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CHARACTERIZATION OF CD11c$^+$ DENDRITIC CELL POPULATIONS IN SPLEENS OF MICE INFECTED WITH *TOXOPLASMA GONDII*

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Abstract

To investigate the morphology, immunophenotype and stimulatory activity of splenic dendritic cells (DC), a procedure was developed to obtain dendritic cell populations by mechanical tissue disruption of spleens followed by metrizamide density gradient centrifugation and cell sorting on the basis of CD11c expression. The resultant low density cell fraction consisted of a non-adherent cell population that could at least be characterized by typical DC morphology, constitutive levels of surface MHC class II and expression of DC specific markers. The capacity for antigen presentation of the isolated CD11c$^+$ DC was carried out using *Toxoplasma gondii* lysate antigen (TLA). The results describing the phenotype and accessory function provide some evidence that DC play a crucial role as antigen presenting cells (APC) with important implications for understanding the complex network regulating antigen uptake, processing and presentation.

Key words: Spleen, Dendritic cell, Mouse, Toxoplasma lysate antigen (TLA).

Introduction

Dendritic cells (DC) are irregularly shaped, motile leukocytes that are involved in initiating primary MHC class II-restricted immune responses (Inaba *et al.*, 1990; Pavli *et al.*, 1990; Makala *et al.*, 1998)); the principal stimulators cells of primary mixed lymphocyte reactions (Steinman *et al.*, 1983; Crow and Kunkel, 1982; Wilders *et al.*, 1983); and are also involved in the induction of T cell-dependent antibody production (Liu and Macpherson, 1993). Liu and Macpherson (1991) showed that DC are able to actively process and present

antigen *in vivo*. Dendritic cells have also been shown to be involved in autoimmune diseases, graft rejection and the pathogenesis of HIV infection (Knight *et al.*, 1992). They represent a small subpopulation of bone arrow-derived leukocytes and have been classified largely on the basis of their tissue location. Isolation of DC is difficult because of their low frequency in tissue and the scarcity of specific markers for these cells. In all tissues that have been studied to date, DC are a small fraction of total cells. Multiple step procedures, often taking a day or more are required for their enrichment. However, in all the lymphoid and non-lymphoid organs DC are present, they are responsible for capturing and presenting antigens to T cells (Steinman, 1991; Bourguin et al., 1998). Austyn *et al.*, 1988 documented the localization and migration patterns of spleen DC. This localization positioned most DC at regions where arterial vessels and T cells enter the white pulp, hence we speculate that it may function to localize these APC at sites that permit access to the circulating pool of resting T cells.

The aim of this study was to optimize the isolation of a functionally active and enriched DC population from mice spleens and to investigate the possibility of establishing an *in vitro* DC culture system. We have adapted previously devised methods for the isolation and purification of tissue DC utilizing a combination of fractionation on metrizamide gradients and sorting on the basis of CD11c expression (Ruedl *et al*, 1996; Makala *et al.*, 1998). Isolation and purification were followed by characterization using the following criteria: morphology; expression of membrane antigens; and capacity for antigen.

Materials and Methods

Animals. Samples were taken from transgenic mice purchased from a commercial supplier (CLEA,Japan, Inc.). All the experimental mice were maintained in conventional conditions on a commercial diet. The age range was 6-10 weeks and both sexes were used.

Tissue culture media. Except where stated, all cell manipulations were carried out at room temperature in Hanks balanced salt solution (HBSS) without calcium or magnesium (Flow Laboratories, Irvine, UK), buffered with 5mM hepes and 0.4 g/l sodium bicarbonate. Tissue culture medium was RPMI 1640 (Dutch modification, Gibco, Paisley, UK) supplemented with 2mM L glutamine (Gibco); 1 mM sodium pyruvate (Flow laboratories); 40μg/ml gentamicin, 100 IU /ml penicillin; 100μg/ ml streptomycin; 2% normal mouse serum; and 10 μM 2-mercaptoethanol (2-ME) unless otherwise stated.

Monoclonal antibodies. Anti-mouse monoclonal antibodies used in this study are summarized in Table 1.

