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Rat bone marrow-derived dendritic cells, but not *ex vivo* dendritic cells, secrete nitric oxide and can inhibit T-cell proliferation

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SUMMARY

The relationships between different dendritic cell (DC) populations are not clearly established. In particular, it is not known how DC generated *in vitro* relate to those identified *in vivo.* Here we have characterized rat bone marrow-derived DC (BMDC) and compared them with DC isolated from spleen (SDC) and pseudo-afferent lymph (LDC). BMDC express typical DC markers and are mostly OX41 positive and CD4 negative. In contrast to *ex vivo* DC, some BMDC express Fc receptors. FcR^+ and FcR^- BMDC express similar levels of major histocompatibility complex class II molecules (MHC) and are B7 positive, but some FcR^- BMDC express high levels of B7. In contrast to freshly isolated or cultured *ex vivo* SDC and LDC, both BMDC subpopulations can express inducible nitric oxide synthase (iNOS) and can secrete nitric oxide (NO) in amounts similar to those secreted by peritoneal macrophages. Despite expressing MHC class II and B7, FcR^+ BMDC stimulate only a very weak MLR and inhibit stimulation by FcR^- BMDC and *ex vivo* DC. Inhibition is only partially NO dependent. FcR^+ BMDC are not macrophages, as judged by adherence and phagocytosis. Both subpopulations are able to present antigen to primed T cells *in vitro* and are able to prime naïve CD4 T cells *in vivo*. However, unlike SDC, BMDC are unable to stimulate cytotoxic T-lymphocyte (CTL) responses to a minor histocompatibility antigen. Thus, BMDC show marked differences to *ex vivo* DC and their relationship to those of *in vivo* DC populations, to date, is unclear.

INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells (APC), which play a pivotal role in the stimulation and regulation of immune responses.[1] In animals, and to a lesser extent humans, DC can be prepared by digestion of peripheral tissues or central lymphoid tissues,[2–4] or by cannulation of afferent or pseudo-afferent lymphatics.[5–7] In mouse, rat and human, cells with phenotypic and functional DC characteristics can also be prepared by culture from different precursors, including bone-marrow stem cells, $CD34^+$ blood cells and monocytes.[8–12] It is clearly important, from both a straightforward biological viewpoint and because of the increasing use of DC in immunotherapy, to understand how these different DC populations relate to each other, and how DC generated *in vitro* relate to DC *in vivo*. It is now apparent that DC *in vivo* can arise from both myeloid and lymphoid precursors[13,14] and there is evidence for distinct lineages in human DC generated from human blood mononuclear cells.[11,15,16] It is also unclear how those DC migrating from tissues under steady-state conditions[5,7,17,18] relate to those accumulating, or stimulated to migrate, by proinflammatory stimuli.[19–21] The understanding of DC is further complicated because within a single lineage, DC exist at different stages of maturation or differentiation.

Relatively few studies have attempted to compare DC generated *in vitro* with those obtained *ex vivo* and, where this has been attempted, the comparisons have been limited.[12,22] In the experiments described in the present report we have generated DC from rat bone marrow using granulocyte–macrophage colony-stimulating factor (GM–CSF) and interleukin (IL)-4 [bone marrow-derived DC (BMDC)], and have compared their phenotypic and functional properties with DC obtained *ex vivo* from spleen (SDC) and pseudo-afferent intestinal lymph (LDC).

We show that BMDC, although superficially similar to *ex vivo* DC, unlike *ex vivo* DC, can be stimulated to synthesize inducible nitric oxide synthase (iNOS) and to secrete large

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Abbreviations: BMDC: bone marrow-derived DC; CLN, cervical lymph node; DC, dendritic cell, LDC, pseudo-afferent lymph DC; iNOS, inducible nitric oxide synthase; SDC, spleen DC.

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amounts of nitric oxide (NO). BMDC are much weaker stimulators of an allogeneic mixed lymphocyte reaction (MLR) than SDC or LDC, and their ability to stimulate an MLR is not increased by several factors associated with DC maturation; indeed, when large numbers of BMDC are used as stimulators, proliferation is inhibited. This inhibition is only partly the result of NO secretion. There are two populations of BMDC distinguishable by FcR expression. FcR^+ BMDC are less stimulatory in an allogeneic MLR and inhibit the ability of other DC populations to stimulate an MLR. FcR^- BMDC augment the MLR stimulation by LDC. Both BMDC and SDC can present antigen (Ag) to sensitized T cells and prime naïve CD4 T cells *in vivo.* In contrast to SDC, however, BMDC were unable to prime $CD8^+$ CTL against a minor histocompatibility Ag. Therefore, BMDC differ functionally from *ex vivo* DC and it remains unclear how they fit into our current picture of DC *in vivo.*

MATERIALS AND METHODS

Animals

Rats were bred and maintained under specific pathogen-free (SPF) conditions at the MRC Cellular Immunology Unit (Oxford, UK). Strains used were PVG ($RT1^c$), DA ($RT1^{av1}$), LEW ($RT1^l$) and F1 crosses of of DA/LEW. All experiments were carried out under the authority of a licence issued by the Home Office.

Antibodies and other reagents

Anti-iNOS monoclonal antibodies (mAbs) (ANOS5 and ANOS11)[23] were used at 10 μg/ml for immunocytochemistry. Most mouse anti-rat antibodies used in this study were gifts from the MRC Cellular Immunology Unit. OX6 [mouse immunoglobulin G1 (IgG1)] recognizes rat major histocompatibility complex (MHC) class II; OX62 (mouse IgG1) recognizes an integrin-like molecule expressed solely on LDC and γδ T cells.[24,25] The mouse anti-rat CD80 mAb (3H5) was a kind gift from Dr Okumura (Jutendo University, Tokyo, Japan).[26] Anti-rat CD11c was purchased from Serotec (Oxford, UK). N^G-monomethyl L-Arginine (NMMA), indomethacin and saponin (from Saponaria spp.) were purchased from Sigma (Poole, UK). GM–CSF (supernatant from an X63-Ag8 line transfected with murine GM–CSF) was a gift from Dr D. Gray (University of Edinburgh, Edinburgh, UK). Rat IL-4 [supernatant from a rat IL-4-transfected Chinese hamster ovary (CHO) line], recombinant rat interferon-γ (IFN-γ) and recombinant rat tumour necrosis factor-α (TNF-α) were gifts from Dr D. Mason (MRC Cellular Immunology Unit).

Cell culture

The media (R5) used was RPMI-1640, supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 U/ml penicillin and 50 μg/ml streptomycin (all from Gibco, Paisley, UK). Lipopolysaccharide (LPS) contamination of the FCS was <0·4 ng/ml (as measured by the supplier). For MLRs, FCS was replaced by 5% DA rat serum. All cell cultures were performed at 37°, in an atmosphere of 5% CO_2.

Generation and isolation of DC

Culture of bone marrow DC was based on a previously described procedure.[8,27] Bone marrow was aspirated from femurs of 10–16-week-old male PVG ($RT1^c$) or DA/l ($RT1^{av11}$) rats. Red blood cells were removed by Geys lysis solution, and lymphocytes/MHC class II^+ cells were removed by rosetting.[28] Cells were cultured in 24-well plates at 10^6 cells/ml/well in complete media [R5 plus 1% vol/vol GM–CSF (30 ng/ml) and IL-4 (2000 U/ml)]. Medium was changed every 2–3 days and cells were harvested after 6 or 7 days. Semi- and non-adherent cells were harvested after 6 or 7 days. Cells used for fluorescence-activated cell sorter (FACS) analysis of iNOS were indirectly depleted of macrophages (Mϕ) on day 6 by harvesting BMDC and replating in 24-well plates at 10^6 cells/ml.

