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Short communication

Toll-like receptor 3 (TLR3): A new marker of canine monocytes-derived dendritic cells (cMo-DC)

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Abstract

Toll-like receptors (TLRs) are a family of functionally important receptors for recognition of pathogen-associated molecular pattern (PAMP) since they trigger the pro-inflammatory response and upregulation of costimulatory molecules, linking the rapid innate response to adaptative immunity. In human leukocytes, TLR3 has been found to be specifically expressed in dendritic cells (DC). This study examined the expression of TLR3 in canine monocytes-derived DC (cMo-DC) and PBMC using three new anti-TLR3 mAbs (619F7, 722E2 and 713E4 clones). The non-adherent cMo-DC generated after culture in canine IL-4 plus canine GM-CSF were labelled with the three anti-TLR3 clones by flow cytometry, with a strong expression shown for 619F7 and 722E2 clones. By contrast, TLR3 expression was low to moderate in canine monocytes and lymphocytes. These results were confirmed by Western blot using 619F7 and 722E2 clones and several polypeptide bands were observed, suggesting a possible cleavage of TLR3 molecule or different glycosylation states. In addition, TLR3 was detectable in immunocytochemistry by using 722E2 clone. In conclusion, this first approach to study canine TLR3 protein expression shows that three anti-TLR3 clones detect canine TLR3 and can be used to better characterize canine DC and the immune system of dogs.

Keywords: TLR3; Dog; Canine monocytes-derived dendritic cells

1. Introduction

Toll-like receptor 3 (TLR3) recognizes viral double-stranded (ds) RNA as well as poly(I:C), a synthetic dsRNA analogue, leading to the activation of the transcription factor NF-κB (Alexopoulou et al., 2001). In human immature Mo-DC, TLR3 molecules were shown to reside inside the cells, presumably with the site of action located in or near intracellular vesicles (Matsumoto et al., 2003). It was also demonstrated that dsRNA activated TLR3 in DC to release type 1 IFN,

Abbreviations: cMo-DC, canine monocyte-derived dendritic cells; DC, dendritic cells; TLR, Toll-like receptor

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inducing tumor cells apoptosis and NK-mediated tumor cytotoxicity (Seya et al., 2003). Recently, the structures of the TLR3 ectodomain (ECD) have been elucidated by X-ray crystallography (Choe et al., 2005; Bell et al., 2006), and it was proposed that RNA binding by the TLR3 ECD leads to dimerization (Bell et al., 2006). As for canine TLR tissue expression, only canine TLR2, TLR4 and TLR9 have been investigated through molecular techniques (Bazzocchi et al., 2005; Ishii et al., 2006; Asahina et al., 2003; Wassef et al., 2004; Hashimoto et al., 2005).

This work investigated the cross-reactivity of three new anti-TLR3 mAbs, the 619F7 clone (anti-human TLR3), the 722E2 clone and the 713E4 clone (multi-species reactivity) (Dendritics, Lyon, France) towards canine PBMC (cPBMC) and cMo-DC by flow cytometry, immunocytochemistry and Western blot.

2. Materials and methods

2.1. Dogs

Blood samples were drawn from the jugular vein of male and female adult beagle dogs ($n = 5$) from the Department of Food Department of the Lyon National Veterinary School. The use of these experimental animals was in accordance with the institutional guidelines.

2.2. Cells and reagents

cMo-DC were generated from adherent monocytes as previously described (Bonnefont-Rebeix et al., 2006). Briefly, canine PBMC were isolated using Ficoll density gradient of 1.070. Adherent monocytes were then cultured for 7 days in the presence of canine GM-CSF plus canine IL-4 (cGM-CSF and cIL-4, respectively, were kindly provided by Merial, Lyon, France) representing 1% of the total culture medium volume.

Human monocytes were isolated from PBMC with a two-step discontinuous density gradient separation as described previously (Duperrier et al., 2000), followed by the CD14 microbeads positive selection technique. Briefly, 40×10^6 PBMC were layered over the two layered-gradient of 50% (6 ml) and 40% (3 ml) dilutions of iso-osmotic solution of Percoll (1.130 g/ml; Pharmacia LKB, Uppsala, Sweden) and centrifuged at $1000 \times g$ for 25 min at 4 °C. Monocytes were harvested at the interface of the two Percoll solutions and washed twice in PBS before the CD14 microbeads positive selection technique (Miltenyi Biotec, Paris, France). Human monocytes were then plated in tissue culture wells at 37 °C in humidified 5% CO_2 in air to induce their differentiation in immature DC. Culture medium containing 10% heat inactivated human serum was supplemented every other day with 200 U/ml rhGM-CSF and 500 U/ml rhIL-4 until day 6, where all non-adherent cells were harvested.