Table I . Anti-mouse monoclonal antibodies used in this study

Clone	Specificity	Isotype	Reference
HL3	CDIIc	IgG	Metlay et al., 1990
AF6-120.1	MHC class II (1-A^b)*	IgG2a	Wall et al., 1983
16-Al	CD80(B7-1)	IgG	Razi-Wolf et al., 1992
GL 1	CD86 (B7-2)	IgG2a	Freeman et. al, 1993
30-F 11	CD45 (LCA)/Ly-5)	IgG2b,k	Leddbetter and Herzenberg, 1979
RA3-6B2	CD45R (B220)	IgG2a,k	Coffman, 1982
C1:A3-1	F4/80	IgG	Austyn and Gordon, 1981
145-2C11	CD3e	IgG	Leo et al., 1987

Abbreviations: CD - Cluster of Differentiatron *The anti-MHC class II (anti-mouse I-A^b($A\alpha^b$) (AF6-120.1) used in this study reacts with the α chain of the I-A^b MHC class 11 allo-antigen. It crossreacts with cells from mice of the H-2^K, H-2^P and H-2^u haplotypes which cover the mice types used in our study.

Preparation and enrichment of splenic dendritic cells. Single cell suspensions from spleens of transgenic mice were made using a modification of a previously described method (Makala *et al.*, 1998). Briefly, spleens were dissected out and carefully removed. Spleens were cut into small pieces and mechanically disrupted by using glass slides in RPMI-1640, without bicarbonate or by passage through a nylon cell strainer (70 µm) (Becton, Dicknison, NJ, USA). Resulting cells were suspended in tissue culture medium and layered unto 14.5% w/v analytical grade metrizamide (Nycomed AS, Oslo, Norway) in RPMI 1640 without bicarbonate, supplemented with 2% normal mouse serum (NMS), at 10 ml of cells to 2 ml of metrizamide and centrifuged at 600g for 10 minutes at room temperature. Low density cells (LDC) were removed from the interface,washed twice and resuspended in tissue culture medium. In some cases 0.83% ammonium chloride was used to lyse red blood cells. These cells were then incubated for 1 hr on ice with phycoerythrin-labeled anti- CD11c (1:200, Pharmingen, San Francisco, CA, USA) in the presence of 5% normal mouse serum, washed and sorted in a Coulter EPICS-ELITE-ESP cell sorter.

Characterization of low density cells from mice spleen

Morphology by light and electron microscopy. For light microscopy slide cell preparations were air dried, fixed and stained using Geimsa's stain. Cells were observed under a light microscope and the proportion of cells showing morphological features of DC were identified using the following criteria: relative size; cytoplasm;nucleus ratio; shape and position of the nucleus; vacuolation of the cytoplasm and cytoplasmic protuberances.

For electron microscopy cells were pelleted and fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 hr, rinsed in buffer, post-fixed in 2% osmium tetroxide in 0.2 M cacodylate buffer for 1 hr, dehydrated through a graded ethanol series and finally embeded in TAAB Epon 812 low viscosity resin. Ultrathin sections (70 nm) were cut using a diamond knife, grid stained with 1% uranyl acetate and lead citrate and analyzed by a Hitachi transmission electron microscope (Hitachi, Ltd, Tokyo, Japan)

Phenotypic characterization by FACS analysis. After sorting, generated $CD11c^+$ cells (10^5) were stained with a panel of monoclonal antibodies to identify typical surface markers of DC obtained from spleens. The cells were washed three times with PBS containing 5% FBS and 0.02% NaN3 for 1 minute at 400 g. Binding was visualized with a single-step technique involving incubation with the following labeled anti-mouse mAb: anti-mouse $I\text{-}A^b$ FITC (AF6-120.1); hamster anti-mouse CD11c/ PE (HL3); anti-mouse CD80/ B7-1 FITC (16-10A1); anti-mouse CD86/ B7-2 (GL1); anti-mouse CD45/ LCA FITC (30-F11); anti-mouse CD45R/ B220 FITC (RA3-6B2); and anti-mouse F4/80 FITC (CI:A3-1). All mAb were obtained from Pharmingen (Pharmingen International, Becton Dickinson Company). After incubation, cells were washed, analyzed and fluorescence quantified using a Coulter EPICS-XL flow cytometer at 488nm. Cells were electronically gated on forward angle light scatter to exclude contaminating erythrocytes and small debris and on 90° light scatter to exclude granulocytes.