Lymph DC were isolated from pseudo-afferent thoracic duct lymph, as described previously.[5] Rats were not irradiated. PVG ($RT1^c$) rats were used routinely, but in some experiments DA/L ($RT1^{av11}$) rats were used; similar results were obtained.

Spleen DC were isolated using a modification of a previously described procedure,[29,30] by digestion with 2 mg/ml collagenase D (Boehringer Mannheim, Mannheim, Germany) and 0·05% DNaseI (Boehringer Mannheim) followed by suspension in Ca^{2+}-free Hanks' balanced salt solution (HBSS) (Gibco) containing 50 mM EDTA, at 4°, to separate clusters. Cells were then centrifuged over Nycoprep Animal 1·077 (Nycomed, Oslo, Norway), washed and incubated in Petri dishes in R5 for 2 hr at 37°. Non-adherent cells were washed away. The remaining cells were cultured overnight and non-adherent cells collected by pipetting. These cells were then rosetted with opsonized sheep red-blood cells (SRBC; TCS Biologicals, Botolph, Claydon, UK) to remove FcR^+ cells. To obtain highly purified SDC, non-adherent cells were incubated with an antibody cocktail to remove lymphocytes, natural killer (NK) cells and Mϕs. The resultant populations were judged 75–85% DC by morphology and immunocytochemistry. To obtain freshly isolated SDC, the overnight adherence was omitted and SDC were enriched using negative selection of lymphocytes, NK cells and Mϕs (used for the antigen presentation *in vivo*, as shown in Fig. 9a).

Opsonization of SRBC and Fc rosetting of BMDC

SRBC (TCS Biologicals) were washed three times in phosphate-buffered saline (PBS), then resuspended in a 5% solution with 20 μg/ml rabbit anti-SRBC hyperimmune serum (Nordic, Tilburg, The Netherlands) in R5 and incubated for 1 hr at 37°, washed three times in R5 and made up to a 5% (vol/vol) solution. BMDC were separated into FcR^+ and FcR^- populations by incubation with opsonized SRBC (10 SRBC : 1 BMDC) for 30 min at 4° on a rotating wheel. Cells were then centrifuged on a Histopaque (Sigma) gradient, FcR^- cells removed from the interface and FcR^+ cells recovered from the pellet after lysis of SRBC.

Isolation of Mϕs

Rats were killed by CO_2 and ice-cold PBS injected into the peritoneal cavity. Resident peritoneal Mϕs were collected by pipette after opening the peritoneal cavity. Thioglycollate-elicited Mϕs were collected from the peritoneal cavity of freshly killed rats injected intraperitoneally (i.p.) with thioglycollate (Difco Brewers thioglycollate; Difco, West Molesey, UK) 3 days previously.

Phagocytosis and endocytosis assays

Phagocytosis of opsonized SRBC was measured using the following procedure. DC or Mϕs were mixed with opsonized SRBC at ratios of 1 : 10 and 1 : 50 cells : SRBC and incubated for 1 hr at 37°. External SRBC were lysed using Geys solution, and cytospins were prepared and labelled with OX6 (anti-MHC class II) or OX42 (anti-CD11b/c). The numbers of SRBC internalized by 200 cells were counted. Endocytosis of fluorescein isothiocyanate (FITC)-conjugated beads was measured as follows. Various sizes of beads (Sigma, supplied as 5% beads, diluted 1 : 100 in the assay) were incubated with BMDC for 30 min at 37 or 4°. Cells were washed three times and the mean fluorescence intensity (MFI) measured using a fluorescence-activated cell sorter (FACscan; Becton-Dickinson, San Jose, CA). Specific uptake was assessed by subtracting the MFI of cells incubated at 4° from the value at 37°, to give the ΔMFI.

Immunocytochemistry and flow cytometry

Cells were resuspended at 5×10^5/ml in R5 media and mixed with an equal volume of 10% bovine serum albumin (BSA) (Sigma) and then spun onto glass slides in a cytospin for 5 min at 400 rpm. Slides were then fixed in ethanol for 10 min and washed in PBS. Slides were labelled with mouse anti-rat mAbs followed by rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). Diamino-diamino-benzene (10 mM) (Kementec, Copenhagen, Denmark) and 0·005% H_2O_2 (Sigma) were then added to the slides for 10 min. Slides were then counterstained with Harris Haematoxylin and dehydrated through ethanol and Histoclear (National Diagnostics, Hessle, UK) and mounted with DePeX (BDH, Dorset, UK).

For flow cytometry, DC were harvested from plates by pipetting (leaving the adherent cells) and resuspended at 2×10^5 cells/200 μl in a 96-well round-bottom plate. Cells were permeabilized on ice for 1 hr with 0·3% saponin (Sigma) and 1% BSA in PBS (PBS/sap/BSA) and then stained for 30 min on ice with anti-iNOS, or with OX21 as a negative control or OX6 as a positive control. Cells were then washed with PBS/sap/BSA. Secondary FITC conjugated rabbit anti-mouse was then incubated with cells for 30 min. For double staining, 50 μl of OX6-biotin was used at 10 μg/ml for 30 min without saponin, then streptavidin Quantum red (Sigma) was used to detect MHC class II molecules. For surface labelling, cells were washed and stained in PBS containing 1% BSA and 10 mM sodium azide. Cells were extensively washed and analysed on a Becton-Dickinson FACscan connected to a BD FACSmate. Plots were analysed using Cell Quest and at least 10 000 events were collected for each sample.

Treatment with cytokines or endotoxin and measurement of NO

Cells were plated out in 24-well plates at 10^6 cells/well with IFN-γ (50 U/ml) and LPS (50 ng/ml), in R5 media. After 24–48 hr, cells were harvested, washed and analysed by FACS or immunocytochemistry.

Various populations of DC or Mϕs were cultured at 5×10^5/150 μl in 96-well round-bottom plates. IFN-γ was added at 50 U/ml, TNF-α at 25 U/ml and LPS at 50 ng/well. After 48 hr of culture, NO release was assessed using the Greiss reagent [1% sulphanilamide (Sigma) in 5% phosphoric acid and 1% *N*-(1-naphtyl)ethylenediamine (NEDA) (Sigma) in H_2O]. For each sample, a negative control was included that contained 250 μg/ml NMMA.

Allogeneic MLR

DC (generally $RT1^{av1\ xl}$, occasionally $RT1^c$) were isolated as described above and graded numbers added to 2×10^5 allogeneic (generally $RT1^c$ occasionally $RT1^{av1\ xl}$) cervical lymph node cells in 96-well round-bottom plates in MLR medium. After 3 days, culture plates were pulsed with [^{3}H]thymidine ([^{3}H]TdR) (Amersham, Little Chalfont, UK), 0·5 μCi/well overnight, harvested onto betaplate filtermats and counted on a betaplate scintillation counter (Wallac, Milton Keynes, UK). In some assays, final concentrations of 0·2 μg/ml indomethacin or 1 mM NMMA were added to wells.