Since human B cells were labelled with the three anti-TLR3 mAbs clones, the human HPA5B B cell line was used as positive control for its TLR3 expression. This human EBV-infected B cell line was obtained by EBV transformation of peripheral blood B cells from normal healthy donor.

2.3. Flow cytometry and mAbs

Flow cytometric acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton Dickinson, Pleasanton, CA, USA). Direct cell staining was performed using FITC- or PE-conjugated mAbs with their isotypic controls (Becton Dickinson, Pleasanton, CA, USA). The mAbs used were anti-CD14-PE (TÜK4, Dako, Glostrup, Denmark; cross reactivity in the dog: Jacobsen et al., 1993), anti-canine Class II-FITC (YKIX 334.2, Serotec, Oxford, UK), anti-human CD86-FITC (FUN-1, Becton Dickinson, Pleasanton, CA, USA; cross reactivity in the dog: Bonnefont-Rebeix et al., 2006). Three anti-TLR3 mAbs (619F7, 722E2 and 713E4 clones, Dendritics, Lyon, France) were generated by immunization of BALB/C mice with hTLR3-pUNO. Hybridoma supernatants were screened for antibody production against hTLR3-HEK293T transfected cells, and their specificity was confirmed by flow cytometry on human mammary carcinoma cell line (CAMA1) and by immunocytochemistry on human tonsils frozen sections (Dendritics, Lyon, France). For intracellular TLR3 staining, cells were permeabilized with 0.25% saponin (Sigma, St Louis, MO, USA) for 1 h then washed. Cells were then incubated for 30 min with anti-TLR3 mAbs, then washed. Staining was revealed with FITC-conjugated F(ab′)2 fragment goat anti-mouse IgG for 15 min (Dako, Glostrup, Denmark).

2.4. Cytocentrifugation and immunocytochemistry

PBMC and cultured cells were adhered to glass slides by Cytospin (Thermo Shandon, Cheshire, UK). Then, adherent cells were stained with May–Grünwald–Giemsa or permeabilized with 0.5% saponin, 1% BSA/PBS for 30 min before immunolabelling with anti-TLR3 mAbs using a streptavidin-biotin-peroxydase method with diaminobenzidine (DAB) as substrate

(Autoprobe II kit, Biomeda, Foster City, CA, USA). Negative control was performed with omission of the primary mAb.

2.5. Western blot

Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% Triton X-100, 10% glycerol, 1% EDTA, 5 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and the protein concentrations were measured by the Bradford method standardized with BSA. Each cell lysate was subjected to denaturation at 75 °C for 5 min in presence of 2% β-mercaptoethanol and 2% SDS. Equal amounts (10–50 μg) of proteins from each sample were subjected to SDS-PAGE (12%) and blotted onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in tris-buffer saline tween (TBST: 25 mM Tris–HCl, 0.15 M NaCl, 0.05% Tween 20) at room temperature (RT) for 1 h, before incubation with 16 μg/ml anti-TLR3 mAbs (respectively 722E2, 713E4 and 619F7 clones, previously adsorbed on a similar membrane) at RT for 1 h and at 4 °C overnight. The blots were developed with an HRP-conjugated sheep anti-mouse IgG antibody (1:50 000) (Sigma–Aldrich, St Louis, MO) at RT for 1 h and visualized using SuperSignal West Pico Chemiluminescent (Pierce, Rockford, IL).