Antigen presentation assay. Lymphocytes were purified from spleens of previously *T. gondii* infected mice cut into small pieces and mechanically disrupted by using glass slides in RPMI-1640, without bicarbonate or by passage through a nylon cell strainer (70 µm) (Becton, Dicknison, NJ, USA). In some cases 0.83% ammonium chloride was used to lyse red blood cells. These cells were then incubated for 1 hr on ice with FITC-labeled anti-

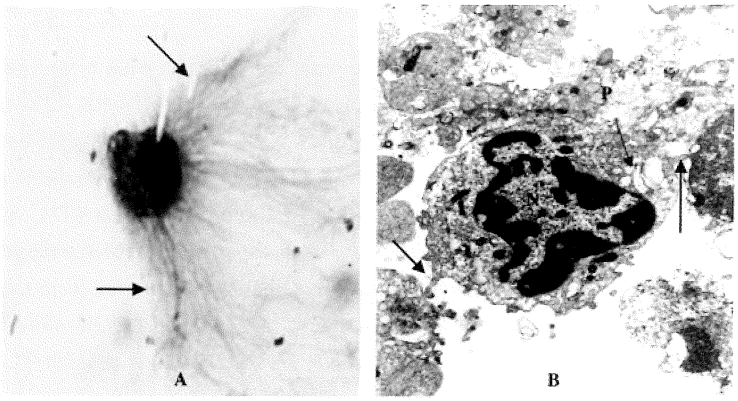


Fig. 1. Microscopic visualization of Giemsa-stained mouse spleen CD11c+ dendritic cells (DC) (A) (x670) and transmission electron micrograph (B) (x7,000) of DC exhibiting typical DC morphology (cytoplasmic protrusions/veils - see arrows, irregularly shaped nucleus (N), autophagosomes P).

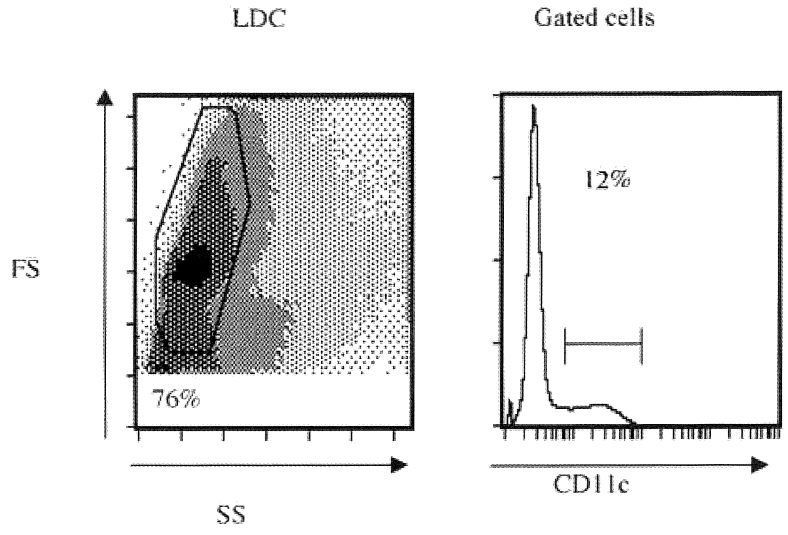


Fig. 2. Sorting conditions for the isolation of CD11c+ cells from the low-density population obtained from mouse spleens. On the left, is a dot-plot showing forward scatter (FS) on the y-axis, as a parameter for cell size and on the X-axis, side scatter (SS), as a measure of granularity. On the right, CD11c staining on the gated cells in the dot plot is shown.

