-transcription polymerase chain reaction (RT-PCR)

Gene sequences were obtained from Gene Bank for IL-12 and IL-10 and primers were custom-made. Isolation of total RNA was done aseptically from MLN tissues using RNA Easy kit (QIAGEN, Inc., Valencia, CA) as per manufacturers protocol. Primers used for the amplification of murine IL-12, IL-10 and GAPDH were as follows: IL-12, 5′-CTT GCC CTC CTA AAC CAC CTC AGT-3′ (forward) and 5′-CCA CCA GCA TGC CCT TGT CTA-3′ (reverse); IL-10, 5′-GAA GAC AAT AAC TGC ACC CAC TTC-3′ (forward) and 5′- ATG GCC TTG TAG ACA CCT TGG