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Generation of large numbers of highly purified dendritic cells from bone marrow progenitor cells after co-culture with syngeneic murine splenocytes

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Abstract

Dendritic cells (DCs) are called the sentinels of the human immune system because of their function as antigen presenting cells (APCs) that elicit a protective immune response. Given that DCs have been used for many years as target cells in a great number of experiments, it became essential to devise a new method for producing DCs in higher quantities and of greater purity. Here we report a novel technique for obtaining more dendritic cells, and with higher purity, from in-vitro co-culture of bone marrow (BM) cells with splenocytes. From a total of 20×10^6 BM cells and 120×10^6 splenocytes, 3×10^6 BM cells along with 20×10^6 splenocytes were co-cultured in petri dishes for DC generation; 120×10^6 splenocytes from one C57BL/6 mouse were also co-cultured in petri dishes for DC generation. The control group were BM cells cultured in the same conditions except for the presence of splenocytes. Purity and maturation state of DCs were checked by lineage surface markers (CD11c, CD11b, CD8 α , and F4/80) and the expression levels of MHCII as well as co-stimulatory molecules (CD86, CD80, and CD40). Endocytosis and thymidine uptake capacity were also used to test the functionality of DCs. The levels of IL-12p70, IL-23, and IL-10 were also checked in the supernatant of cultured cells by ELISA.

The number of DCs derived from co-culture of BM and splenocytes (DCs^{TME}) was at least twice that of BM-derived DCs in the absence of splenocytes. In addition, the purity of DCs after co-culture of BM and splenocytes was greater than that of DCs in the control culture (90.2 % and 77.2%, respectively; $p < 0.05$). While functional assays showed no differences between co-culture

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and control groups, IL-10 levels were significantly lower in DCs^{TME} compared to BM-derived DCs in the absence of splenocytes (193 pg/ml and 630 pg/ml, respectively; $p < 0.05$).

The results of the present study show that the generation of DCs from BM progenitors is accelerated in the presence of syngeneic splenocytes. Given the larger number of generated DCs, and with higher purity, in this technique, DCs^{TME} could be more advantageous for DC-based immunotherapy and vaccination techniques.

Keywords

Dendritic cells; bone marrow; spleen; co-culture; purity; large number

Introduction

Dendritic cells are the most important antigen-presenting cells (APCs) in the immune system (Yuan & Liu, 2010). They detect foreign antigens in the peripheral tissues and present the processed antigens to naïve T cells (Inaba et al., 1992). These characteristics of DC make them a major immune cell target for immunotherapy and vaccination.

One of the common techniques for in vitro generation of mouse DCs is culture of bone marrow (BM) progenitor cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for 7 to 10 days (Son et al., 2002). In this culture method, a mixture of cells, including monocytes and macrophages as well as DCs, is usually generated (O'Neill et al., 2004). The other DC-generating method is called conditioned medium culture. In this method spleen cells are cultured in the presence of 30% conditioned medium from the NIH/3T3 cell line and 20ng/ml GM-CSF (Periasamy et al., 2009; Despars et al., 2008).

Another method that is used to develop DCs is long-term culture (LTC) technique. The main goal of this method is to study hematopoiesis (O'Neill et al., 2004). It has been reported that most of the BM-based LTC systems support granulopoiesis. In contrast, murine spleen-based LTC contributes to the generation of dendritic-like cells (Geijtenbeek et al., 2003; Scheicher et al., 1992; Harada et al., 2009). Interestingly, in mouse LTC systems, a splenic stromal cell line such as STX3 is commonly used to support DC generation from the progenitor cells of BM (O'Neill, Ni, & Wilson, 1999) or spleen (Periasamy et al., 2009). However, in LTC a smaller number of DCs are generated over a longer time period— from 14 to 17 days compared to 7 to 10 days' culture for differentiation of DCs from BM progenitors in the classic technique of DC generation (Periasamy et al., 2009).

Currently, many methods used in immunotherapy or vaccination take advantage of DCs after their isolation and culture from BM (Toscano et al., 2010). For mouse DC production, one of the best methods is the culture of BMDC progenitors supplemented with growth factors like GM-CSF, which over a period of 7 to 10 days differentiate to DCs (Inaba et al., 1992). This established culture system has been modified to increase the yield and purity of DCs, but to date the disadvantage of limited yield and purity of generated DCs has not yet been resolved. Given that the splenic DC pool is derived from the migration of BMDC progenitors via blood and from the local expansion of DC precursors in secondary lymphoid tissues (Auffray, 2008), co-culture of spleen- and bone marrow-derived cells may eliminate the above-mentioned disadvantages.

