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Major Histocompatibility Complex Class II and Co-stimulatory Molecule CD80 Expression in Splenic Dendritic Cells from BALB/c Mice Infected with *Neospora caninum*

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Abstract

We determined the expression of major histocompatibility complex class II (MHC) and the co-stimulatory molecule CD80 between dendritic cells (DCs) from spleens of non-infected and *Neospora caninum*-infected mice. BALB/c mice were infected intraperitoneally with a 0.1 ml suspension containing 5×10^4 trachyzoites of the NC-1 strain of *N. caninum* sacrificed on days 3, 5, and 7 post-infection, and splenic DCs were isolated with 14.5% w/v metrizamide gradient and characterized by expression of high levels of surface membrane antigen; CD205. The expression of MHC class II and CD80 in the splenic DCs of non-infected and *N. caninum*-infected mice was examined by flow cytometry. Our results demonstrated for the first time that MHC class II on DCs was significantly increased level during *N. caninum* infection. Furthermore, an up-regulation of CD80 was shown two-fold level at 7 days' post-infection. Both MHC class II and CD80 are important for antigen presentation, these results agree with the accepted concept that DCs participate in generating of Ag-specific immunity during infection.

Keywords: co-stimulatory molecule CD80, dendritic cell, major histocompatibility complex class II, *Neospora caninum*

Introduction

Dendritic cells (DCs) represent a rare population of antigen-presenting cells (APC) in the blood, non-lymphoid and lymphoid tissues [1], linking the innate immune system with the adaptive specific immune system [2]. DCs are the most potent, such as being the principal stimulators of allogeneic and syngeneic mixed lymphocyte reaction (MLR) [3], in the induction of T-cell dependent antibody production [4], and initiating primary MHC class II restricted immune responses [5]. The membrane-bound co-stimulatory molecule CD80 (B7-1), a 60 kD protein, a member of the immunoglobulin (Ig) superfamily, was the first ligand to be identified for CD28 [6]. CD 80 expression is almost exclusively restricted to lymphoid tissues, primarily on "professional" APC, including Langerhans cells,

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monocytes and activated B cells [7]. DCs can induce immune responses against parasites based on well-known studies of *Leishmania major* [8] and *Toxoplasma gondii* [9]. *Neospora caninum* is an intracellular parasites, which was originally identified as a *T. gondii*-like parasite causing canine neuromuscular paralysis and bovine infertility and abortion [10]. However, MHC class II and co-stimulatory molecule expression patterns in DC during *N. caninum* infection have not yet been well elucidated; thus, the present study aimed to clarify the expression of MHC class II and CD80 in DCs from spleens of BALB/c mice during acute neosporosis to better understand DC function and host resistance to *N. caninum* infection.

Materials and methods

Mice and *N. caninum* infection

Inbred female BALB/c mice 4-6 weeks old were purchased from a commercial supplier (CLEA, Japan). All mice were kept under SPF condition with a 12:12 hr light-dark cycle and housed in cages (8 mice/cage) with free access to sterilized tap water and a standard commercial diet (Ca-1, CLEA Inc, Japan). For infection of mice, 24 BALB/c mice were divided into three groups of eight and infected intraperitoneally (ip) with 5×10^4 *N. caninum* tachyzoites in 0.1 ml of PBS. Spleens were collected from mice on 3, 5, and 7 days' post-infection (dpi) for preparation of DCs and unfractionated splenocytes. Serum was also collected for specific *N. caninum* antibody. Non-infected mice were sacrificed and used as controls.

Parasites culture and purification

N. caninum tachyzoites of the Nc-1 strain [11] were maintained in vero cells in sterile tissue culture medium; Minimum Essential Medium Eagle-MEM, SIGMA supplemented with 8% heat-inactivated fetal bovine serum (FBS) (BroWhittacker, Maryland, USA) and 50 µg/ml of kanamycin. Tachyzoites were purified from parasites and host cell debris following centrifugation in cold phosphate buffered saline (PBS). The resulting pellet was re-suspended in cold PBS, passed through a 27-gauge needle and filtered using a 5.0 µm pore filter (Millipore, Massachusetts, USA).

Antibodies

All antibodies used for purification and staining were anti-mouse monoclonal antibodies (mAbs) and were purchased from PharMingen (San Diego, USA); anti-MHC class II (clone SF1-1.1); anti-CD80 (clone 16-10A1) and anti-CD16/CD32 (clone 2.4G2). Anti-CD205 (clone NLDC-145) was purchased from Serotec (Oxford, England).

