The following pages constitute the final, accepted and revised manuscript of the article:

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Immunol Lett. 2005 Nov 15;101(2):193-201

Publisher: Elsevier.

Use of alternative location to go to the published version of the article requires journal subscription.

Alternative location: http://dx.doi.org/10.1016/j.imlet.2005.05.012

Activation of purified allogeneic CD4+ T cells by rat bone marrow-derived dendritic cells induces concurrent secretion of IFN-γ, IL-4 and IL-10

Shorena Janelidze[1], Karin Enell[1], Edward Visse[1], Anna Darabi[1], Leif G Salford[1] and Peter Siesjö[1]

[1]*Glioma Immunotherapy Group, The Rausing Laboratory, Division of Neurosurgery, Department of Clinical Neuroscience, University of Lund*

Short title: Rat bone marrow-derived dendritic cells

Key Words: dendritic cells, bone marrow, endocytosis, MLR, T_H1/T_H2 polarization

Address correspondence and reprint requests to:

Shorena Janelidze, Glioma Immunotherapy Group, The Rausing Laboratory, BMC I12, 221 84 Lund, Sweden, Tel: +46 46 2221440, Fax: +46 46 2224606, E-mail:

Shorena.Janelidze@neurokir.lu.se

SUMMARY

Dendritic cells (DCs) are highly specialized antigen-presenting cells that play a key role in the initiation and regulation of immune responses. The ability of DCs to process antigens and the outcome of their interaction with T cells are largely dependent on phenotype as well as maturation state of DCs. In this study we generated DCs from rat bone marrow precursors. Bone marrow cells cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, and Flt-3 ligand (FL) produced immature DCs that expressed intermediate levels of major histocompatibility complex (MHC) class II, low levels of CD80 and CD86 molecules and displayed a high capacity of endocytosis. Bone marrow-derived DCs (BMDCs) matured in the presence of lipopolysaccharide (LPS) upregulated expression of MHC class II, CD80 and CD86, while their phagocytic capacity was dramatically reduced. Mature BMDCs stimulated vigorous proliferation of purified allogeneic CD4$^+$ T cells in a primary mixed leukocyte reaction (MLR) and elicited a mixed cytokine profile in allogeneic CD4$^+$ T cells: DCs activated CD4$^+$ T cells to produce interferon (IFN)-γ, IL-4, and IL-10. Thus, rat BMDCs effectively internalize antigens and stimulate T cell proliferation but fail to induce an unidirectional polarization of T helper (T$_H$) cells and in this respect differ from both human and mouse DCs.

INTRODUCTION

Dendritic cells (DCs) constitute a rare cell population present in most tissues at a frequency ranging from 0.1 to 2% of the total cell number. DCs reside in peripheral tissues in an immature state where they can efficiently capture and process antigens. Internalization of foreign antigens together with pro-inflammatory microenvironmental signals trigger maturation of DCs and their migration to the lymphoid organs. In lymphoid organs DCs induce clonal expansion of antigen-specific T cells, thereby initiating immune responses. Under physiological steady-state conditions DCs bearing self-antigens or apoptotic cell bodies continuously migrate to the draining lymph nodes but instead of immunity peripheral tolerance against self-antigens is maintained [1-3]. It is now apparent that DCs also tightly regulate polarization of T helper (T_H) cells [4-7].

Due to the pivotal role DCs play in controlling immunity, DC-based treatment strategies are increasingly recognized to have therapeutic potential in many immunopathological conditions including cancer, autoimmunity and allogeneic transplantation. But, until recently, difficulties in isolation and culture and the paucity of DCs in tissues have impeded their application in animal models of immune diseases or in clinical settings. During the last decade, however, numerous in vitro systems have been developed allowing the production of large quantities of DCs. Standardized protocols exist for generation of human and mouse DCs from circulating monocyte [8-10] and bone marrow or blood progenitor cells [11-15]. It was originally demonstrated that DCs could be successfully grown from mouse bone marrow or blood cultures supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF). In contrast to the mouse system, low numbers of DCs (< 5% of the starting cell number) were generated from rat bone marrow cells in the presence of GM-CSF [16,17].