Antigen presentation in vitro *and* in vivo

Various DC populations were isolated, pulsed with 100 μg/ml keyhole limpet haemocyanin (KLH) (Sigma), washed extensively with PBS containing 50 mM EDTA and then injected into the footpads of syngeneic rats. After 10 days, popliteal lymph node cells were removed from primed rats, made into single-cell suspensions and incubated in 96-well plates with soluble antigen (100 μg/ml KLH in R5 media with 5% normal rat serum replacing FCS). Four days later, proliferation was measured, as described above, for MLR.

Various populations of DC were incubated with 100 μg/ml KLH for 3 hr at 37°, washed extensively and then graded numbers were cultured with 2×10^5/well nylon wool-enriched splenic T cells from rats primed to KLH 2 weeks previously. After 5 days, proliferation was measured as described for the MLR experiments.

Generation of cytotoxic T cells in vivo

Cytotoxic T-lymphocyte (CTL) responses were primed to a maternally transmitted mitochondrial-associated minor histocompatibility antigen (MTA; ref. 31). In short, MTA^- rats were injected i.p. with lymphocytes from MTA^+ rats. After at least 3 weeks, rats were killed, spleen and lymph nodes were removed and single-cell suspensions were prepared, Primed MTA^- cells were cultured at 2×10^5 cells per well in 96-well plates with 2×10^5 MTA^+ irradiated (20 Gy) spleen or lymph node cells. Five to 6 days later, cells were harvested and tested for CTL activity against MTA^- and MTA^+ concanavalin A (Con A)-stimulated blasts in a 3-hr JAM cytotoxicity assay.[32] Con A blasts were produced by culturing spleen or lymph node cells with Con A (2·5–10 μg/ml) for 24 hr and then pulsing overnight with [^{3}H]TdR. Cells were washed, centrifuged over Histopaque to remove dead cells and plated at 10^4 cells/well in 96-well plates. Effector cells were added in graded doses and, after 3 hr, cells were harvested and residual radioactivity measured as described above. The percentage specific cytotoxicity was calculated as follows:

$$\% \text{ Specific cytotoxicity} = [(S - E) \div S] \times 100,$$

where E = counts per minute (c.p.m.) retained in the presence of effector cells and S = c.p.m. retained in the absence of effector cells.

RESULTS

BMDC comprise FcR$^+$ and FcR$^-$ subpopulations and are not Mϕs

Bone marrow cells were cultured for 7 days in the presence of GM–CSF and IL-4, and the resulting cells (BMDC) were examined by immunoperoxidase labelling on cytospins or by flow cytometry. Most BMDC displayed typical DC morphology and expressed MHC class II, intracellular adhesion molecule-1 (ICAM-1), B7 and CD80 molecules (Fig. 1). BMDC did not express the rat lymph DC marker OX62 (data not shown, also reported in ref. 12). In contrast to lymph DC, most BMDC expressed OX41 but not CD4 (data not shown). A subpopulation of LDC have been shown to express both of these markers.[17] Flow cytometry showed that the BMDC expressed heterogeneous levels of MHC class II molecules, suggesting different maturation stages.

As immature DC may express Fc receptors,[33] BMDC were separated by Fc rosetting using opsonized SRBC (no mAb to rat FcR is available). Two populations were recovered: after 7 days of culture about 75% of BMDC were FcR$^+$, but by 14 days, this proportion had dropped to 35–40%. Flow cytometry showed that FcR$^-$ BMDC and FcR$^+$ BMDC expressed similar levels of MHC class II and CD11c. FcR$^+$ BMDC expressed low levels of B7 (visualized by CTLA-4 fusion protein and anti-CD80),[26] but expression of B7 by FcR$^-$ BMDC was biphasic, with a proportion expressing relatively high levels (Fig. 1b). Although expression of MHC class II was heterogeneous, on cytospins it appeared that in FcR$^+$ BMDC, a larger proportion of MHC class II was cytoplasmic (Fig. 1a, and data not shown).

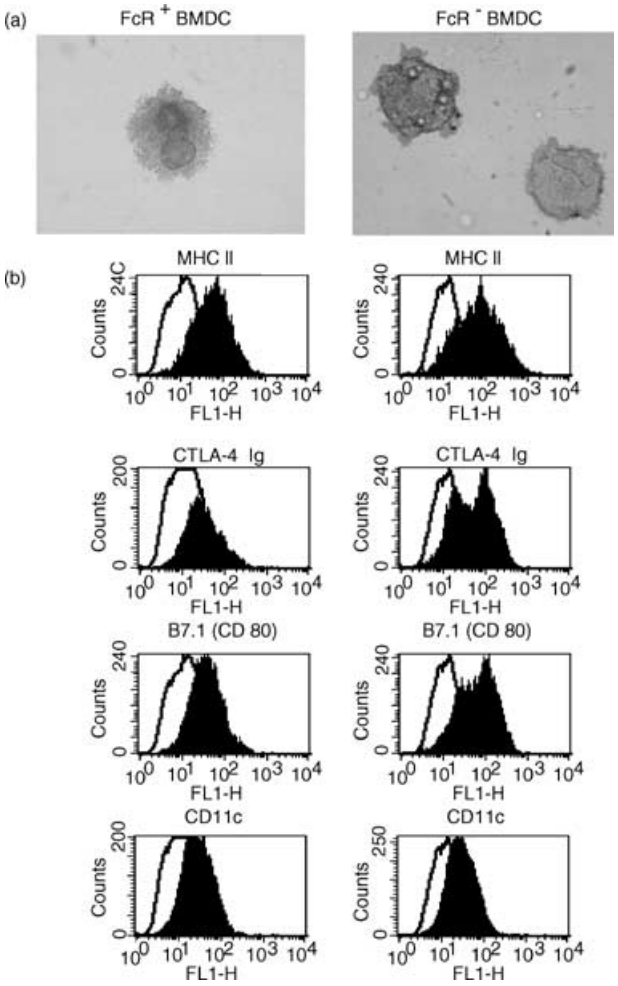


Figure 1. Phenotype of bone marrow-derived dendritic cells (BMDC) separated into FcR$^+$ and FcR$^-$ populations and labelled by (a) immunocytochemistry [labelling of anti-major histocompatibility complex (MHC) class II; magnification × 25] and (b) flow cytometry. BMDC were cultured from lymphocyte/MHC class II-depleted bone marrow cells. After 7 days, cells were harvested and separated into FcR$^+$ and FcR$^-$ subpopulations by rosetting. Cells were labelled for MHC class II, B7 (CTLA-4 Ig), B7.1 (CD80) or CD11c molecules (filled histograms) or irrelevant antibody (open histograms).

