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Dendritic Cell – T Cell Interactions: CD8 $\alpha\alpha$ Expressed on Dendritic Cells Regulates T Cell Proliferation

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1. SUMMARY

Expression of the CD8 $\alpha\alpha$ homodimer has been used to differentiate lymphoid (CD8 α^+) from myeloid (CD8 α^-) dendritic cells (DCs). We have reported that CD8 α^+ and CD8 α^- DCs have differential abilities to stimulate proliferation in allogeneic T cells. However, no specific function has been attributed to DC-derived CD8 α . The current study examines the hypothesis that CD8 $\alpha\alpha$ expression on DCs regulates DC-induced T cell activation. CD8 α -transduced bone marrow-derived DCs were more potent stimulators of T cell proliferation, and produced significantly greater quantities of IL-12 in co-culture with T cells. LCK, a kinase whose expression is reported to be T cell-restricted and known to bind to the cytoplasmic tail of CD8 $\alpha\beta$ in T cells, was detected readily in primary CD8 α^+ splenic DCs and at greater levels than CD8 α^- DCs from the same tissues. LCK also co-precipitated with CD8 α on immunoblots strongly suggesting its role in CD8 α^+ DC-induced T cell activation. Collectively, these data show that CD8 α expressed on DC may not only be a lineage/maturation marker but also contribute to DC function.

2. INTRODUCTION

Dendritic cells (DC) are the most potent antigen presenting cells in all tissues. Reports from our laboratory and others show unique functions for tissue DC that express CD8 α [1–3]. The CD8 $\alpha\alpha$ homodimer differentiates lymphoid (CD8 α^+) from myeloid (CD8 α^-) murine DC, and is distinct from the CD8 $\alpha\beta$ heterodimer expressed on T cells. Both CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ molecules are able to bind MHC class I, and CD8 $\alpha\beta$ interacts with MHC class I which contributes to activation of CD8 $^+$ T cells. However, there are no reports indicating that DC-derived CD8 $\alpha\alpha$ may have a role in DC-induced T cell activation. Instead, CD8 $\alpha\alpha$ on DC has been regarded as a cell lineage marker rather than a molecule that could contribute to functional differences between myeloid and lymphoid DC [4]. We have recently reported that compared to CD8 α^+ DCs, CD8 α^- lung DC have differential abilities to stimulate proliferation in allogeneic T cells [1]. In the current study we investigated the role of CD8 α molecules in DC-induced T cell responses. Moreover we asked if CD8 molecules expressed on DC utilized the downstream signaling molecule, Lck, also known to be used in T cell signaling.

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3. MATERIALS AND METHODS

Mice

Female C57BL/6 mice (6–8 wks) were purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN) and Jackson Laboratories (Bar Harbor, ME). Mice made genetically deficient in CD8 α (B6.129S2-*Cd8a*^{+m1MAK}) and wild type litter mates (8–10 weeks of age) were purchased from the Jackson Laboratory. Mice were euthanized by intramuscular injection of a ketamine cocktail (79.3% Ketaject, 17.5% atropine, and 3.2% acepromazine). Mice were housed in specific-pathogen free facilities in the Laboratory Animal Resource Center at Indiana University School of Medicine. All experiments were performed in accordance with procedures approved by Indiana University School of Medicine Animal Use and Care Committee.

Isolation of Splenic Dendritic Cells

Splenic mononuclear cells were isolated using a modified procedure that has been previously reported [5]. Spleens were harvested from C57BL/6 mice and processed into single cell suspensions. Erythrocytes were lysed by hypotonic shock in 0.84% NH₄Cl. The remaining cells were washed twice with cRPMI, and then resuspended in the same medium. Anti-CD11c microbeads (Miltenyi Biotec) were used to isolate splenic DC from the mononuclear cell populations. Purity of all cells was >90%. Flow cytometry using the following fluorochrome-conjugated anti-mouse antibodies: FITC CD11c, PE CD80, PE CD86, PE CD40, and PE I-A^b (PharMingen, San Diego, CA) revealed that splenic DC were identical to those reported in our prior studies [5].