CD3e (1:200, Pharmingen, San Francisco, CA, USA) in the presence of 5% normal mouse serum, washed and sorted in a Coulter EPICS-ELITE-ESP cell sorter, thus purifying them for $CD3^+$ T cells. Low-density cells were additionally prepared from these mice. Cultures were established in 96 well flat-bottomed tissue culture plates (Falcon). Individual wells contained 2 x 10^5 $CD3^+$ spleen T cells together with a range of concentrations of $CD11c^+$ DC with or without TLA at 100 μg/ml in a total volume of 200 μl. Responder $CD3^+$ spleen T cells were cultured with a range of concentrations of $CD11c^+$ DC to give final responder: stimulator ratios of 1:1, 10:1, and 100:1. Control cultures contained responder cells or stimulator cells alone. Cultures were established in three replicate wells. Control cultures were arranged as follows: responder $CD3^+$ T cells alone or with antigen; unfractionated spleen cells alone or with TLA; and stimulator $CD11c^+$ DC alone or with TLA. Cultures were incubated at 37 °C in 95% air, 5% CO_2 for 96, 120 or 144 hr. Four hours before the termination of cultures, cells were pulsed with a non radio active cell counting kit-8 (Dojindo, Japan). Cell were then harvested and using an ELISA reader absorbance was determined at 415 nm.

Statistics. Statistical analysis of the results of the antigen presentation assay was carried out as a matrix of pairwise comparison probabilities (P) in multiple analysis of variance.

Results

Yield, viability. The yield of cell populations routinely from PP of 10-12 mice by mechanical disruption was up to 1 X 10^8–1.3 X 10^8 cells. After density centrifugation the cell suspension consisted approximately 1 X 10^7-3 X 10^7 cells. After cell sorting on the basis of CD11c staining, recovery ranged from 3 X 10^5-6 X 10^5 cells. The viability as determined by trypan blue exclusion was always greater than 90%.

Morphology of dendritic cells. Low density cells were characterized by morphology under light microscopy. Using the criteria of relative size, cytoplasm:nucleus ratio, shape and position of nucleus and cytoplasmic protuberances, the morphological features of LDC were analyzed. Mouse alveolar macrophages were used as controls (data not shown). As identified microscopically, a nonadherent cell population could be observed. Subjectively DC were generally smaller than macrophages and these cells showed evidence of cytoplasmic protuberances or dendrites (Fig.1A). These cells were observed under light microscopy continually extending, retracting and reorienting their cellular processes or veils in live cultures when observed over a longer period of time (data not shown).

At the ultrastructural level the isolated $CD11c^+$ cells provided further evidence of typical DC morphology consistent with the phase contrast microscope observations, for example, the above mentioned cytoplasmic veils (V), irregularly-shaped excentric nuclei (N), autophagosomes (AP) and multivesicular bodies (MVB) as illustrated in Fig. 1B.

Expression of surface antigens. The $CD11c^+$ DC population was sorted using the conditions shown in Fig. 2. Flow cytometry results are illustrated in Fig. 3. Flow cytometry of the $CD11c^+$ DC showed that approximately 90% were $CD11c^+$. This population included both $CD11c^{high}$ and $CD11c^{low}$ sub-populations. All the sorted cells expressed the common leukocyte CD45 epitope and high levels of MHC class II products. Moreover, a subpopulation of the freshly isolated $CD11c^+$ DC cells reacted with the anti-macrophage marker, F4/80. The co-stimulatory receptors B7.1 and B7.2 could also be detected at constitutive levels, where as CD45R/B220, a pan B cell marker and a member of the protein tyrosine phosphatase family was detectable at even higher levels. Taking expression of high levels of