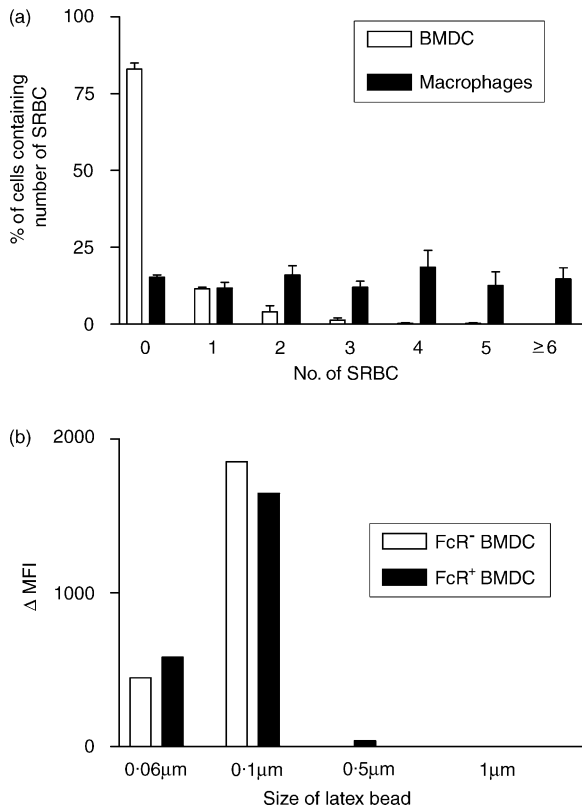


Figure 2. Phagocytosis and endocytosis by bone marrow-derived dendritic cells (BMDC) and macrophages. BMDC were harvested after 7 days of culture and used either as whole cells or as subpopulations separated by FcR rosetting. (a) BMDC or thioglycollate-elicited peritoneal macrophages (Mϕs) were cultured with opsonized sheep red blood cells (SRBC), non-phagocytosed SRBC were lysed and cytospins were prepared and labelled with OX6 [anti-major histocompatibility complex (MHC) class II] or OX42 (anti-CD11b/c). The numbers of SRBC within 200 cells were counted. (b) FcR$^-$ and FcR$^+$ BMDC were cultured with 0·06–1 μm fluorescein isothiocyanate (FITC)-labelled latex beads for 30 min at 37° or 4° and, after washing, the mean fluorescence intensity (MFI) was measured by flow cytometry. Numbers represent the difference between the MFI at 4° and 37°. The data shown represent the results from a representative experiment repeated four times.

To show that the FcR^+ cells were not Mϕs, phagocytosis of opsonized SRBC and endocytosis of latex beads were assessed (Fig. 2a). Although 20–30% of FcR^+ BMDC phagocytosed SRBC, positive cells contained only one or two SRBC (Fig. 2a). In contrast, thioglycollate-elicited peritoneal Mϕs (Thio-Mϕs) phagocytosed much larger numbers of SRBC. Both FcR^+ and FcR^- BMDC were only able to endocytose small beads of $\leq 0{\cdot}1$ μm in diameter (Fig. 2b). We have previously found that peritoneal Mϕs endocytose large numbers of particles of a range of sizes tested. In addition, FcR^+ BMDC did not adhere to plastic.

BMDC, but not LDC, express iNOS

BMDC harvested after 6 days were separated into FcR^+ and FcR^- populations and cultured overnight with or without IFN-γ and LPS. Expression of iNOS by BMDC was examined by immunocytochemistry, and by flow cytometry following permeabilization. Both FcR^- and FcR^+ BMDC expressed iNOS when cultured overnight with IFN-γ and LPS (Fig. 3, stimulated), but not when cultured with media alone (Fig. 3, resting). In contrast, LDC cultured in an identical manner, with or without IFN-γ and LPS, did not express detectable iNOS (Fig. 3, resting and stimulated LDC). The expression of iNOS by BMDC was found to vary, with 30–60% of cells being positive in different experiments. iNOS-positive cells displayed typical DC morphology. Flow cytometry of BMDC cultured with IFN-γ and LPS showed that 60% of BMDC co-expressed iNOS and MHC class II molecules (Fig. 4a) and that iNOS-positive BMDC also expressed the typical DC marker, CD11c (data not shown). LDC cultured under the same conditions did not express detectable iNOS (Fig. 4b). To confirm the specificity of the anti-iNOS mAbs, BMDC were cultured with or without IFN-γ and LPS, and cell lysates were immunoblotted using ANOS5 and ANOS10 mAbs. Both mAbs recognized a band migrating at 150 kDa under reducing conditions (the expected molecular mass of iNOS[23]) in lysates from stimulated, but not resting, BMDC (data not shown).

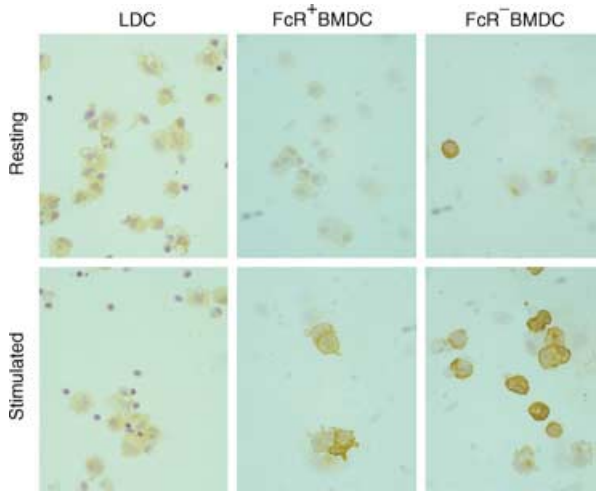


Figure 3. Immunocytochemical detection of inducible nitric oxide synthase (iNOS) expression by dendritic cells. FcR^+ and FcR^- bone marrow-derived dendritic cells (BMDC) (harvested on day 7), and fresh pseudo-afferent lymph DC (LDC), were cultured overnight with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) (stimulated), or medium alone (resting), and cytospins were labelled with anti-iNOS mAbs (ANOS 5 and 11). Fresh DC show little, if any, reactivity. Cultured BMDC, but not LDC, show conspicuous up-regulation of expression. The data shown represent the results from a representative experiment repeated at least four times. Magnification: ×25.

BMDC, but not LDC or SDC, secrete NO in a regulated manner

To show that iNOS expression correlated with NO secretion, BMDC were cultured for 48 hr with different stimuli, in parallel with peritoneal Mϕs, LDC and SDC. NO secretion was assessed by nitrite accumulation in the supernatant (Fig. 5). Mϕs and BMDC secreted similar amounts of NO, whereas LDC and SDC did not secrete detectable NO. NO secretion was completely inhibited by NMMA (Mϕ data not shown). Separated FcR^- and FcR^+ BMDC were cultured for 48 hr with IFN-γ and LPS, and nitrite production was measured. SDC were cultured in an identical manner. Both FcR^- and FcR^+ BMDC secreted similar amounts of NO, whereas SDC did not secrete detectable NO (Fig. 5b).

Therefore, both FcR^+ and FcR^- BMDC, as well as peritoneal Mϕs, can be stimulated to secrete similar amounts of NO, but LDC and SDC are unable to secrete detectable NO.

BMDC are weak stimulators of an allogeneic MLR

BMDC harvested at 7 days were compared with SDC and LDC as stimulators in an allogeneic MLR (Fig. 6). Consistently, LDC and SDC were almost always considerably more potent than BMDC. However, small numbers of BMDC show relatively efficient stimulation, whereas when larger numbers are used ($>5 \times 10^3$), thymidine incorporation was reduced. Direct cell counting showed that this reduction reflects decreased cell proliferation (data not shown). In contrast, SDC and LDC do not show such inhibition, even when 2×10^5 LDC are used as stimulators. These differences are not explained by kinetics of the MLR, as proliferation stimulated by all DC peaked at 3 days (data not shown).

As the weak stimulatory ability of BMDC might reflect immaturity, we attempted to mature the DC by replating and culturing them overnight,[12] with or without LPS and/or IFN-γ. BMDC treated as such remained weak stimulators at low numbers in comparison with SDC and still inhibited proliferation at high numbers (data not shown).