However, cells yields were dramatically increased, albeit not in the range of mouse and human BMDCs, when interleukin (IL)-4 or IL-4 plus Flt-3 ligand (FL) were used in combination with GM-CSF [18-20]. Several observations may provide a plausible explanation for this phenomenon. For example, successful generation of human DCs from blood monocytes requires the presence of IL-4 along with GM-CSF in culture medium. IL-4 has been shown to suppress macrophage outgrowth thus allowing formation of DCs from human peripheral blood [9]. It has recently been demonstrated that, while GM-CSF ensures terminal differentiation of DC precursors, FL sustains their long-term expansion [21]. In addition, GM-CSF and FL are known to increase the total DC numbers in mice and humans when administered *in vivo* [22-24].

Given the ability of DCs to induce both immunity and tolerance and to regulate T_H1/T_H2 responses, opposite outcomes of DC-based treatments may be desired, depending on the nature of immune pathology. Therefore, the generation of large numbers of DCs is not per se sufficient for the development of optimal therapeutic approaches. Detailed functional characterization of *in vitro* propagated DCs is of utmost importance.

The objective of this study was to examine the phenotype of DCs generated from rat bone marrow cultures supplemented with GM-CSF, IL-4, and FL and, most importantly, their functional properties, such as the ability to internalize antigens, activate $CD4^+$ T cells, and direct T_H1/T_H2 polarization.

MATERIALS AND METHODS

Animals

Male Fisher 344 and Wistar rats (Scanbur BK AB, Sollentuna, Sweden) were maintained under specific pathogen-free conditions at the Biomedical Center Animal Facility, University of Lund. Rats between 8 to 12 weeks of age were used in all experiments. All animal procedures were performed according to the practices of the Swedish Board of Animal Research and were approved by the Committee of Animal Ethics in Lund-Malmö.

Cell culture medium and other reagents

The culture medium (R10) was RPMI 1640 supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES, 50μg/ml gentamicin, 50μM 2-Mercaptoethanol (all from Invitrogen AB, Sweden), and 10% FBS (fetal bovine serum, Biochrom AG, Berlin, Germany). Ag8653 myeloma cells transfected with murine GM-CSF was a gift from Dr. Mary Jo Wick (University of Lund, Sweden). The concentration of GM-CSF in culture supernatants was measured using a GM-CSF ELISA kit (BD Biosciences Stockholm, Sweden). Recombinant rat IL-4 was purchased from PeproTech (London, UK). Recombinant mouse FL was purchased from R&D Systems (Abingdon, UK). LPS (Escherichia coli 055:B5) was purchased from Sigma-Aldrich (Stockholm, Sweden).

Generation of BMDCs

BMDCs were generated as described previously [15] with some modification. Bone marrow was collected from the femurs of Fisher 344 rats, passed through a 50μm Filcon filter (DakoCytomation, Solna, Sweden) to remove debris and clumps and resuspended at 2×10^6cells/ml in R10s (R10 supplemented with 20ng/ml IL-4, 50ng/ml GM-CSF, and 25ng/ml FL). Bone marrow cells (10ml) were then plated into 100mm bacteriological Petri dishes and maintained at 37°C in 5%CO2/95% air. Three days after initiation of the culture, 10ml of fresh R10s were added to each Petri dish. On day 6, 50% of the spent medium was collected, centrifuged, the cell pellet was then resuspended in 10ml fresh R10s and returned into the original Petri dish. On day 7, non-adherent and semi-adherent cells were harvested by flushing the Petri dishes with R10. To induce maturation cells were resuspended at 1×10^6 cells/ml in R10 supplemented with 10ng/ml IL-4, 25ng/ml GM-CSF, and 0.1μg/ml LPS and replated in tissue culture plastic plates. Non-adherent mature BMDCs were harvested on day 8 for phenotypic and functional analysis.