Isolation and purification of dendritic cells from spleens

Single cells suspension from BALB/c mice spleens were isolated as previously described by Makala and colleagues [12-13]. Briefly, immediately after slaughter, the skin covering the abdomen was removed. Organs were removed aseptically, the serosa and fat were dissected away, then placed in a glass petri-dish with tissue-culture medium, cut into tiny pieces and disrupted mechanically using a 5 ml syringe head and passage through a nylon cell strainer (70 µm-pore) (Becton Dickinson, New Jersey, USA). 0.83% ammonium chloride was used to lyse contaminating red blood cells by incubation for 5 min at 37°C. Cells were washed and re-suspended in tissue-culture medium to identify unfractionated splenocytes. For DC, the resulting cells were re-suspended in 8 ml of tissue-culture medium in a glass centrifuge tube and layered onto 2 ml of 14.5% w/v analytical grade metrizamide (Nycomed AS, Oslo, Norway) in RPMI 1640 without bicarbonate, supplemented with 2% FBS. The suspension was centrifuged at 600×g (with brakes off) for 10 min at room temperature. The low-density cells (LDC) were removed from the interface, washed twice and re-suspended in PBS containing 3% FBS. These were resident splenic DC and were kept on ice until used.

The FcBlock™ anti-CD16/CD32 was used to prevent non-specific binding of mAbs to Fc receptors. In some cases, morphological analysis by light microscopy was carried out to further characterize the isolated DC. Briefly, cytospin preparations were prepared using a cytocentrifuge (Shandon Southern, Pittsburgh, USA) at 200×g for 5 min at room temperature. The cytospin slide cells were air-dried, fixed and stained using Giemsa.

Cells were observed under light microscope and the proportion of cells showing morphological features of DC were identified using the following criteria: size, cytoplasm : nucleus ratio, shape and position of nucleus, and cytoplasmic protuberances.

Flow cytometry

The levels of *N. caninum* antigen-specific membrane surface antigens MHC class II, CD80 and CD205 from splenic DCs and unfractionated splenocytes were determined by flow cytometry. Briefly, cells were stained with fluorescein isothiocyanate (FITC) labeled anti-CD205 and anti-CD16/CD32 (Fc III/II receptor, FcBlock™), phycoerythrin (PE) labeled anti-CD80, and PE labeled anti-MHC class II at 4°C for 45 min, washed and re-suspended with PBS. The cells (1×10^4) were collected and fluorescence quantified using a Coulter EPICS-XL flow cytometer (Beckman Coulter, Florida, USA). Cells were electronically gated on forward angle light scatter to exclude contaminating erythrocytes and small debris and on 90 light scatter to exclude granulocytes.

Detection of serum *N. caninum* antibody by indirect immunofluorescent-antibody test (IFAT)

To confirm *N. caninum*-infected mice, *N. caninum* tachyzoites were mounted onto glass slides, dried and fixed with acetone before use. Bound murine antibodies were detected with fluorescein-conjugated goat anti-mouse IgG and IgM (Southern Biotechnology, Birmingham, USA) diluted at 1 : 200 in PBS supplemented with 3% FBS. The specific antibody in the serum sample that reacted with *N. caninum* tachyzoite antigen was observed using fluorescence microscopy.

Results

Dendritic cell yield, purity and viability

The yield of cells from spleens of 8 non-infected and *N. caninum*-infected BALB/c mice isolated after mechanical disruption of spleen and ammonium chloride lysis, but before metrizamide gradient centrifugation ranged from 0.8×10^8-1.2×10^8 cells. After metrizamide density gradient centrifugation, the cell suspension consisted of approximately 0.9×10^7 - 1.2×10^7cells. The isolated cells consisted of a cell population characterized by expression of high levels of the dendritic cell marker CD205, compared to unfractionated splenocytes before metrizamide density gradient centrifugation. The purity of the gradient cells, as determined by flow cytometry using CD205 expression, was always > 90% in both non-infected and *N. caninum*-infected mice (Table 1 and Fig 1). The cell viability, as determined by trypan blue exclusion, was always > 90% (data not shown).