FcR^+ BMDC do not stimulate a significant MLR and inhibit stimulation by other DC

BMDC harvested at 7 days were separated by Fc rosetting and used to stimulate an allogeneic MLR with either CLN cells or purified $CD4^+$ T cells as responders. In all assays the FcR^- BMDC were far more potent than FcR^+ BMDC (Fig. 7a), but purified FcR^- BMDC still inhibited T-cell proliferation at high DC : T-cell ratios (Fig. 7a). Direct counting of cells showed that the reduced thymidine incorporation reflects decreased

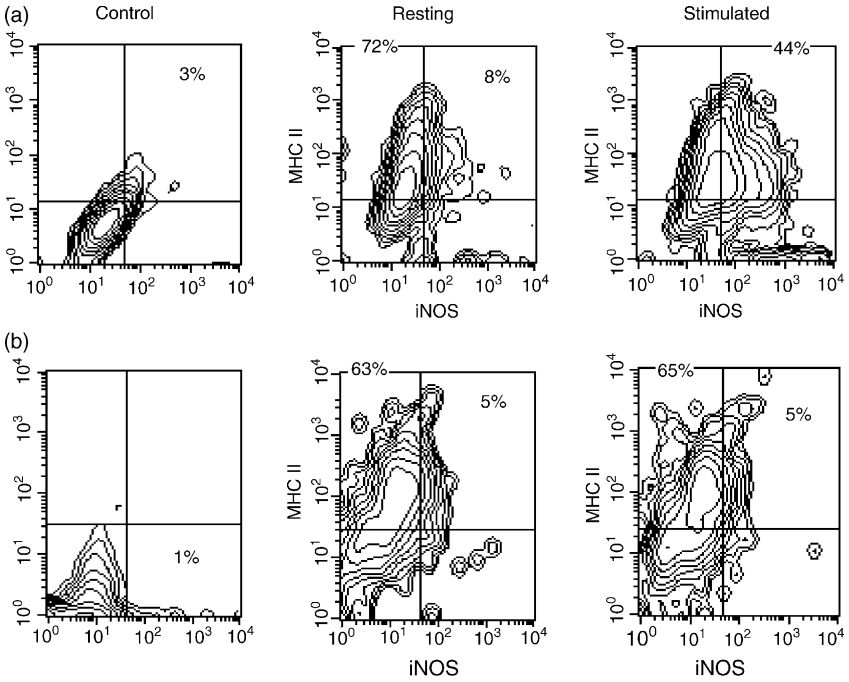


Figure 4. Flow cytometric analysis of inducible nitric oxide synthase (iNOS) expression by DC. Bone marrow-derived dendritic cells (BMDC) (a) and pseudo-afferent lymph DC (LDC) (b), prepared as described in the Materials and methods, were cultured overnight in the presence (stimulated) or absence (resting) of interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Cells were permeabilized and then labelled with anti-iNOS monoclonal antibodies (mAbs) and rabbit anti-mouse-conjugated fluorescein isothiocyanate (FITC), followed by biotin-conjugated OX6 [major histocompatibility complex (MHC) class II] and streptavidin-Quantum Red. Resting cells show little, if any, iNOS reactivity. After culture with IFN-γ and LPS, MHC class II$^+$ BMDC, but not LDC show marked up-regulation of expression.

proliferation and the culture media does not become exhausted (data not shown). Examination of cultures showed that there was little or no clustering of FcR$^+$ BMDC and lymphocytes in comparison with FcR$^-$ BMDC, LDC or SDC (not shown). When graded numbers of FcR$^+$ BMDC were added to a constant number of LDC in the MLR, dose-dependent inhibition of proliferation was seen (Fig. 7b). A similar experiment, using a fixed number of LDC and adding graded numbers of FcR$^-$ BMDC, resulted in enhanced proliferation (Fig. 7b). Inhibition of proliferation at high numbers might be the result of NO or prostaglandin secretion.[34,35] Both NMMA, an inhibitor of iNOS, and indomethacin, when added to a BMDC-stimulated MLR, partially reduced the dose-dependent inhibition (Fig. 8a, 8b). A combination of the NMMA and prostaglandin did not remove inhibition (Fig. 8c). Addition of NMMA and/or indomethacin did not result in FcR$^+$ BMDC stimulating significant proliferation (data not shown). We conclude that the inhibition of T-cell proliferation by high numbers of FcR$^-$ BMDC is partly caused by NO and prostaglandin secretion, but that other, as-yet-unidentified, factors are also involved.

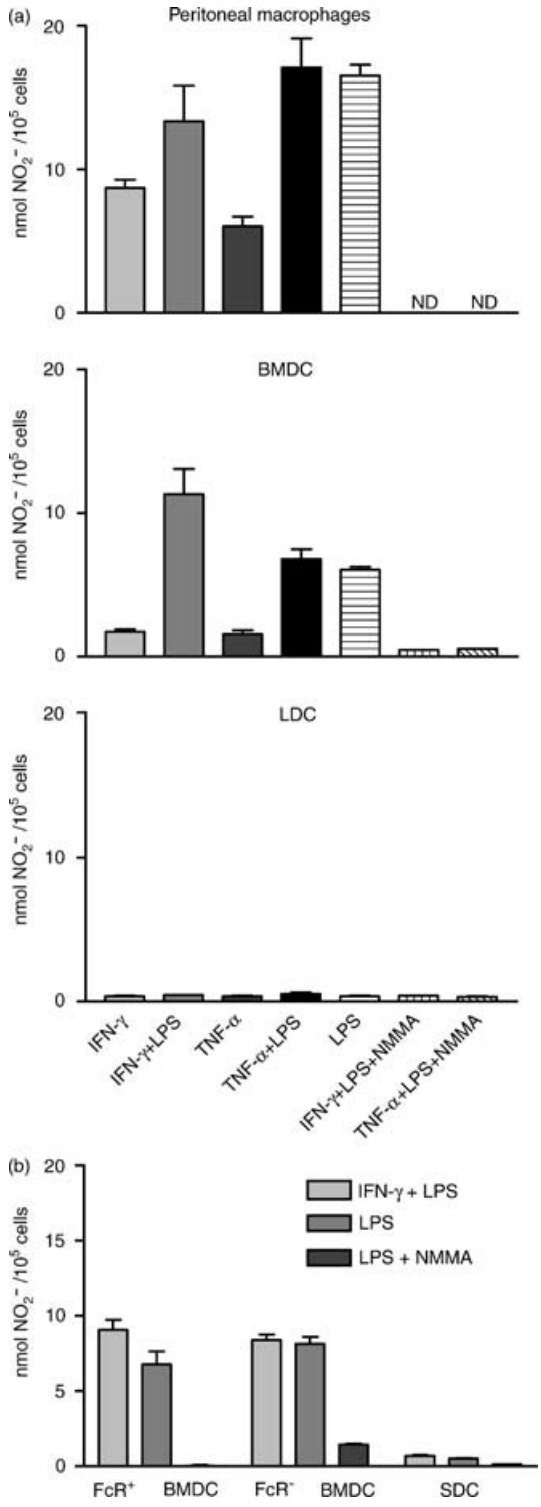
BMDC can present Ag to T cells *in vitro* and *in vivo*

BMDC and SDC were compared as APC for naive T cells *in vivo*. BMDC were harvested at 7 days, separated into FcR$^+$ and FcR$^-$ populations, pulsed with KLH and injected into the footpads of syngeneic naïve rats. Freshly isolated SDC were treated similarly as a positive control. Ten days later, rats were killed and popliteal lymph node cells incubated with KLH, HSA or no Ag, and proliferation measured. SDC, FcR$^+$ and FcR$^-$ BMDC are all able to prime T cells to KLH (Fig. 9a). SDC and both subpopulations of BMDC are able to prime naïve T cells to comparable levels. To show that the effects of BMDC were not the result of cross-priming (host processing), parental strain (DA) DC were used to prime (LEW × DA)F1 recipients. Purified popliteal node T cells were then restimulated with KLH-pulsed SDC from LEW or DA donors. Only DC from DA donors stimulated significant proliferation, showing that priming was restricted to DA MHC and must have occurred by direct presentation of Ag by DC (data not shown). The high background for FcR$^-$ BMDC in Fig. 9(a), was not seen in other experiments (data not shown).