3. Results and discussion

After culture of canine adherent monocytes in the presence of cGM-CSF and cIL-4 for 7 days, the non-adherent cMo-DC expressed CD86+, Class II+ and CD14+ as previously described using flow cytometry (Bonnefont-Rebeix et al., 2006). Here, TLR3 expression was strongly revealed in cMo-DC using two anti-TLR3 clones, the 722E2 and the 619F7, and more weakly with the clone 713E4 (Fig. 1, Table 1, $n = 5$). The same profile of TLR3 expression was obtained with the labelling of the human EBV-infected B cell line, whereas TLR3 expression was low to moderate in canine monocytes and lymphocytes depending on anti-TLR3 clone, 722E2 clone remaining always more intense (Table 1). Moreover, canine monocytes and cMo-DC expressed TLR2 and TLR4 (detected by flow

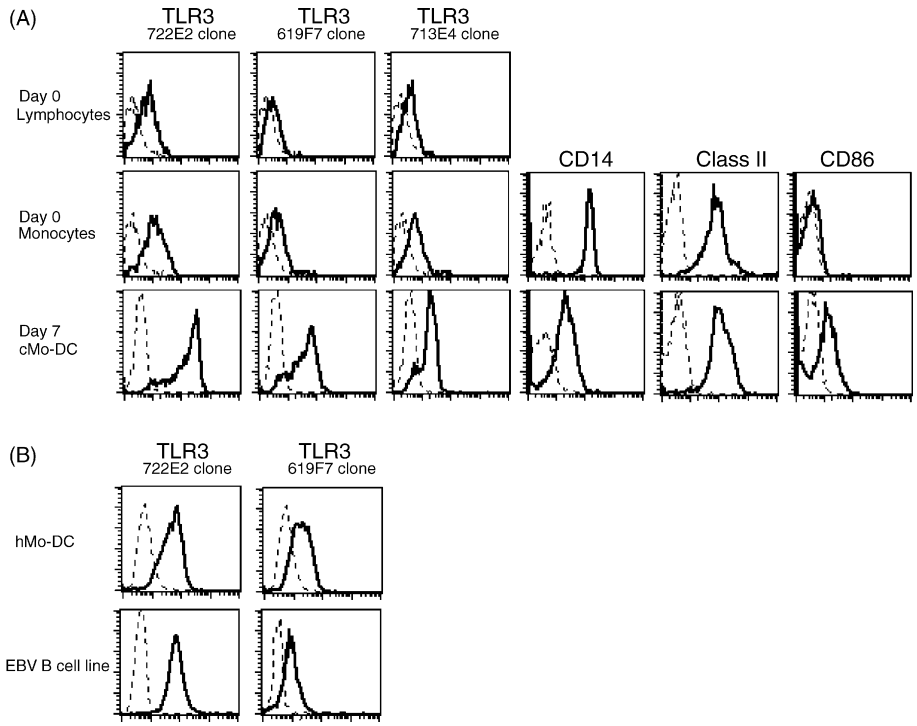

Fig. 1. FACS profiles of (A) canine monocytes and lymphocytes at day 0 and cMo-DC at day 7 after culture in cGM-CSF + cIL-4; (B) human EBV-infected B cell line and human immature DC. Direct cell staining was performed using FITC- or PE-conjugated mAbs with their isotypic controls, whereas permeabilized cells were stained with anti-TLR3 mAb and FITC-labelled secondary Ab. Each histogram represents an overlay of the respective mAb (bold profiles) and an isotype-matched control Ig or FITC-labelled secondary Ab (dotted profiles) for direct staining or intracellular indirect staining, respectively. The data shown in (A) are representative for five independent experiments.

Table 1
Intracellular expression of TLR3 in canine lymphocytes, monocytes, and cMo-DC, evaluated by flow cytometry with three anti-TLR3 mAbs (722E2, 619F7 and 713E4 clones)

	722E2		619F7		713E4	
	MFI	%	MFI	%	MFI	%
Lymphocytes	13 ± 9	85 ± 3	7 ± 3	38 ± 30	7 ± 6	16 ± 26
Monocytes	22 ± 11	85 ± 2	11 ± 4	51 ± 18	9 ± 7	43 ± 36
cMo-DC	127 ± 21	95 ± 1	39 ± 11	76 ± 6	26 ± 12	76 ± 10

Data are shown as mean ± standard deviation ($n = 5$). %, percentage of positive cells; MFI, mean channel fluorescence intensity. Differences in the level of expression of TLR3 between cMo-DC and monocytes was measured with p values: $p < 0.01$ with 722E2 and 619F7 clones, and $p < 0.05$ with 713E4 clone. This expression in cMo-DC was also significant when compared with lymphocytes (identical p values), whereas the differences were not significant between monocytes and lymphocytes ($p > 0.05$).

cytometry with anti-TLR2 TL2.1 clone and anti-TLR4 HTA125 clone, respectively, eBioscience, San Diego, CA, USA), with large intra-individual variations (data not shown).