Flow cytometry

For surface labeling 0.5×10^5 or more cells were washed in PBS (phosphate-buffered saline, Invitrogen AB, Sweden) supplemented with 1%BSA (bovine serum albumin, Roche Diagnostics, Bromma, Sweden). To block FcγII receptors cells were incubated with rat Fc block (BD Biosciences, Stockholm, Sweden) at 4°C for 30min. Cells were then incubated with antibodies against cell surface molecules. The staining was carried out at 4°C for 30min. For phenotypic analysis of BMDCs following FITC-,

PE-, or biotin-conjugated antibodies (clone name given in parentheses) were used: anti-TCRαβ (R73), anti-granulocyte (HIS48), anti-CD45RA (OX33), anti-IgM (G53-238), anti-NKR-P1A (10/78), anti-MHC II RT1B (OX-6), anti-CD40 (HM40-3), anti-CD80 (3H5), anti-CD86 (24F) (all from BD Biosciences, Stockholm, Sweden); anti-ED2 (ED2), anti-CD11c (8A2), anti-OX62 (OX62) (all from Serotec, Oslo, Norway); anti-granulocytes (RK-4) (BMA, Augst, Switzerland). Whenever biotin-conjugated antibodies were used, staining was visualized using streptavidin-FITC or streptavidin-PE (BD Biosciences, Stockholm, Sweden). Dead cells were excluded on the basis of propidium iodide or actinomycin D (Sigma-Aldrich, Stockholm, Sweden) staining. Appropriate isotype-matched antibodies were included in all experiments. Cell fluorescence was measured using a FACSCalibur cytometer (Beckton Dickinson, Heidelberg, Germany). Data analysis was performed using CellQuest software (Beckton Dickinson, Heidelberg, Germany). Gates were set based on the isotype matched antibody staining. The percentages of positive cells and mean fluorescence intensities (MFI) were calculated by the subtraction of the background from the isotype control staining. Results were expressed as mean ± SD.

In vitro antigen uptake

BMDCs (0.1×10^5 cells/ml) were incubated with 1μg/ml FITC-albumin (Molecular Probes, Leiden, Netherlands) at 4 or 37°C for 1 hour. Cells were then washed with cold PBS containing 1%BSA. Autofluorescence of extracellular particles was quenched by incubation with trypan blue (Invitrogen AB, Stockholm, Sweden) for 2 minutes on ice. Cells were thereafter washed with BSA-PBS and analyzed on a FACSCalibur flow cytometer.

Mixed leukocyte reaction

CD4^{+} T cells were purified from spleens of Wistar rats using the MACS technique. Red blood cells were lysed with sterile water. Spleen cells were depleted of macrophages and dendritic cells by plastic adherence and stained with anti-granulocyte (HIS48), anti-NKR-P1A (10/78), anti-MHC II RT1B (OX-6), and anti-CD8 (OX8) antibodies followed by incubation with goat anti-mouse antibody conjugated to magnetic microbeads. The labeled cell suspension was then passed through a magnetic column according to the manufacturer's recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4^{+} T cells were isolated to >90% purity as assessed by flow cytometry.

Allogeneic CD4^{+} T cells (1×10^5) were co-cultured with graded doses of irradiated BMDCs (2000 rads) in 96-well U-bottomed microplates for 5 days. Cells were then pulsed with 1 μCi/well ^{3}H-thymidine for the last 18 hours of co-cultures, harvested onto glass fiber filters and ^{3}H-thymidine incorporation was quantified in a beta counter (Wallac Microbeta, Turku, Finland). All samples were assayed in at least quadruplicate. Proliferation was evaluated by calculation of the stimulation index (SI) according to the following formula:

$$SI = \frac{cpm(Tcells + BMDCs) - cpm(BMDCs)}{cpm(Tcells)}$$

Results were expressed as mean ± SD.

Cytokine production by T cells

A total of 1×10^6 allogeneic CD4^{+} T cells were cultured with 1×10^5 irradiated BMDCs in a final volume of 1ml in 24-well plates. After 5 days of stimulation cells

were harvested, washed and the cell concentration was adjusted to 0.2×10^6 cells/ml. Cells were then restimulated in 24-well plates coated with 0.5μg/ml anti-CD3 antibody (G4.18, BD Biosciences, Stockholm, Sweden) in the presence of 2μg/ml anti-CD28 antibody (JJ319, BD Biosciences, Stockholm, Sweden) or cultured untreated in R10. Twenty-four hours later cell culture supernatants were collected and frozen for subsequent analysis by ELISA.