Ag presentation by DC *in vitro* to primed CD4$^+$ T cells was assessed by restimulation of T cells primed to KLH *in vivo* 2 weeks previously. Both FcR$^-$ and FcR$^+$ BMDC were able to stimulate primed T cells, but FcR$^-$ BMDC were again much more potent than FcR$^+$ BMDC (Fig. 9b). Interestingly, in this assay SDC were less potent than FcR$^-$ BMDC when small numbers were used, but were more potent when larger numbers were used. FcR$^-$ BMDC inhibited proliferation when large numbers were used. FcR$^+$ BMDC were less potent than FcR$^-$ BMDC, but similar to SDC when small numbers were used.

SDC, but not BMDC, can prime CTL to minor histocompatibility Ags

In view of the discrepancy between the ability of BMDC to stimulate an MLR and to activate T cells to KLH, we examined the ability of BMDC to prime CTL to a mitochondria-associated



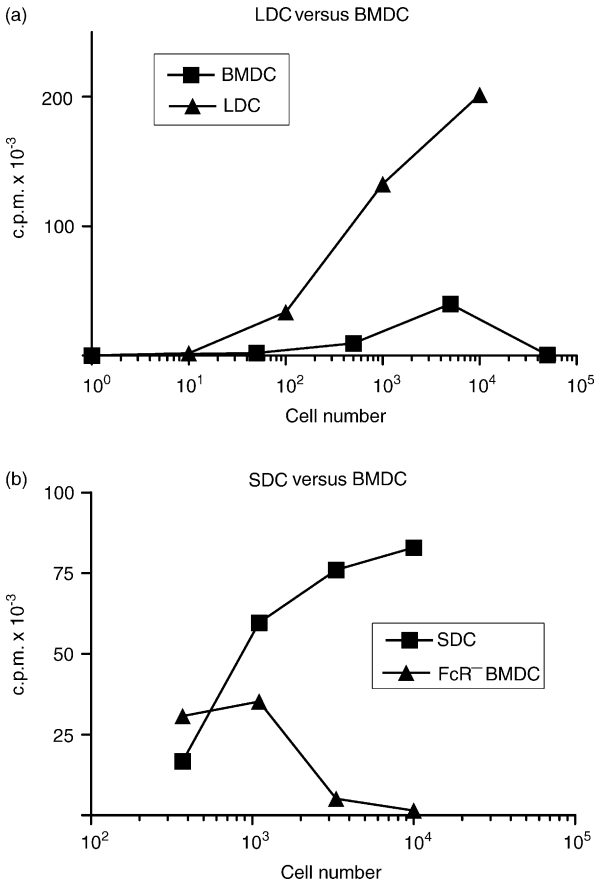


Figure 6. Allogeneic mixed lymphocyte reaction (MLR) stimulation by *ex vivo* DC and bone marrow-derived dendritic cells (BMDC). Graded numbers of irradiated BMDC (harvested on day 7), pseudo-afferent lymph DC (LDC) and spleen DC (SDC) were added to 2×10^5 allogeneic CLN cells. Three days later, [^{3}H]thymidine ([^{3}H]TdR) was added for 16 hr, following which the cells were harvested and [^{3}H]TdR measured by scintillation counting. (a) Comparison of LDC and BMDC. (b) SDC and FcR$^-$ BMDC. BMDC are weaker stimulators of an MLR than LDC and SDC, and when larger numbers of BMDC are used, [^{3}H]TdR incorporation is progressively reduced. c.p.m., counts per minute.

Figure 5. Release of nitric oxide (NO) by DC and macrophages (Mϕs). Bone marrow-derived dendritic cells (BMDC) (harvested on day 7), pseudo-afferent lymph DC (LDC), spleen DC (SDC) and peritoneal Mϕs were cultured for 48 hr in the presence of interferon-γ (IFN-γ) and lipopolysaccharide (LPS), or with medium alone. NO release was assessed (using the Greiss reagent) as nitrite accumulation in the supernatants. BMDC and Mϕs, but not LDC or SDC, show considerable N^G-monomethyl L-Arginine (NMMA)-inhibitable nitrite accumulation. (a) Peritoneal Mϕs, BMDC, and LDC. (b) FcR$^+$ BMDC, FcR$^-$ BMDC, and SDC. The data shown represent the results from a representative experiment repeated at least three times. ND, not done in a particular experiment. NMMA reproducibly inhibited NO release by Mϕs in other experiments.

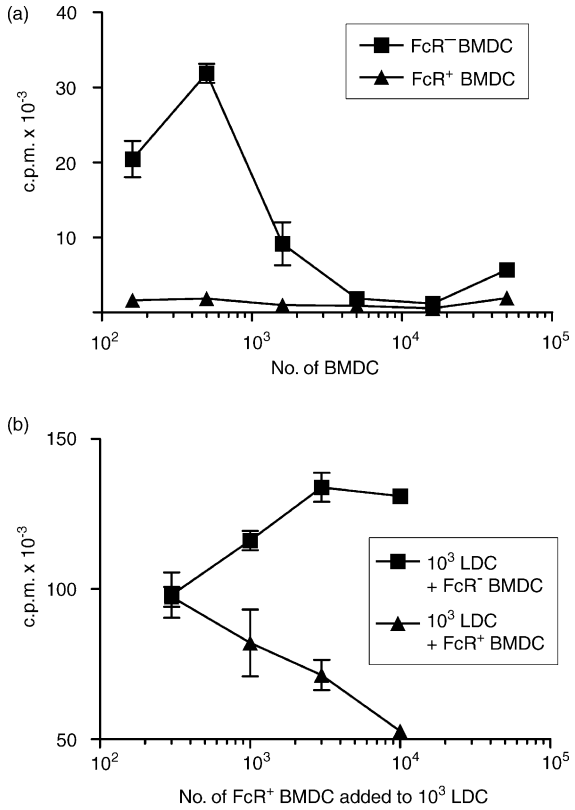


Figure 7. Allogeneic mixed lymphocyte reaction (MLR) stimulation by FcR^+ and FcR^- bone marrow-derived dendritic cells (BMDC). (a) Graded doses of irradiated FcR^- and FcR^+ BMDC were added to 2×10^5 allogeneic CLN cells and cultured for 96 hr. [^{3}H]Thymidine ([^{3}H]TdR) was added for the final 16 hr. (b) Graded doses of FcR^- BMDC and FcR^+ BMDC were added to a fixed number (10^3) of pseudo-afferent lymph DC (LDC) and cultured with allogeneic CLN cells, as described in the Materials and methods. c.p.m., counts per minute.