Using the streptavidin-biotin-peroxidase procedure on cytospin preparations, TLR3 expression in cMo-DC was also revealed strongly with the 722E2 clone (Fig. 2), very weakly with the 619F7 clone, but was not detectable with the 713E4 clone. TLR3 expression in cPBMC was only barely detectable with 722E2 clone. Concomitant with this, Western blot analysis of canine PBMC and cMo-DC does not allow visualizing any protein with the 713E4 clone, whereas proteins were detected with the 619F7 and 722E2 clones (Fig. 3). One main polypeptide of approximately 65-kDa was identified in human and canine Mo-DC, human EBV-infected B cell line and PBMC, but not in cPBMC and human monocytes. A second polypeptide of approximately 50-kDa was visualized in human PBMC and EBV-infected B cell line. Additional polypeptides were observed exclusively with 722E2 clone in canine and human PBMC and Mo-DC at various molecular weights.

These results are in accordance with those in human, where TLR3 was shown to be exclusively expressed in DC but absent in precursor monocytes, by means of total RNA extraction and Northern blot analysis (Muzio et al., 2000). Nevertheless, human PBMC respond to stimulation with dsRNA, the TLR3 ligand, by producing IP-10/CXCX10 protein (Proost et al., 2003), and also to stimulation with poly(I:C) by producing cytokines (Re and Strominger, 2004), suggesting that TLR3 is present in lymphocytes.

Interestingly, Western blot analysis of human TLR3 mainly concerned its extracellular domain (hTLR3 ECD) and showed a major polypeptide band of approximately 110-kDa, much larger than the predicted mass of 75-kDa (Sun et al., 2006). The authors attribute

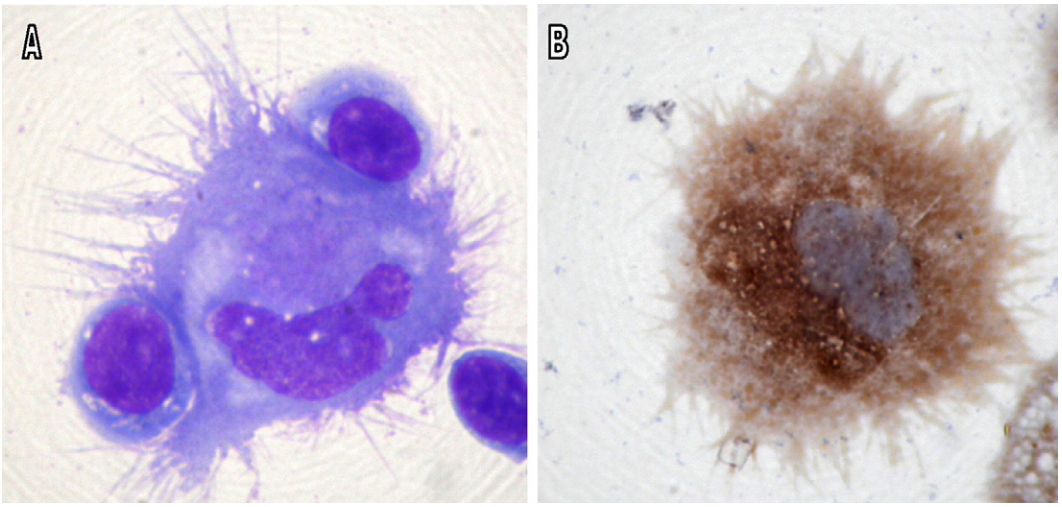

Fig. 2. Representative photograph of stained cytospin preparations of cMo-DC after 7 days of culture in the presence of cGM-CSF + cIL-4. (A) May–Grünwald–Giemsa-stained cMo-DC. (B) Immunolabelling of cMo-DC with anti-TLR3 clone 722E2, using a standard streptavidin-biotin-peroxidase procedure. Original magnifications: A and B, 1000×.