Isolation of Bone Marrow Derived Dendritic Cells

BMDC were generated as previously described [6] with modifications. Bone marrow cells were obtained from C57BL/6 mice by flushing femoral cells with a 23-gauge needle with RPMI. Low-density mononuclear bone marrow cells were isolated by Ficoll and centrifuged. Cells (5×10^6) were cultured in 10cm tissue dishes with the fresh CRPMI with recombinant GM-CSF(100 ng/ml) and IL-4 (100 ng/ml). Media was changed every 2–3 days and replaced with fresh media supplemented with GM-CSF(100 ng/ml)and IL-4 (100 ng/ml). On day 11, the adherent cells were stained with anti CD11C-PE antibody and analyzed by flow cytometry. All cells were utilized for assays on day 12.

Cell Lines

D10.G4.1 T cell hybridomas were purchased from ATCC and were cultured in cRPMI supplemented with 10% T cell stimulatory media (Sigma) and IL-1 (10 pg/ml) (BD PharMingen, San Diego, CA). The DC2.4 and FS2C dendritic cell lines were generously provided by Dr. Kenneth Rock and Dr. Cheong-Hee Chang, respectively and have been previously described [7,8].

Mixed Lymphocyte Reaction (MLR)

DC were gamma-irradiated (2000 cGy) and used as stimulators and Balb/c CD90⁺ pure splenic T cells (I-a^d) (3×10^5), r D10.G4.1 T cells (1.5×10^5) [9] were used as responders. The D10.G4.1 cell line has been reported to proliferate vigorously in response to I-a^b) [9]. CD90⁺ and CD4⁺ T cells were isolated using magnetic beads (Miltenyi Biotec) as reported [5]. These cells were co-cultured in a 37°C, 5% CO₂ incubator in triplicate wells in 96-well flat bottom plates. After two days, the cells were pulsed with 0.5 μ Ci/well of [³H] thymidine for 18 hrs, and proliferation determined as the mean ³H incorporation in triplicate cultures.

Immunoblotting

Splenic DC were washed with 10ml ice-cold HBSS, then lysed with 2X SDS-PAGE loading dye (4% SDS, 100mM Tris-HCL, pH 6.8, 20% glycerol, 2% w/v 2-mercaptoethanol, and 0.1% bromophenol blue). The cell lysates were then resolved on a 10% SDS-PAGE gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Anti-LCK antibodies (Santa Cruz) were used to detect LCK by western blotting. Also the lysates from CD11c⁺CD8 α ⁺ and CD11c⁺CD8 α ⁻ cells were immunoprecipitated (IP) with anti-CD8 α antibody (BD Pharmingen) then western blotted with anti-LCK antibody (Santa Cruz). The blots were developed using chemiluminescence before exposure on film.

Transduction of bone marrow (BM) cells

A. Preparation of Retrovirus: Phoenix Eco cells (~80% confluent) were plated on 10cm tissue culture dish 10ml of DMEM (high glucose) with 10% FCS. Then the plasmids (MIEG3, MIEG3CD8 alpha) were added (10 μ g DNA/dish) using Lipofectamine reagent. Eighteen hours later the medium was changed to DMEM containing 10mM sodium butyrate and 10% FCS. Then, eight hours later, the cells were gently washed twice with warmed PBS, and 10mL fresh DMEM with 10% FCS was added and the plates were incubated at 32–37°C. Two to four days later the supernatant was collected every 24 hours, filtered with 0.45 μ m filter, and stored at –80°C. Freshly thawed supernatant was used for transduction. B. Transduction of bone marrow cells: To set up for transfection, non-tissue culture 6-well plates were incubated with RetroNectin 50 μ l (1mg/ml) (TakaRa BIO) overnight at 4°C. The next day supernatant was decanted and the wells were blocked with 2% BSA in PBS for 30 min at room temperature and wash twice with PBS. Next, prestimulated cells (2 $\times$ 10⁶ cells/well) were incubated with either MIEG3 or MIEG+CD8 α retroviral supernatant. After incubation at 37°C overnight, transductions are repeated on the same plates the next day. Transduction efficiencies were assessed by determining the proportion of EGFP positive cells using FACS analyses 48 h after the transduction. After 48h, both suspension cells and adherent cells were treated with Cell Dissociation Buffer and then culture in the fresh CRPMI with GM-CSF(100 ng/ml) and IL-4 (100 ng/ml). On day 8, the GFP positive cells are sorted by FACS and place in culture again in CRPMI with GM-CSF and IL-4. On day 11, the cells are stained with anti CD11C-PE and anti CD8 alpha antibody and analyzed by flow cytometry. On day 12, then set up experiment: MLR (mixed lymphocyte reaction) and IL-12 production.