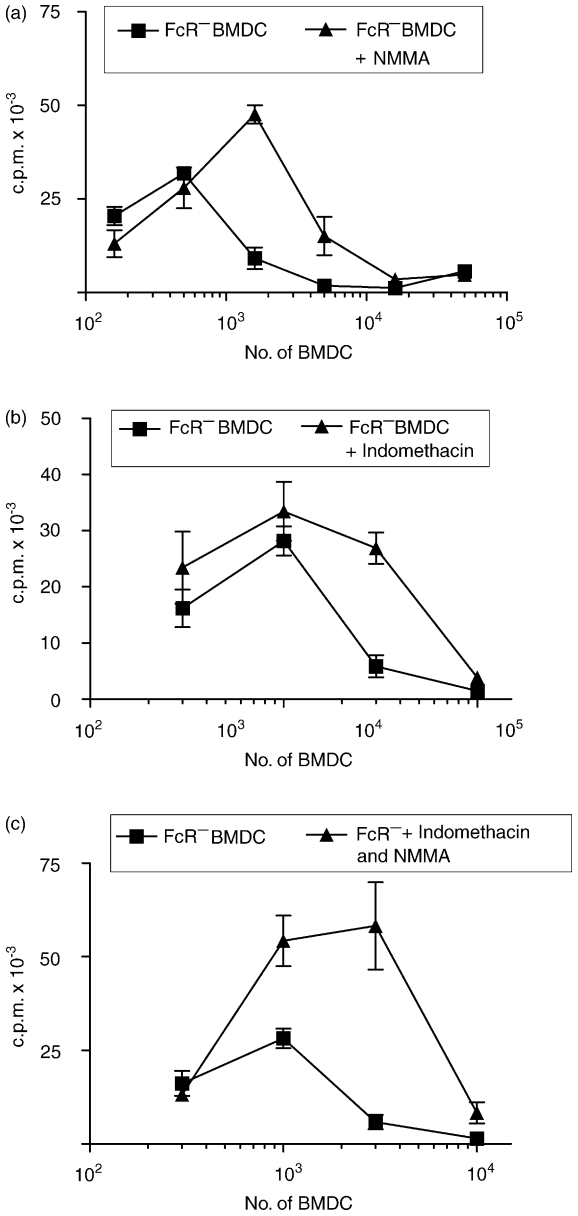


Figure 8. Partial abrogation of mixed lymphocyte reaction (MLR) inhibitory effects of bone marrow-derived dendritic cells (BMDC) by N^G-monomethyl L-Arginine (NMMA) and indomethacin. Graded doses of irradiated FcR^- BMDC were added to 2×10^5 allogeneic CLN cells and cultured for 96 hr, with or without the addition of (a) NMMA, (b) indomethacin or (c) NMMA+indomethacin. Proliferation was measured as described in the Materials and methods. The data shown represent the results from representative experiments repeated at least four times. c.p.m., counts per minute.

minor histocompatibility antigen (miHA) MTA.[31] Injection of MTA^- rats with MTA^+ SDC stimulates CTL which are able to kill MTA^+ Con A blast targets, but not MTA^- targets. When 2×10^6 SDC or 4×10^7 spleen cells were used to prime this CTL response, significant killing was observed, yet when BMDC were used under identical conditions, no priming of CTL was seen (Fig. 9c).

DISCUSSION

DC have central roles in the induction and regulation (both positive and negative) of specific immune responses.[1,36] In keeping with the complexity of their roles, it is apparent that at least two distinct lineages of DC exist *in vivo*, and that within these lineages DC may exist in different stages of maturation or differentiation, expressing distinct functional and phenotypic characteristics. To help understand DC properties and life histories, systems have been developed for generating DC *in vitro*, including growth from BM precursors.[8,12] While BMDC show many functional and phenotypic similarities to DC extracted from animals,[8] our preliminary studies in the rat suggested that BMDC showed significant differences from *ex vivo* DC. Because we felt that it was not clear how BMDC relate to any population of DC identified *in vivo*, we performed a detailed comparison of BMDC with DC extracted from spleen or intestinal pseudo-afferent lymph.[5,30]

BMDC express some, but not all, markers expressed by *ex vivo* DC (e.g. BMDC express OX41, but not OX62). BMDC, identified by morphology and MHC class II expression, are

themselves functionally heterogeneous. A proportion of BMDC bound, but did not internalize, IgG-coated SRBC and we were thus able to separate BMDC using FcR expression. Surprisingly, there were few differences in surface marker expression between the two populations; in particular, levels of surface MHC class II were similar. FcR^+ cells did, however, express low levels of B7 whereas some FcR^- BMDC expressed high levels. LDC, in contrast to BMDC, express higher levels of B7.[17] In contrast to Mϕs, neither BMDC population was able to phagocytose significant numbers of opsonized SRBC. Both populations were only able to endocytose small (0·1 μm) latex beads. Although it has not been formally proven that FcR^+ BMDC are precursors of FcR^- BMDC, this is probably the case because when cultured, FcR^+ BMDC give rise to populations containing both FcR^+ and FcR^- DC (T. J. Powell & G. G. MacPherson, unpublished). Two subpopulations of lung DC have been identified that differ in Fc receptor expression and function with respect to stimulation of naïve and memory T cells.[37]

DC can exist at different stages of maturation and, as well as expression of FcR (characteristic of some immature DC),[33] expression of MHC class II and B7 suggests that most FcR^+ BMDC, and some FcR^- cells, are immature. In contrast to another report,[12] a variety of potential maturation signals (replating, TNF-α, LPS) did not lead to up-regulation of surface MHC class II expression or B7, neither did they increase the ability of BMDC to stimulate an allogeneic MLR in comparison with SDC or LDC.

BMDC also differ markedly from *ex vivo* DC in that BMDC, but not *ex vivo* DC, can be stimulated to express iNOS and secrete NO. Secretion of NO by DC is controversial. Murine BMDC have been shown to express iNOS and secrete NO when stimulated with IFN-γ and LPS.[34,35] We found that rat BMDC behaved similarly, and that the amounts of NO they secreted were comparable to those secreted by peritoneal Mϕs. Interestingly, both FcR^+ and FcR^- BMDC expressed similar levels of iNOS and secreted similar amounts of NO. BMDC that expressed iNOS also expressed the characteristic DC marker, CD11c. A recent report has found that FLT3 ligand increases yields of rat BMDC, but comparisons with SDC were not attempted[38] and there is no evidence that FLT3 ligand affects NO secretion by BMDC. Rat GM–CSF was not available to us when we carried out these studies.

The secretion of NO by *ex vivo* DC appears to be very variable. Langerhans' cells have been stimulated to express[39] or not express[40] iNOS/NO, and thymic DC appear to express iNOS constitutively.[41] DC obtained from rats recovering from experimental allergic encephalomyelitis can secrete NO,[42] while freshly isolated human blood DC express iNOS and can secrete NO.[43] In contrast, we could not detect significant