ELISA

Measurements of IL-4 and IL-10 were done using BDOptEIA ELISA sets (BD Biosciences, Stockholm, Sweden) according to the manufacturer's recommendations. For IFN-γ, ELISA plates were coated with 2μg/ml capture antibody (polyclonal, BioSource Europe, Nivelles, Belgium) at 4°C overnight. Plates were next incubated with 10% FBS in PBS for 2 hours to block unspecific binding. Standards (recombinant rat IFN-γ, BD Biosciences, Stockholm, Sweden) and samples were added to the plates and incubated at 4°C overnight. Biotin-conjugated detection antibody (DB-1, BioSource Europe, Nivelles, Belgium) was applied to the plates at a final concentration of 1μg/ml for 45 minutes, followed by 30 minutes incubation with 2.5μg/ml avidin-conjugated peroxidase (Sigma-Aldrich, Stockholm, Sweden). Color was developed using 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, Stockholm, Sweden) and plates were read at 405 nm. All steps were performed at room temperature unless otherwise indicated. The sensitivity of the assay was 1.6pg/ml for IL-4, 15.6pg/ml for IL-10 and 31.3pg/ml for IFN-γ.

RESULTS

Phenotype of rat BMDCs

Phenotype of cells derived from BM cultured in the presence of GM-CSF, IL-4 and FL was examined by flow cytometry. Few cells were positive for T cell (3.4±2%, TCR$\alpha\beta$), NK cell (6.7±2.6%, NKR-P1A) or macrophage (1.3±0.2%, ED2) markers (data not shown). A small fraction (5.9±1.1%) of cells expressed the B cell-specific antigen CD45RA, but did not express surface IgM (data not shown). The CD45RA$^+$IgM$^-$ cells probably represent pro- or pre-B cell populations.

A subpopulation of BMDCs (32.1±5.5%) stained for OX62, an alpha E2 integrin expressed by a fraction of rat DCs (Fig.1A). Only 16.8±5.3% of BMDCs were positive for CD11c and the staining intensity was generally low (Fig.1B). LPS treatment did not induce changes in the expression of OX62 (33.8±1.6%, Fig.1C) or CD11c (18.3±3.5%, Fig.1D).

BMDCs collected on day 7 of culture exhibited an immature phenotype. A minor subset of cells expressed the costimulatory molecules CD80 and CD86 (3.2±2.2%, Fig.2A). On the average 54.2±6.9% of cells were MHC class II positive. MHC class II$^+$ cells could be further subdivided into two groups based on the expression of the granulocyte marker RK4: MHC II$^+$RK4$^+$ (19.6±4.6%) and MHC II$^+$RK4$^-$ (34.6±7.8)(Fig.2A). In response to LPS treatment of BMDCs, the number of CD80$^+$CD86$^+$ cells increased to 27.8±8.2% (Fig.2B). The total amount of MHC II positive cells reached 73.9±5.7%, while the amount of MHC II$^+$RK4$^+$ cells remained almost unchanged (18.9±3.2%). Interestingly, the population with high MHC II

expression became apparent first after LPS treatment (Fig.2B) and these cells represented 26.5±5.6% of the total cell number.

Endocytic capacity of rat BMDCs is downregulated during maturation

To assess the endocytic capacity of BMDCs, macropinocytosis of FITC-albumin was studied. As expected, immature BMDCs were highly endocytic compared to mature cells (Fig.3A). Mean fluorescence intensity (MFI), which reflects the amount of FITC-albumin particles taken up by BMDCs, decreased from 193.1±34.2 in immature to 85.4±29 in mature cells (Fig.3B).

BMDCs induce CD4$^+$ T cell proliferation in a mixed leukocyte reaction

The ability of DCs to induce proliferation of T cells in a mixed leukocyte reaction (MLR) assay is commonly used for the evaluation of their function. The basis of the assay is the capacity of T lymphocytes to recognize allogeneic MHC molecules as intact structures on the surface of foreign antigen-presenting cells such as DCs. Thus, to further characterize BMDCs, their ability to stimulate allogeneic CD4$^+$ T cells was investigated. Graded numbers of mature BMDCs were incubated with a fixed number of purified allogeneic CD4$^+$ T cells. Background counts for BMDCs alone were ‹150 cpm and for CD4$^+$ T cells alone were ‹3000 cpm. The cpm value for the maximal response was 139019. The background proliferation level of CD4$^+$ T cells alone differed between experiments. Therefore, the SI was used as an estimate of the allostimulatory activity of BMDCs. As shown in Fig.4, as few as 1000 BMDCs (BMDC:T cell ratio of 1:100) induced vigorous proliferation of CD4$^+$ T cells with the SI reaching 46.9.