ELISA

IL-12 (p70) production from the supernatants of co-cultured cells was determined by standard ELISA. The standard was obtained from BD Pharmingen (Cat.No. 554592). The purified 9A5 antibody (Cat.No.554658) and biotinylated C17.8 anti(Cat. No. 554476) were purchased from BD Pharmingen as well.

Statistical Analyses

Student's t-test (two-tailed, unpaired) was performed using Graphpad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Data were considered significant if p<0.05.

4. RESULTS

We first determined the ability of DCs from wildtype (WT) or CD8 α -deficient mice to induce proliferation in T cells. WT and CD8 α deficient BMDC were co-cultured in varying ratios with the D10 T cell (CD4⁺) T cell line (Fig. 1A) or pure allogeneic splenic T cells (CD4⁺) (BALB/c – I-a^b) (Fig. 1B) in mixed leukocyte reactions. Notably, BMDC from WT mice induced greater proliferation in T cells from both sources compared to knockout mice. Since BMDC from WT and KO mice should only differ in CD8 α expression, then the functional differences

between these cells could be attributed to DC-derived CD8 α . Indeed, BMDC from each source were comparable in expression of co-stimulatory molecules required for T cell activation (data not shown) suggesting that phenotypic differences other than CD8 α should not account for differential abilities to induce T cell proliferation. As expected, BMDC from CD8 α -deficient mice did not express this marker (data not shown). However, figure 1C shows that 3.1% of BMDC from wildtype mice were CD8 α ⁺. These cells, though few in number, may have had role in explaining the differential abilities of the DC subsets to induce T cell proliferation.

To further examine the role of CD8 α on DC we transduced BMDC with CD8 α followed by assessing the function of these cells. CD8 α was amplified from C57BL/6 splenocytes and cloned it into a retroviral vector, MIEG3, which also expresses an enhancing green fluorescence protein (EGFP) as shown in Fig. 2A. Sequence analysis confirmed 100% homology of cloned CD8 α with WT CD8 α as described in Methods. Next we transduced murine bone marrow progenitors cells with either the control vector or with CD8 α . Expression of CD8 α transcripts were confirmed by RT-PCR (Fig. 2B), and Western Blot confirmed protein expression (data not shown). Cells that co-expressed GFP and CD8 α were sorted by flow cytometry into CD8 α ⁺ and CD8 α ⁻ fractions, which further confirmed surface expression of CD8 α .

To assess their functional differences, CD8 α ⁺ and CD8 α ⁻ BMDC were irradiated and co-cultured in varying ratios with D10 T cells followed by an assessment of T cell proliferation (Fig. 2C). Notably, compared to CD8 α ⁻ BMDC, CD8 α ⁺ BMDC induced significantly greater dose-dependent proliferation (four fold increase, *p<0.05) in D10.G4.1 T cells in mixed leukocyte reactions

DC-derived IL-12 has a key role in DC-induced T cell activation/proliferation. Therefore, we next determined if CD8 α -transduced DC produced greater amounts of IL-12 in co-culture with T cells. Fig. 2D shows that CD8 α ⁺ BMDC produced significantly greater IL-12 (p70) (six fold increase, *p<0.01) in co-culture with T cells (ratio of DC: D10.G4.1 T cells = 0.5:1) compared to CD8 α ⁻ BMDC in co-cultures. Very low levels of IL-12 were detected in supernatants from BMDC, alone; BMDC transfected with MIEG3; or BMDC transfected with CD8 α (Fig. 2D).

CD8 α -mediated signaling in T cells is in part mediated by binding the kinase LCK. Therefore, we next determined if LCK, thought to be restricted to T cells [reviewed in [10]], is expressed in CD8 α ⁺ primary tissue DC. To conduct these studies, CD8 α ⁺ and CD8 α ⁻ splenic DC were isolated from C57BL/6 (Fig. 3A), lysed and cell lysates examined for LCK expression by immunoblotting. Interestingly, CD8 α ⁺ splenic DC expressed greater quantities of LCK than CD8 α ⁻ DC (Fig. 3B) Notably, the DC2.4 cell line, which is a myeloid type DC [7], and FSDC which is a fetal skin derived DC did not express LCK, and therefore were negative controls for these studies. As expected LCK was detected in bulk splenic T cells, and D10.G4.1 T cells (Fig. 3B). We next determined if LCK was bound to CD8 α in DCs. Indeed, LCK co-precipitated with CD8 α suggesting that DC not only express LCK but it is bound to CD8 α (Fig. 3C).