Table 1 Single-percentage expression of surface membrane marker CD205, MHC class II and CD80 on splenic dendritic cells (DCs), and unfractionated splenocytes from non-infected (day 0) and *N. caninum*-infected BALB/c mice. Data are expressed as mean percentages of three experiments ± standard deviation (± SD).

Specificity	Cell type	% Expression (Mean ± SD)			
		day 0	3 dpi	5 dpi	7 dpi
CD205	splenic DC	90.8 ± 2.4	91.8 ±5.4	93.0 ± 2.9	95.3 ± 1.3
	splenocyte	69.7 ± 2.0	77.8 ± 3.6	79.6 ± 9.8	87.6 ± 4.5
MHC class II	splenic DC	77.7 ± 0.8	92.3 ± 4.9	93.2 ± 6.2	91.0 ± 1.8
	splenocyte	56.4 ± 9.5	90.9 ± 2.5	90.4 ± 5.5	91.5 ± 0.3
CD80	splenic DC	6.3 ± 0.5	8.2 ± 1.4	8.0 ± 1.0	13.7 ± 2.3
	splenocyte	4.8 ± 1.2	8.2 ± 2.3	7.6 ± 1.6	9.3 ± 1.5

MHC class II = major histocompatibility complex; CD = cluster of differentiation; DCs = dendritic cells; dpi = days post-infection.

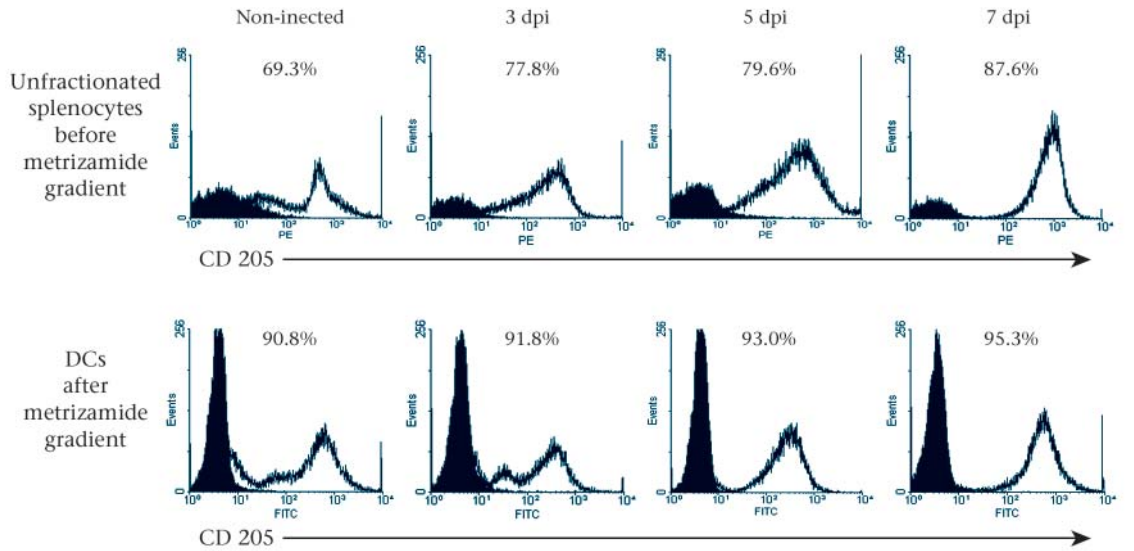

Fig 1 One-parameter histograms showing expression of CD205 on unfractionated splenocytes before and after metrizamide gradient fractionation of non-infected and *N. caninum*-infected BALB/c mice. Percentages of positive cells are given in each panel. The Y-axis represents frequencies showing numbers of positive cells and the X-axis is the log intensity of green (FITC) fluorescence. Data represent three experiments and show the respective control staining (filled histograms) and specific monoclonal antibody binding (open histograms).

Morphology of the isolated dendritic cells

Low density cells obtained by mechanical tissue disruption followed by metrizamide density gradient centrifugation were characterized by morphology under light microscopy. Using the criteria of relative size, cytoplasm : nucleus ratio, shape and position of nucleus and cytoplasmic protuberances/veils, the morphological features of the harvested DC were analyzed. The isolated DC showed irregular shapes when observed over a longer period of time in culture. Moreover, the isolated cells seemed continually to retract and reorient their cytoplasmic processes (Fig 2).