Interestingly, at high BMDCs numbers (BMDC:T cell ratio of 1:2) the SI decreased to 12.1.

T_H1/T_H2 polarization of CD4$^+$ T cells activated by BMDCs

Upon induction of the immune response naïve T_H cells can differentiate to at least two functional subsets: IFN-γ secreting T_H1 cells and IL-4 secreting T_H2 cells. In addition to their role in initiating immune responses, DCs have been shown to direct polarization of T_H cells towards T_H1 or T_H2 populations. In order to assess the ability of rat BMDCs to regulate T_H cell differentiation, allogeneic CD4$^+$ T cells were cultured with mature BMDCs for 5 days. Cells were then extensively washed and restimulated polyclonally with anti-CD3 and anti-CD28 antibodies for additional 24 hours. To evaluate the background production of cytokines, BMDC-T cell co-cultures were kept in R10 for the last 24 hours. As shown in Fig.5 BMDCs elicited mixed cytokine profiles in allogeneic CD4$^+$ T cells. Both IFN-γ and IL-4 were detected in culture supernatants. CD4$^+$ T cells produced large quantities of IFN-γ: 9.5±6.2ng/ml (Fig.5A). The amount of IL-4 was much lower: 57.5±10.9pg/ml (Fig.5B). In addition to IFN-γ and IL-4, BMDCs activated CD4$^+$ T cells also secreted IL-10: 3.6±1.4ng/ml (Fig.5C).

DISCUSSION

In this study we generated cells with typical DC phenotype from rat bone marrow cultures supplemented with FL, GM-CSF, and IL-4. Only few of the bone marrow-derived cells expressed antigen specific markers for T, B, NK cells or macrophages. BMDCs expressed intermediate levels of MHC II and low levels of CD80 and CD86.

DCs can mature by various stimuli such as ligation of pattern-recognition receptors, pro-inflammatory cytokines, necrotic cells or only cluster disruption. As a result, DCs undergo complex changes including upregulation of MHC class II and costimulatory molecules. Indeed, rat BMDCs upregulated expression of MHC II, CD80, and CD86 molecules in response to LPS treatment. A subpopulation of BMDCs was positive for the rat DC marker OX62. The levels of OX62, NKR-P1A, MHC II, CD80, and CD86 staining in our study were generally lower than those reported by Brissette-Storkus et al [19]. The blocking of Fc receptors prior to staining in our experiments could explain this discrepancy and warrants caution in the interpretation of previous data from flow cytometry of BMDCs. We found that a subset of MHC II$^+$ BMDCs was RK4 positive. RK4 is rat pan granulocyte marker that binds to a cell surface antigen of unknown specificity. We were unable to use the widely accepted rat granulocyte antibody HIS48, because the IgM isotype control of the HIS48 gave a strong unspecific staining (data not shown). It has been reported that FL induces human and mouse plasmacytoid DCs in vivo and in vitro and that in some mouse strains plasmacytoid DCs express the granulocyte marker Gr-1 [25-30]. It is at present unclear whether the MHCII$^+$RK4$^+$ subpopulation of BMDCs represents the rat equivalent of human and mouse plasmacytoid DCs.

To further characterize rat BMDCs a number of functional test have been performed. Immature DCs efficiently internalize antigens [31] but downregulate their ability to capture antigens during maturation [8,32]. This study is the first to show that rat immature BMDCs have the capacity to internalize FITC-albumin, which they to a large extent loose after LPS treatment. Thus, also in the rat downregulation of

endocytosis reflects the transformation of BMDCs from an immature to a mature phenotype.