5. DISCUSSION

Studies from several groups have shown that ligation of the CD8 $\alpha\beta$ heterodimer (expressed on T cells) by MHC I results in the initiation of a p56 LCK kinase-mediated intracellular signaling cascade that aids in the initiation and maintenance of T cell activation. In fact, Src-family kinases, Lck and Fyn, play a critical role in T-cell development and activation. However, not much is known about the functional role of the CD8 $\alpha\beta$ homodimer expressed on DC. It has been reported that CD8 α ⁺ DC promote Th1 responses, and are responsible for inducing peripheral tolerance to tissue-associated antigens [reviewed in [11]]. Recently it has been shown that CD8 α expression is plastic and may be associated with the stage of DC maturation [4]. Therefore in these studies we asked if CD8 α contributed to DC function. The current data show that expression of CD8 α on BMDC may play a functional role in enhancing BMDC's

ability to activate T cells. Furthermore, the expression of CD8 α on BMDC may induce upregulation of DC-derived mediators, such as IL-12, that enhance T cell activation. These data and that shown in Figure 3 indicate that CD8 α ⁺ DC highly express LCK suggest that CD8 α is not simply a marker of DC subsets, but contributes to DC-T cell interactions. To the best of our knowledge this study is the first to report a potential functional role for CD $\alpha\alpha$ expressed on DCs in DC-induced T cell proliferation. In addition, these data show that LCK expression is not limited to T cells. The specific nature of DC-derived CD8 α -mediated T cell effects will be examined in future studies.

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Fig 1.

Expression of CD8 α on BMDC and allogeneic T cell proliferation induced by BMDC. (A) BMDC were stained with CD11c antibody (left) panel. CD11c⁺ cells were then stained with CD8 α -specific Ab (center), or isotype control Ab (right) and analyzed by flow cytometry. Wildtype and CD8 deficient (CD8 α ^{-/-}) BMDC were irradiated and cocultured with D10 T cells (B) or allogeneic (BALB/c) splenic CD4⁺ T cells (C) at the indicated ratios. Data shown from a representative of three experiments.

Fig 2.

Construction of CD8α expressing retroviral vector and CD8α⁺ transduced BMDC induce greater T cell proliferation. (A) CD8α cDNA was amplified from C57BL/6 mice splenocytes by RT-PCR. Sequencing analysis confirmed 100% homology to murine CD8α. The cDNA was cloned into a retroviral vector, MIEG3, which also expressed an enhancing green fluorescence protein (EGFP). Vectors with or without CD8α were used to transduce murine bone marrow progenitor cells isolated from C57BL/6 mice. BMDC were propagated in the presence of GM-CSF and IL-4 and sorted by FACS. A pure population of sorted EGFP⁺ CD8α⁺ BMDC was expanded further by cell culture. (B) CD8α mRNA expression in these transduced cells were confirmed by RT-PCR. (C) BMDC were transduced with an empty vector (BMDC-MIEG3) or with a vector expressing CD8α (BMDC-CD8α), then irradiated and co-cultured with D10.G4.1 T cells in varying ratios. (D) Proliferative responses were determined as mean counts per minute (CPM) ± s.d. of thymidine incorporated into the T cells in triplicate cultures. All data are representative of at least three experiments yielding comparable results. (E) Detection of IL-12 (p70) in cell culture supernatants from proliferation assays as determined by ELISA. BMDC:T cell ratios of 0.5:1 were used for all assays. All data are representative of at least three experiments yielding comparable results.

Fig 3.

CD8 α + Splenic DC express LCK. (A) Splenic CD11c+ cells from C57BL/6 mice were isolated by magnetic beads and CD8 α positive cells were sorted by FACS. Data show greater 97% purity of CD8 α + fraction. (B) Western blots were performed to examine LCK expression in T cells (D10.G4.1) and DC2.4 DC or FSDC. (C) Splenic dendritic cell lysates were immunoprecipitated - IP with anti-CD8 α antibody and western blotted-WB with an anti-LCK antibody. All data are representative of at least three experiments yielding comparable results.