Infection of BALB/c mice with *N. caninum*

In order to demonstrate infection, the presence of serum-specific antibody to *N. caninum* from BALB/c mice infected ip with 5×10^4 *N. caninum* tachyzoites was monitored by indirect immunofluorescent antibody test (IFAT) using *N. caninum* tachyzoite antigen following infection (Fig 3). Specific antibodies to *N. caninum* were first detectable 5 dpi, whereas none were detectable in non-infected mice. The infected mice did not show clinical signs of neosporosis.

Infection up-regulates MHC class II glycoproteins and the co-stimulatory molecule CD80 in splenic DC

Since studies have shown that the up-regulation of MHC class II products and co-stimulatory molecules in DC after activation is essential for DC activation/maturation [14], we were prompted to analyze the expression pattern of the surface antigen in DCs from non-infected and *N. caninum*-infected BALB/c mice 3, 5, and 7 dpi. The results of the expression of MHC class II glycoproteins and the co-stimulatory molecule CD80 following *N. caninum* infection are summarized in Table 1 and Fig 4. Flow cytometric analysis showed that MHC class II products on DC increased significantly following *N. caninum* infection. Moreover, expression of the co-stimulatory molecule CD 80 was up-regulated consistently exhibiting a two-fold increase at 7 dpi.

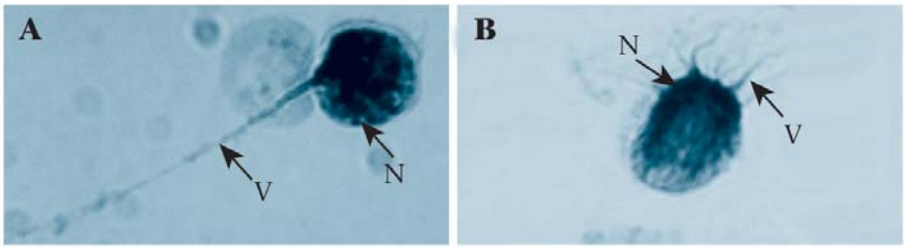

Fig 2 Giemsa-stained light microscopic visualization of isolated splenic dendritic cells (DCs) from BALB/c mice (A and B) exhibiting typical DC morphology: cytoplasmic protuberances (V), irregularly shaped nuclei (N), and large cytoplasmic : nucleus ratio (×670).

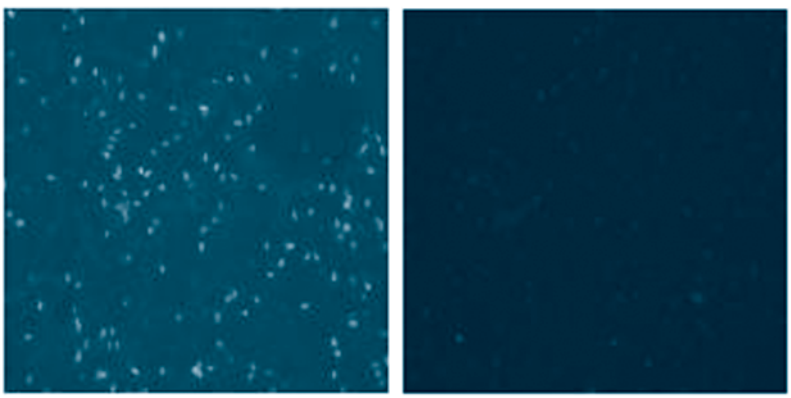

Fig 3 Indirect immunofluorescent antibody test (IFAT) for *N. caninum*-specific antibody in serum collected from *N. caninum*-infected BALB/c mice at 7 dpi (A) and non-infected (B) using *N. caninum* tachyzoite antigen.

Discussion

The aim of the present study was to assess the expression of MHC class II and the co-stimulatory molecule CD80 in immune competent cells obtained from spleens of non-infected and *N. caninum*-infected BALB/c mice at 3, 5, and 7 dpi by flow cytometric analysis. We successfully used color staining to demonstrate the surface marker of splenic DCs; CD205 was shown to be > 90%, as in previous studies, which identified high CD205 in DCs from afferent cattle lymph [15], central nervous system (CNS) of mice [16], and human skin [17].