We also found that mature BMDCs induced a strong proliferation of purified allogeneic $CD4^+$ T cells at low stimulatory: target cell ratio with the SI reaching 46.9. Interestingly, at high BMDCs numbers the SI decreased dramatically. This decrease in proliferation cannot be explained by high cell numbers in the MLR assay, since we were able to detect a SI of 180 when $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies (data not shown). A similar reduction in SI was observed for DCs derived from $CD34^+HLA-DR2^-$ cells incubated with GM-CSF and TNF-α [21]. DCs generated from rat bone marrow using GM-CSF and IL-4 have been shown to be week stimulators of an allogeneic MLR [33]. However, the addition of FL to the culture medium increased the allostimulatory capacity of rat BMDCs at least 10 times [19]. It is difficult to compare the results of MLR between different studies due to the differences in the experimental procedures. Nevertheless, the highest values for ^{3}H-thymidine incorporation in our assay were close to data reported by Brissette-Storkus et al, although this study did not use purified $CD4^+$ T cells.

DCs are a heterogeneous cell population. Several phenotypically distinct cell subtypes have been described in humans and mice. Although, functional plasticity is the general feature of all DC subsets some degree of specialization, yet, exists. For example, mouse $CD8^+$ DCs have been shown to preferentially induce T_H1 polarized responses in $CD4^+$ T cells, while $CD8^-$ DCs tend to induce T_H2 responses [4,5,7]. Human monocyte-derived DCs were found to direct T_H1 differentiation, whereas DCs derived from plasmacytoid cells drive T_H2 development [6]. Two subtypes of rat DCs, $CD4^+SIRP^+$ and $CD4^-SIRP^-$, have been identified in lymph from the small intestine [34].

Similar populations have been described among OX62$^+$ DCs of rat spleen, thymus and lymph nodes [35]. This study failed, however, to detect CD8 expression on OX62$^+$ splenic DCs. In contrast, de la Mata et al [36] reported that between 19 to 48% of spleen DCs were CD8$^+$ and that some of CD8$^+$ cells co-expressed OX62. In addition, several subtypes of DCs have been identified in rat tissues that do not fall into CD4$^+$SIRP$^+$ and CD4$^-$SIRP$^-$ categories described in lymph nodes, spleen or thymus (reviewed in Ref. 37). Despite the abundance of human and mouse studies on functional specialization of DCs, few data exist in respect to rat cells. The CD4$^-$SIRP$^-$ subpopulation of OX62$^+$ splenic DCs has been shown to induce a T$_H$1 response in allogeneic CD4$^+$ T cells, characterized by production of large amounts of IFN-γ. In contrast, the CD4$^+$SIRP$^+$ DC subpopulation induced mixed T$_H$1/T$_H$2 responses as evident by the presence of both IFN-γ and IL-13 in the culture supernatants. Interestingly, although IL-4 was never detected, both CD4$^-$ and CD4$^+$ DCs stimulated T cells to produce a comparable amount of IL-10 [38]. In another study rat respiratory tract DCs preferentially stimulated T$_H$2 responses in antigen-stimulated splenocytes [39]. We found that rat BMDCs induced concurrent production of IFN-γ, IL-4 and IL-10 in allogeneic CD4$^+$ T cells. IFN-γ and IL-4 are typically secreted by T$_H$1 and T$_H$2 cells, respectively. One possible explanation for the observed mixed T$_H$1/T$_H$2 responses in our study is that BMDCs consist of phenotypically diverse DC subtypes with different T$_H$ polarization properties. Although, we were unable to detect either CD4 or CD8 expression on BMDCs (data not shown), the subdivision into MHCII$^+$RK4$^+$ and MHCII$^+$RK4$^-$ populations may prove to be useful for the differentiation between functionally distinct rat BMDCs.

In addition to IFN-γ and IL-4, CD4$^+$ T cells secreted IL-10 when activated by BMDCs. IL-10 was originally described in mice as exclusively T$_H$2 cytokine [40].