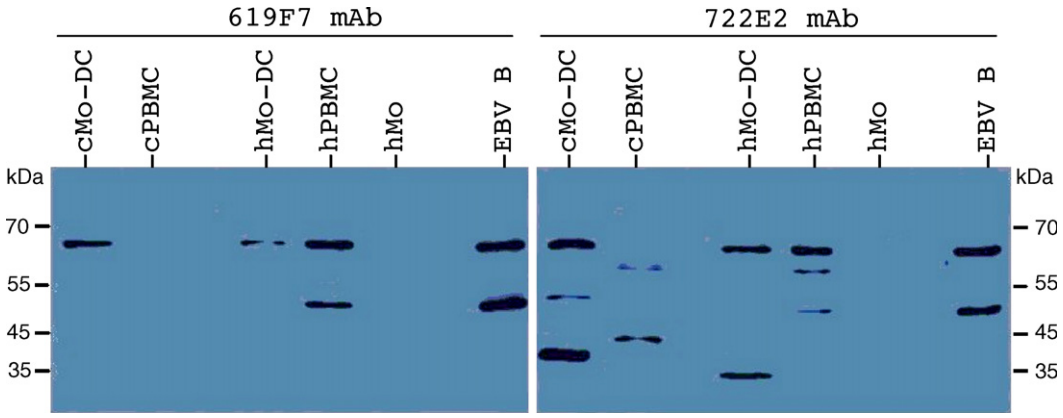

Fig. 3. Western blot expression of TLR3 in canine and human PBMC and Mo-DC, as well as in human monocytes (hMo) and EBV-infected cells (EBV B) using two different TLR3-specific mAbs, the 619F7 and the 722E2 clones. Equal amounts of proteins from each denatured sample were subjected to SDS-PAGE (12%) and blotted onto a nitrocellulose membrane. Molecular weight size markers are indicated along the left and right sides.

the difference to posttranslational modifications, bringing additional modifications other than N-linked glycosylations. Moreover, alignment of human TLR3 ECD amino acid sequence with the predicted canine counterpart shows 83% sequence similarities, numerous potential glycosylation sites and well-conserved protein and RNA interacting sites (Ranjith-Kumar et al., 2007). Thus, the lower polypeptide band of approximately 50-kDa specifically observed in human and canine PBMC and in human EBV-infected B cell line, and those only detected with 722E2 clone could either correspond to different glycosylation states, or could suggest a cleavage of the TLR3 molecule. The level of each TLR3 polypeptide may reflect a different processing of the TLR3 protein, depending of the cell state as well as of the modulations of activating factors. In the same way, up to six TLR2 polypeptides were described in human, with a functionally active soluble form and a corresponding intracellular pool in monocytes (LeBouder et al., 2003).

Recently, it was assessed that hTLR3 ECD predominantly exists as a dimer in vitro (Ranjith-Kumar et al., 2007), but all the cells were used in denatured conditions in this work, so dimers could not be conserved.

Since poly(I:C) was shown to induce maturation of human immature DC (imDC) with upregulation of CD80, CD83 and CD86 and stimulation of a proliferative response by allogeneic lymphocytes (Matsumoto et al., 2003; Kariko et al., 2004), cMo-DC were cultured from days 6 to 7 for 24 h with 20 μg/ml poly(I:C). Then, a mixed leukocyte reaction (MLR) was performed with graded numbers of cMo-DC (from 500 to 10 000) to stimulate 10^5 allogeneic cPBMC as responder cells. Poly(I:C) had no significant effect neither on CD86 expression, nor on lymphocytes proliferation (data not shown). TLR3 intracellular expression measured by flow cytometry was also unchanged, whereas a slight upregulation was reported in human DC.

These three clues could indicate that the cMo-DC were generated in the presence of cGM-CSF and cIL-4 could have already initiated their maturation since they were found to express CD86 and to be competent to stimulate lymphocyte proliferation in MLR, as previously discussed (Bonnefont-Rebeix et al., 2006). Indeed, the lack of canine specific available markers, such as CD83 and the DC-Lamp in humans, does not allow defining if these cMo-DCs are mature or immature. Nevertheless, the strong TLR3 expression observed in cMo-DC could suggest that they are still immature, since Visintin et al. (2001) revealed by Northern analysis that the high mRNA expression level observed in immature human DC generated in the presence of IL-4 and GM-CSF is strongly repressed after maturation with LPS or TNFα.

In summary, this first approach of canine TLR3 protein expression shows that the 619F7 and the 722E2 clones detect canine TLR3 and can be used for flow cytometric analysis, immunocytochemistry and Western blot, whereas the 713E4 clone can only be used for flow cytometric analysis, to investigate canine innate immune defence and its role in adaptative immunity.

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