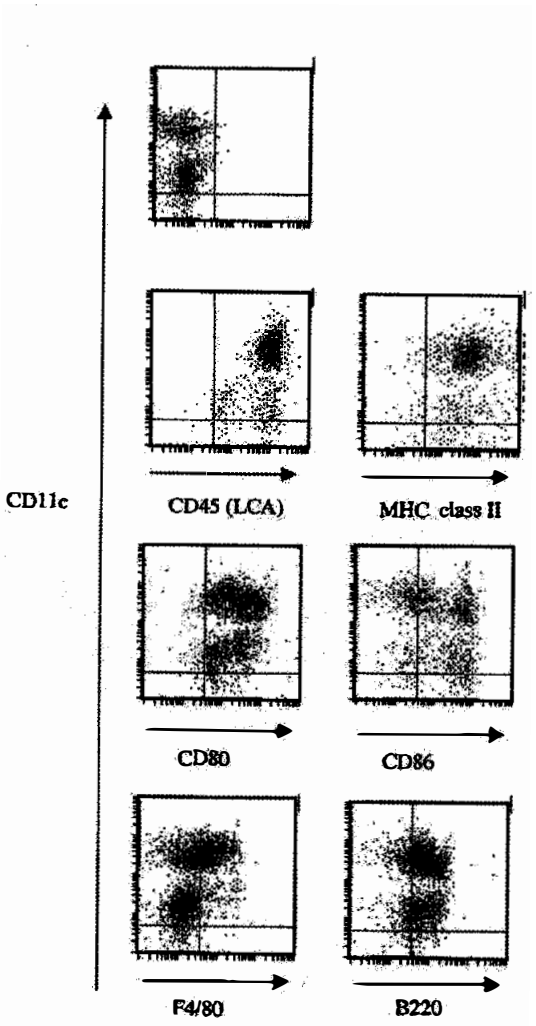


Fig. 3. Flow cytometric analysis of freshly isolated low-density CD11c+ mouse splenic dendritic cells (DC). The surface phenotype of the low-density cell population was analysed using the following panel of monoclonal antibodies, CD11c, CD45 (LCA), CD80 (B7-1), CD86 (B7-2), F4/80, CD45R (B220). Fluorescence is expressed as percentages (%).

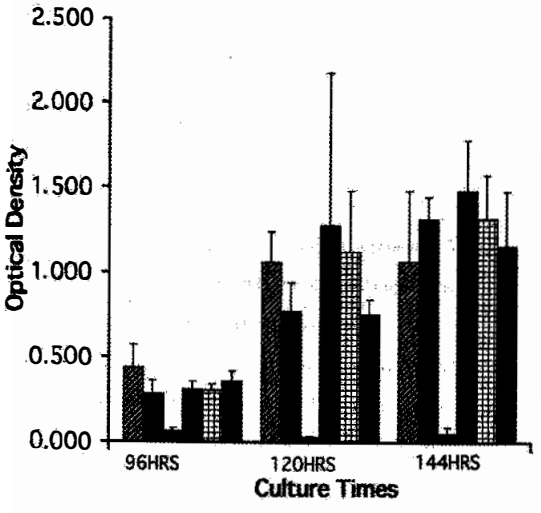
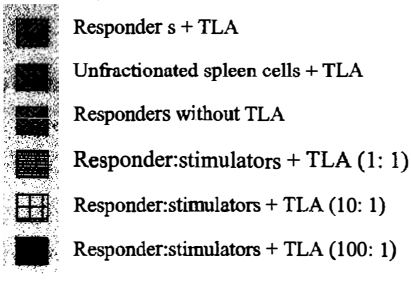


Fig. 4. Proliferation of CD3+ T cells to Toxoplasma lysate antigen (TLA) using CDI Ic+ spleen dendritic cells (DC) as antigen presenting cells compared to the proliferation of unfractionated spleen cells. CD3' spleen T cells from Toxoplasma gondii infected mice were used as responders. Bars represent mean OD from triplicate wells, plus standard deviation (+SD).



MHC class II products as the main identifying criterion for DC, the majority of the $CD11c^+$ DC showed very high levels of MHC class II molecules on their surface.

Antigen presentation by $CD11c^+$ spleen DC. The $CD11c^+$ DC population freshly isolated were tested for their capacity to present antigen to T cells. $CD3^+$ spleen T cells from mice which had been infected with *Toxoplasma gondii* were co-cultured with $CD11c^+$ spleen DC with or without TLA, over a range of stimulator:responder ratios. The results are summarized in Fig 5. Data from three responder:stimulator ratios were pooled between each experiment and multiple analysis of variance was applied. The $CD11c^+$ DC were able to present TLA to $CD3^+$ spleen T cells. Moreover, TLA also significantly stimulated control T cell cultures.