However, Yssel et al [41] showed that human T_H0- and T_H1-like $CD4^+$ T cell clones produce IL-10 at the late stages of activation. It was therefore hypothesized that, at least in humans, IL-10 acts as a negative regulator of immune responses. This interpretation was further supported by the findings that human plasmacytoid and myeloid DCs induce T_H cells secreting both IFN-γ and IL-10 [42,43]. IL-10 is also known to be produced by regulatory T cells [44,45]. It has been recently proposed that DCs may exist in three developmental stages: immature, semi-mature and fully mature cells [46]. While immature and semi-mature DCs are implicated in the initiation of tolerance, only fully mature DC can induce immunity. For example, the generation of IL-10-producing $CD4^+$ T cells with regulatory properties by repetitive stimulation with immature allogeneic DCs was reported by Jonuleit et al [47]. If we assume that BMDCs in our study contain cells at different maturation states, then the presence of IL-10 in the culture supernatants can be the result of the induction of regulatory T cells by immature or semi-mature DCs. Additional studies are warranted in order to determine whether IL-10 is produced by T_H2 cells, regulatory T cells or is a part of the negative feedback loop.

In conclusion, rat BMDCs generated from bone marrow cells cultured in the presence of GM-CSF, IL-4, and FL displayed a high endocytic capacity, stimulated vigorous proliferation of allogeneic T_H cells, but induced mixed rather than polarized T_H1/T_H2 responses. Therefore, caution must be taken with the therapeutic application of these cells in animal models of immune-associated diseases. Further studies are required in order to assure the induction of adequate therapeutic responses by BMDCs. These studies should focus on phenotypic and functional characterization of distinct rat BMDC subsets as well as on the establishment of optimal conditions for maturation of BMDCs.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1

Expression of DC-specific markers on the surface of BMDCs. Rat bone marrow cells were cultured in the presence of GM-CSF, IL-4, and FL. After 7 days, BMDCs were collected. Cells were stained for flow cytometry (immature BMDCs) or stimulated with 0.1μg/ml LPS for additional 24h before flow cytometric analysis (mature BMDCs). Staining was performed for OX62 (immature A, mature C) and CD11c (immature B, mature D) molecules (thick lines) or for isotype controls (thin lines). The results shown are representative of three independent experiments for immature cells and one experiment for mature cells, three animals per experiment.

Figure 2

LPS-induced maturation of BMDCs. Rat bone marrow cells were cultured in the presence of GM-CSF, IL-4, and FL. After 7 days, BMDCs were collected. Cells were stained for flow cytometry (immature BMDCs, 2A) or stimulated with 0.1μg/ml LPS for additional 24h before flow cytometric analysis (mature BMDCs, 2B). Double labeling was performed using anti-CD80 Ab and anti-CD86 Ab, anti-MHC class II Ab and anti-RK4 Ab, or matched isotype control Abs. The results shown are representative of three independent experiments, three animals per experiment.

Figure 3

FITC-albumin uptake by BMDCs. Immature and mature BMDCs were generated

as described in Fig.2. (A) Cells were incubated with $0.1\mu g/ml$ FITC-albumin at 4 (open histograms) or 37°C (filled histograms) for 1 hour. Histograms are representatives of two independent experiments, three animals per each experiment. (B) Mean fluorescence intensity (MFI) of immature versus mature BMDCs incubated with $0.1\mu g/ml$ FITC-albumin at 37°C for 1 hour. MFI was calculated with the subtraction of the background binding of FITC-albumin at 4°C. Results are expressed as mean ± SD of two independent experiments, three animals per experiment.

Figure 4

BMDC-induced proliferation of T cells in a mixed leukocyte reaction (MLR). Graded numbers of irradiated mature BMDCs were cultured with $1x10^5$ allogeneic CD4^{+} T cells for 5 days. Cells were pulsed with ^{3}H-thymidine for the last 18 hours of co-cultures. Stimulation index (SI) was calculated according to the formula in the "Materials and Methods". Results are expressed as mean ± SD of two independent experiments, three animals per experiment.