We demonstrated that *N. caninum*, a coccidian parasite that is morphologically similar to *T. gondii*, can stimulate antigen presentation characterized by the production of MHC class II and CD80 during infection. BALB/c mice showed no clinical signs of neosporosis, and Class II MHC expressions in the splenic DC from *N. caninum* infected mice were up-regulated to statistically significant levels, which peak all 3, 5, and 7 dpi and CD80 levels increased two-fold at 7 dpi. Our results are consistent with other findings that showed increased levels of MHC class II and CD80 on splenic DCs from malaria-infected mice [18]. By contrast, down-regulation of MHC class II and up-regulation of CD80 were found on DCs derived from Peyer's patch macrophages (DPP-DC) of *T. gondii*-infected mice [13]. The loss of MHC class II may express that functions of DCs may be inhibited by *T. gondii* infection, whereby a *T. gondii* survival strategy to long-term persistence.

Since APC play an initial role in the immune response against pathogens, we compared their

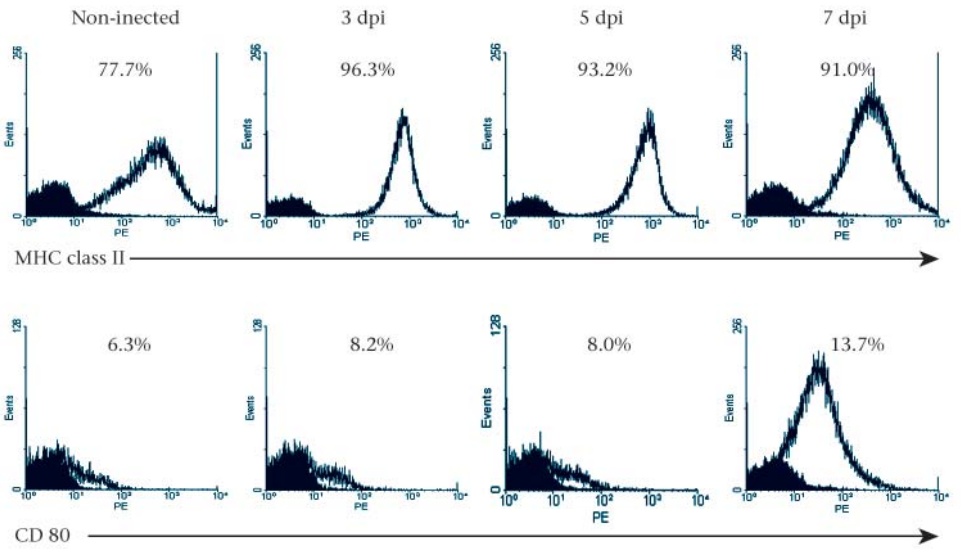

Fig 4 One-parameter histograms showing expression of MHC class II products and co-stimulatory molecule CD80 in splenic DCs obtained from non-infected and *N. caninum*-infected BALB/c mice. Percentages of positive cells are given in each panel. The Y-axis represents frequencies showing numbers of positive cells and the X-axis is the log intensity of red (phycoerythrin) fluorescence. Data represent three experiments and show the respective control staining (filled histograms) and specific monoclonal antibody binding (open histograms).

function in non-infected and *N. caninum*-infected mice. One of the main functions of APC, which in this study were splenic DC, is their parasite antigen-presenting activity in association with MHC class I or II glycoproteins, as well as co-stimulatory ligands. MHC class II glycoproteins and the co-stimulatory ligand CD80, both important for antigen presentation, were significantly increased in DC from *N. caninum*-infected mice. The up-regulation of MHC class II and the co-stimulatory molecules in CD205 positive DC agrees with the published literature because these ligands are widely discussed as a measure of DC activation/maturation [19]. CD80 and CD86, when up-regulated, bind to T-cell surface molecules CD28 and CTLA-4, which may be critical in prolonging primary T-cell responses [20]. It has been suggested that the presence of CD80 favors the activation of Th1 lymphocytes, whereas CD86 favors priming of Th2 cells [21-22]. From this viewpoint, DC may, therefore, play an important role not only in initiating adaptive immunity to *N. caninum*, but also in the early induction of innate immunity to the parasite. Moreover, the up-regulation of MHC class II expression on splenic DC during *N. caninum* infection may be associated with host survival of parasite infection.

Whether or not the mechanisms of immune protection against *N. caninum* in mice are active in hosts that develop natural disease, such as cattle, horses, or dogs or immuno-compromised humans, is the big question. However, the present study does indicate a direction to address future investigations of protective immunity to *N. caninum* infection. Future studies should identify intracellular cytokine expression in splenic DCs that may play an important role in the resolution of infection.

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