The aim of the current article is to introduce a new culture system, consisting of whole spleen and BM cells from one C57BL6 mouse, which is more efficient at producing a large

number of highly purified DCs than the current method of DC generation from BM progenitors.

MATERIALS AND METHODS

1. Mice

Eight-week-old female mice from strains C57BL/6, C57BL/6-Tg (CAG-EGFP) 10sb/J [a transgenic mouse line with an “enhanced” GFP (EGFP) cDNA], and 2D2 mice [T cell receptor (TCR) transgenic mice for myelin oligodendrocyte glycoprotein (MOG 35–55)] were purchased from Jackson Laboratory. Animals were kept under pathogen-free conditions in filter top cages at the Thomas Jefferson University animal care facility. All animal studies were performed according to the guidelines of the Animal Care and Use Committee at Thomas Jefferson University.

2. Co-culture method

2.1. Determination of optimal BM/spleen cell ratio for co-culture—Different ratios of BM to spleen cells (1/2, 1/5, 1/7, 1/10, and 1/20) were tested to obtain the optimal ratio for co-culture. To reach this goal, 2×10^6 , 3×10^6 , and 6×10^6 BM cells along with different numbers of splenocytes were added to each 100 mm bacteriological petri dish (the mixed cells were called “bone marrow spleen cells” or BMSPs). Cultures of 2×10^6 , 3×10^6 , and 6×10^6 BM cells were also used as controls for the efficacy of DC generation from BM in the absence of splenocytes.

2.2. Generation of DC^{TME} from co-culture of BM cells and splenocytes—BM and spleen of one C57BL/6 mouse were aseptically removed. One ml of digestion mix [collagenase and DNase I (Roche, USA)] was gently injected into the spleen until spleen color changed from maroon to reddish-orange. Spleen tissue was cut into small pieces, teased with a syringe plunger and incubated in a 37°C incubator for 20 minutes (Periasamy et al., 2009). At the same time, BM cells were removed as described above. Spleen and BM tissues were separately passed through nylon meshes (Fisher Scientific, USA). Cells were collected, spun down and RBCs were removed with RBC lysis buffer (Biolegend, USA). Based on the optimal ratio obtained (section 2.1), spleen and BM cells were mixed with each other. Complete culture medium contained RPMI-1640 (Mediatec, USA), 10% fetal bovine serum (FBS) (Fisher Scientific, USA), 2mM glutamine (Mediatec, USA), and 10ng/ml GM-CSF (PeproTec, USA). Non-adherent cells were collected and after re-suspension in fresh complete medium were plated in new petri dishes every 2–3 days over 10 days. To obtain activated DCs, 20ng/ml of LPS was added for the last 24h of culture (on day 9) to the plates.

3. Phenotypic analysis of DC^{TME}

Cultured cells were analyzed for their surface markers using rat anti-mouse CD40, CD80, CD86, and MHCII conjugated with phycoerythrin (PE), PerCP-Cy5.5, PE, and PE, respectively. Lineage surface markers were also stained with conjugated rat anti-mouse CD11c, CD11b, F4/80, and CD8α labeled with allophycocyanin (APC), PerCP-Cy5.5, PE, and fluorescein isothiocyanate (FITC), respectively. All antibodies were purchased from BD Biosciences, USA, and cells were stained according to the manufacturer’s instructions. Stained cells were analyzed with BD FACSAria (BD Biosciences) using BD FACSDiVa software.

4. Endocytosis assay

Non-adherent BMSP or BM cells (5×10^5) were collected in polypropylene tubes and after keeping them on ice for 10 minutes, 2μl of endocytic tracer, ovalbumin (OVA)-FITC (2mg/

ml), was added. Control tubes were kept on ice, and sample tubes were incubated in 37°C for 30 minutes. After washing, uptake of OVA-FITC was measured by flow cytometry.