Figure 5

Cytokine production by allogeneic CD4^{+} T cells stimulated with BMDCs. Mature irradiated BMDCs were cultured with allogeneic CD4^{+} T cells at 1:10 ratio for 5 days. Cells were then restimulated with the plate-bound anti-CD3 antibody in the presence of anti-CD28 antibody (closed bars) or cultured untreated in R10 culture medium (open bars). Twenty-four hours later cell culture supernatants were collected and the levels of IFN-γ (A), IL-4 (B), and IL-10 (C) were measured using cytokine-specific ELISA. Results shown represent two independent experiments, three animals per experiment.
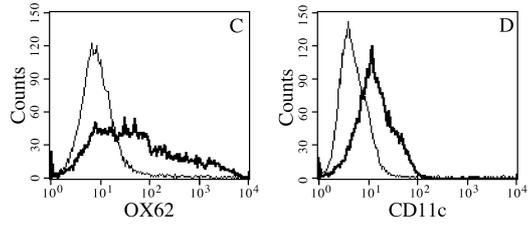
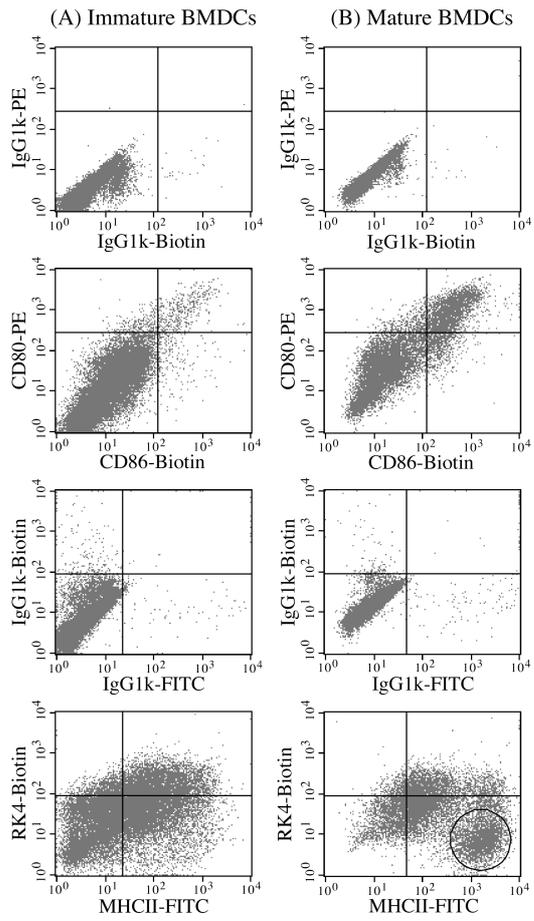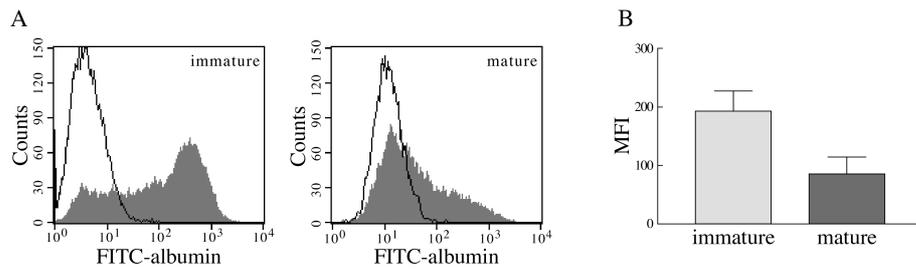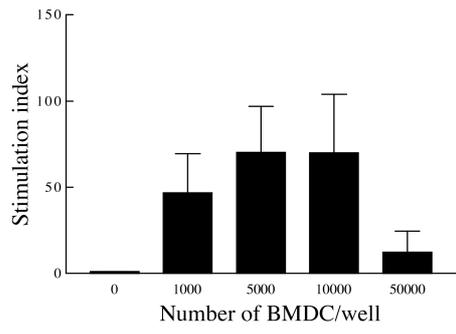Immature BMDCs
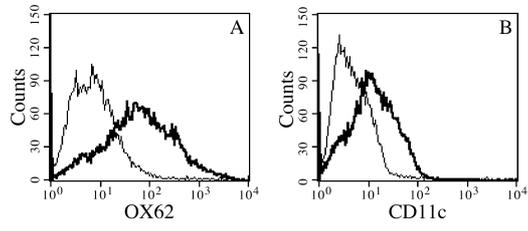
Mature BMDCs

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5