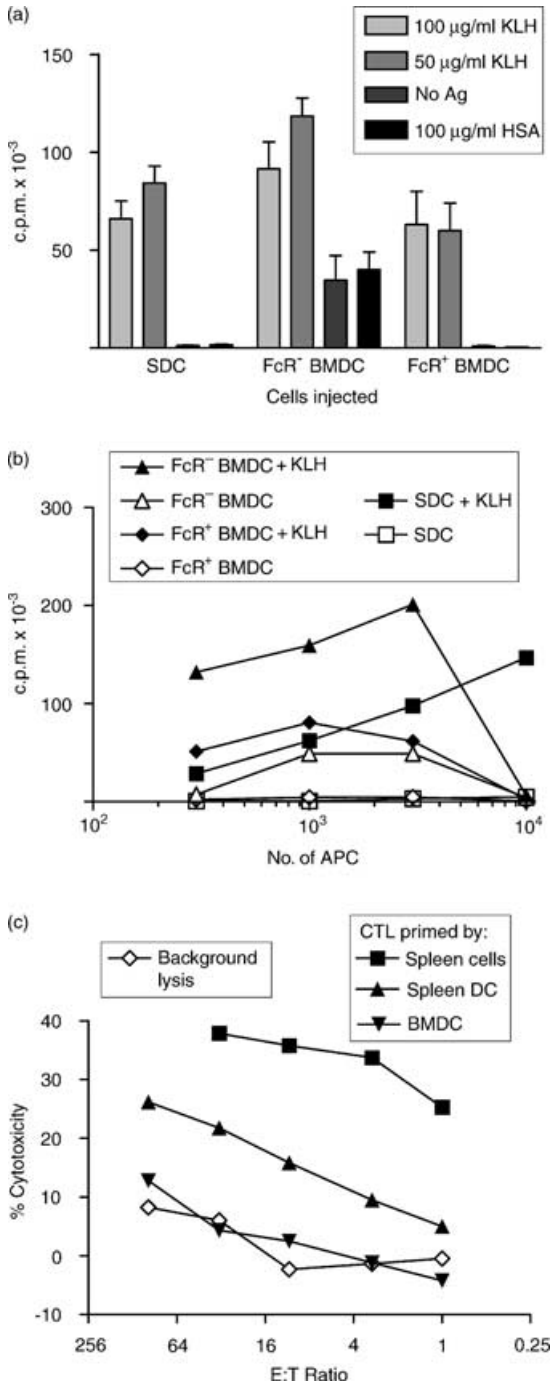


Figure 9. Presentation of antigen (Ag) by bone marrow-derived dendritic cells (BMDC) *in vivo* and *in vitro* to $CD4^+$ and $CD8^+$ T cells. (a) Freshly isolated spleen DC (SDC) (no overnight culture), FcR^- BMDC and FcR^+ BMDC were pulsed with keyhole limpet haemocyanin (KLH) for 1 hr, washed and injected subcutaneously into naïve rats. Ten days later, rats were killed and 2×10^5 CLN cells were cultured with KLH, no antigen, or human serum albumin (HSA), for 5 days, with [^{3}H]thymidine ([^{3}H]TdR) added for the final 16 hr. [^{3}H]TdR incorporation was measured by scintillation counting. This assay showed a non-specific background with FcR^- BMDC that was not seen in subsequent experiments. (b) DC were pulsed with KLH and cultured with nylon wool-enriched T cells from spleens of rats primed with KLH 2 weeks previously, for 5 days with a pulse of [^{3}H]TdR for the final 16 hr. The counts per minute (c.p.m.) were obtained by scintillation counting. (c) [Lewis (female) × DA (male)]F1 rats were primed with [DA (female) × Lewis (male)]F1 BMDC, SDC or spleen cells by subcutaneous injection. Three weeks later, lymph node cells were harvested and restimulated *in vitro* with [DA (female) × Lewis (male)] spleen cells. Cytotoxicity was assessed in a JAM assay.

iNOS expression or NO secretion by freshly isolated SDC or LDC, or after their culture with LPS and IFN-γ. These *in vivo and ex vivo* differences may reflect different lineages or activation states of DC. Apart from the special case of the thymus, those DC in or isolated from solid tissues, which can secrete NO, are associated with inflammation, whereas DC isolated in the absence of inflammation cannot secrete NO. There is a rapid influx of DC into inflamed tissues in the rat[21,44] and these 'inflammatory' DC may be able to secrete NO. It may also be relevant that it has been shown recently that $CD2^+$ monocytes are DC precursors;[45] these may represent the source of 'inflammatory' DC.

BMDC have always been considered to be a paradigm for '*in vivo*' DC and direct comparison has indicated that MLR stimulation by BMDC and spleen DC are similar.[8,12] However, we found that rat BMDC differ markedly from SDC and LDC as APC. BMDC are almost always relatively weak stimulators of an allogeneic MLR compared with SDC or LDC. In addition, the two subpopulations of BMDC differed markedly as stimulators. Whole BMDC inhibited T-cell proliferation when large numbers were used, and separation of FcR^+ and FcR^- cells showed that despite expressing MHC class II and low levels of B7, the FcR^+ DC could barely stimulate an MLR at all, and inhibited stimulation by FcR^- BMDC and SDC or LDC. This was unlikely to be caused by the presence of Mϕs in the FcR^+ population as it did not contain cells capable of significant FcR-mediated phagocytosis or of adhesion to plastic. In contrast, FcR^- BMDC stimulated a moderate MLR and did not inhibit stimulation by other DC populations. The molecular basis of inhibition is partially explained by NO and prostaglandin secretion by BMDC. Murine BMDC can be stimulated to secrete NO, which inhibits an allogeneic MLR,[34,35] but blocking NO secretion by NMMA only partially restored MLR stimulation by rat BMDC. Similarly indomethacin, a prostaglandin inhibitor, had only a minimal effect and the two agents together did not remove inhibition. When BMDC were tested as APC *in vitro* to primed T cells, FcR^- BMDC performed as well as or better than SDC in their ability to stimulate the proliferation of T cells. FcR^+ BMDC were weaker stimulators of primed T cells compared with FcR^- BMDC, but showed similar amounts of proliferation to SDC at lower cell numbers. However, there was still inhibition at high numbers of BMDC that was not present with SDC. It is generally accepted that only DC can activate naïve T cells, whereas activated T cells can be stimulated by many MHC class II^+ cells. FcR^+ DC cannot activate naïve T cells *in vitro* although they do indeed stimulate naïve T cells *in vivo*. Overall, we show that FcR^+ BMDC are generally worse than other DC at stimulating T cells.

In contrast to these *in vitro* results, when BMDC were used to prime naïve T cells *in vivo*, both subpopulations were able to generate T cells that proliferated strongly in a recall response. This could have been a result of the phenomenon of cross-priming.[46] When parental strain A BMDCs were used to prime (A × B)F1 recipients, the sensitized T cells only responded significantly to Ag presented by parental strain A DC, showing that sensitization was by direct presentation by the injected DC and not a result of cross-priming. The most probable interpretation of these data is that FcR^+ DC received maturation signals *in vivo* that we have not been able to identify or reproduce *in vitro*. However, recent reports that MHC class II molecules may be transferred intact between APC[47–49] bring into question the validity of this approach to identifying cross-priming. In addition, rat monocyte-derived DC have been shown to home to peripheral lymph nodes.[50]

In contrast to the priming of $CD4^+$ T cells described above, neither population of BMDC was able to stimulate CTL to a minor histocompatibility antigen. The reasons for this are unclear but may reflect low levels of the mitochondrial peptide expressed by the BMDC.

In conclusion, rat BMDC, grown in IL-4 and GM–CSF, express some (but not all) surface markers, similar to those of *ex vivo* SDC and LDC, but differ markedly in that BMDC are weak APC and can secrete NO. At present it is not possible to determine how BMDC relate to any DC populations identified *in vivo*, but we suggest that they may be equivalent to monocyte-derived DC and/or the DC that accumulate at inflammatory sites. These results illustrate the complexity of DC subpopulations and the need for caution when extrapolating results generated *in vitro* with BMDC to concepts *in vivo*.

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