5. Proliferation assay

CD4⁺ T cells were purified from spleens of 2D2 mice with magnetic micro beads (Miltenyi-Biotec, USA). Purity of CD4⁺ T cells was > 90% by CD4 specific immunostaining. T cells (10⁵) were cultured with DCs (5 × 10³) derived from BM or BMSP in the presence of 20 µg/ml of MOG. After 48 h cells were pulsed with 0.5 µCi ³H-thymidine for the last 18h. Thymidine incorporation was measured using a scintillation counter. Stimulated cells with Anti-CD3 and Anti-CD28 (1µg/ml) were used as positive control, and non-stimulated cells in medium were used as negative control. All proliferation assays were run in triplicates.

6. Cytokine assay (ELISA)

Culture supernatants were collected on day 10, and IL-12p70, IL-23, and IL-10 were measured with ELISA kits (R&D, USA) according to the manufacturer's recommendations.

Statistical analysis

Difference between groups was analyzed by unpaired two-tailed parametric Student's *t*-test using Excel 2007 and Graphpad 5. Data were expressed as means ± S.D. Differences were considered statistically significant if *P*-values < 0.05.

RESULTS

Optimal BM/spleen cells ratio for co-culture

From different ratios of BM to spleen cells (1/2, 1/5, 1/7, 1/10, and 1/20), the ratio of 1/7 was selected as the optimal ratio of production of DC^{TME} in co-culture. At this ratio, (110±5.6) ×10⁶million non-adherent BMSP cells produced (99±5) ×10⁶ of DCs^{TME}, while (46±4.6) ×10⁶ BMDCs were detected among (60±8.3) ×10⁶ non-adherent BM cells.

Morphological analysis of DC^{TME}

Photomicrographs were taken from BMSP and BM cultures from day 0 to day 10 (Fig. 1, Parts I and II). Small cells were detectable in both culture systems up to day 4 (Fig. 1, Part I -A & B and Part II -A & B). On day 4, a few cells with DC-like morphology appeared in both BMSP and BM cell cultures (Fig. 1, Part I-C and Part II-C). However, the number of cells with DC phenotype was higher in the BMSP co-culture system compared to BM controls from day 7 (Fig. 1, Part I -D & E and Part II -D & E), and this difference was more significant on day 10 of culture.

Phenotypic assay of DC^{TME}

Flow cytometry was used to analyze the expression of lineage and maturation specific markers (Fig. 3). As shown in Figure 3.C, among different lineage specific markers, the expression of CD11c was significantly higher in BMSP compared to BM cells (90.2± 3% and 77.2± 5 %, respectively; *p*<0.05). In this respect, on day 10, the absolute number of CD11c⁺ DCs was also significantly higher in BMSP than in BM cells (99± 5)×10⁶ DC^{TME} and (46± 4.6)×10⁶ BMDCs, respectively; *p*=0.0002.; Table 1).

As shown in Figure 3.B, prior to activation with LPS, the percentage of positive cells for MHCII, CD86, CD80, and CD40 in the BMSP group (24.5%, 20.8%, 18%, and 0.7%, respectively) and in the BM control group (26%, 25.6%, 28.5%, and 0.2%, respectively) was not significantly different. However, after LPS activation, a comparison of CD11c, MHCII, CD86, CD80, and CD40 positive cells in the BMSP group and the BM control group

showed that the percentage of CD11c (90.2 and 77.2, respectively; $p=0.026$) and CD86 (77.4 and 59.2, respectively; $p=0.016$) was significantly different in these two groups.

Cytokine assays

On day 10, levels of IL-12p70, IL-23, and IL-10 were determined in the supernatants of LPS-stimulated and un-stimulated BMSP and BM cultures (Fig. 4). While there was no significant difference between the levels of the above-mentioned cytokines in the supernatant of un-stimulated BMSP and BM cells, the levels of IL-10 were significantly higher in LPS-stimulated BMDCs compared to those of activated DC^{TME} (630 ± 60 pg/ml and 193 ± 59 pg/ml, respectively; $p=0.018$).

Functionality of DC^{TME} and BMDCs

Endocytic capability and the level of ³H-thymidine uptakes were checked to determine the functionality of DC^{TME} and BMDCs (Fig. 5). As shown in Figure 5, the percentage of endocytosed FITC-OVA was not statistically different between LPS-stimulated DCs^{TME} and BMDCs ($81.5\pm3.5\%$ and $80.3\pm4.1\%$, respectively; $p=0.719$).