Discussion

We have optimized a DC isolation and enrichment procedure from mouse spleens involving the combined use of mechanical tissue disruption followed by fractionation of isolated cells on metrizamide density gradients and cell sorting on the basis of CD11c expression. We chose to use the surface marker CD11c, a hamster anti-mouse p150/90 reagent (Metlay et al., 1990) because of the reported massive presence of positive cells in the T cell dependent areas of the spleen's white pulp and the rim of marginal zone between the red and white pulp (Metlay et al., 1990). Density gradient centrifugation, fractionating cells according their buoyant density, has been used to isolate cells of dendritic morphology with antigen presenting capability from murine and human tissues (Steinman and Cohn, 1974, Knight *et al.*, 1987).

The isolated cells were morphologically heterogenous and exhibited the cytoplasmic processes and motility characteristic of dendritic cells (Fig. 1.). They were observed under light microscopy to continually extend, retract and reorientate their cellular processes and veils. Transmission electron microscopy provided further evidence of typical DC characteristics. By light microscopy, low-density cells appeared similar to veiled cells isolated from pig or rat (Drexhage *et al.*, 1979). However, other cell types are capable of developing dendritic morphology in response to chemotactic stimuli (Zigmond *et al.*, 1981). The development of dendritic morphology relates to contact with highly positively charged plastic or glass surfaces and can be inhibited by drugs that disrupt microtubule and microfilament function and by mAb to LFA-1 (CD11a/CD18)(Rothlein and Springer, 1986). Therefore, development of dendritic cell morphology is not unique to one cell type and this feature alone cannot be used to distinguish DC from other APC. We therefore used additional identifying criteria, namely expression of surface membrane markers.

Metlay *et al.*, (1990) described a monoclonal antibody that precipitates a p150/90 leukocyte β_2 integrin, the murine counterpart of human CD11c (Metlay *et al.*, 1990). This mAb reacted with splenic DC. Dual labeling $CD11c^+$ spleen cells with CD11c/PE and MHC class II/FITC included significant numbers of cells expressing MHC class II at high levels (Fig. 4.). In addition our FACS analysis revealed a $CD11c^+$ cell population expressing CD80 (B7.1), CD86 (B7.2), CD45R, LCA and the macrophage marker F4/80. The observation that $CD11c^+$ spleen DC, express the macrophage-specific marker F4/80 may suggest a lineage relationship with macrophages and monocytes. This result is in agreement with the findings of Romani and Schuller, (1989), Crowley et al., (1989) and Kraal et al., (1986), that other DC types, for example, Langerhans' cell express F4/80, supporting the postulation that an identical lineage relationship between DC and macrophages exists.

The isolated $CD11c^+$ spleen DC were able to present TLA to $CD3^+$ spleen T cells from *T. Gondii* infected mice. This result is not suprising given the well documented capacity of DC for antigen presentation *in*

vitro and *in vivo* (Inaba *et al.*, 1990). This may also indicate that the isolated CD11c$^+$ spleen DC are mature DC with a mature antigen enzyme profile, thus explaining the successful uptake and presentation of antigen. Moreover, TLA, significantly stimulated control T cells, suggesting a superantigen activity of TLA. This result is consistent with previous studies carried out by Denkers *et al.*, 1994. They demonstrated that live *T. gondii* tachyzoites as well as parasite extracts have stimulatory activities consistent with the presence of a superantigen. The ability of *T. gondii* parasite extracts to stimulate a strong response from CD3$^+$ spleen T cells alone and co-cultures of the same with CD11c$^+$ spleen DC may provide the mechanisms by which this parasite induces a dominant cell-mediated immune response after infection.

Larsen *et al..*, 1990, found out that DC can migrate from other tissues, for example, the heart to the spleen via blood. Given these facts it is possible that some of the DC that are found in the spleen are migrating there from non-lymphoid tissues via the blood. Once in the spleen, cytokines derived from macrophages, endothelial cells or T cells may enhance viability and function thus amplifying immune responses. Whether antigen sensitized DC move into T cells areas or migrate to other tissues to induce cell mediated immune responses remains to be the subject of future investigations.

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