On the other hand, the ability of DCs^{TME} or BMDCs to stimulate CD4⁺ T cells of 2D2 mice was evaluated by ³H-thymidine uptake assay. As shown in Figure 5, no significant differences were detected between LPS-stimulated DCs^{TME} and BMDCs [$128\pm4(\times 10^3\text{cpm})$ and $120\pm6(\times 10^3\text{cpm})$, respectively; $p=0.264$] or between untreated DCs^{TME} and BMDCs [$(128\pm4)\times 10^3\text{cpm}$ and $(120\pm6)\times 10^3\text{cpm}$, respectively; $p=0.264$] or between untreatedx 10 DCs^{TME} and BMDCs ($63\pm28)\times 10^3\text{cpm}$ and $(55\pm20)\times 10^3\text{cpm}$, respectively; $p=0.774$] in their ability to activate MOG-specific CD4⁺ cells from 2D2 mice.

Contribution of BM- and spleen-derived progenitor cells in the generation of DC^{TME}

In order to determine the origin of DC^{TME} in BMSP co-culture, C57BL/6-derived BM cells were mixed with spleen cells from a C57BL/6-Tg (EGFP) mouse. On day 9 of culture, non-adherent cells of BMSP culture were examined by fluorescence microscopy (Fig. 6). The results showed that the contribution of spleen- and BM-derived progenitors in DC^{TME} generation was $43\pm3.7\%$ and $47\pm3.5\%$, respectively.

Discussion

In the present study, a new co-culture system with the capability of producing a huge number of highly purified DCs was introduced. The results of this study showed that the percentage of CD11c⁺ cells, as a typical murine DC marker, was significantly higher in the BMSP co-culture than in traditional BM culture for generation of DCs ($90.2\pm3\%$ and $77.2\pm5\%$, respectively; $p=0.026$). In addition, the BMSP co-culture system showed the ability to produce a greater absolute number of DCs compared to the BM culture system. In fact, among 110 ± 5.6 million non-adherent cells produced in BMSP culture 99 ± 5 million of the cells were DCs^{TME}, while in the BM culture 46 ± 4.6 million BMDCs were detected among 60 ± 8.3 million non-adherent cells. Of interest, the presence of splenocytes increased the viability of cells given that assessment of cell viability at day 5 of culture showed more than 99% viability in BMSP culture while the viability for BM culture in the same conditions was 75% (data not shown). In addition, BMSP and BM cultures showed no difference in the distribution of CD11b⁺ (myeloid cell marker), F4/80⁺ (monocyte marker), and CD8α⁺ (T cell lineage marker) cells after 10 days of culture. According to these findings, both BMDCs and DC^{TME} cells might have originated from myeloid cells. In addition, there was no difference in endocytic capability and antigen presentation activity of BM- and BMSP-derived DCs.

However, after LPS activation, the level of CD86 co-stimulatory molecule was significantly higher on DCs derived from BMSP culture compared to those from BM culture ($77.4 \pm 2.9\%$ and $59.2 \pm 5.6\%$, respectively; $p = 0.016$) while the levels of IL-10 production were lower in DC^{TME} compared to DCs derived from the traditional culture system (193 ± 59 pg/ml and 630 ± 60 pg/ml, respectively; $p = 0.018$). Therefore, it might be concluded that DC^{TME} cells derived from BMSP co-culture are more potent than BMDCs derived from traditional culture in activation of non-regulatory T helper cells and induction of immunity in vaccination protocols. Nevertheless, the results of 3H-thymidine uptake assay did not show any significant difference between DC^{TME} and BMDCs ($p = 0.264$).

In conclusion, the results of the present study show that co-culture of BM and spleen cell precursors greatly increases the number of generated DCs. Therefore, the ability of this new co-culture technique to generate a large number of highly purified DCs will facilitate the use of DCs in future immunotherapy and vaccination protocols.

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Fig. 1. Photomicrographs of BMSP (Part I) co-culture and BM cell culture (Part II) were taken on days 0, 2, 4, 7 and 10 (10× or 20×).

Fig. 2. Photomicrographs of unstimulated and stimulated DC-like cells with LPS showing the same morphology as BMDCs
(A) Unstimulated DC-like cells showed large cytoplasmic veils rather than dendrites. (B) Activated DC-like cells with LPS showed long dendrites, which is a characteristic of mature DC-like cells.

Fig. 3. Flow cytometric analysis of DCs^{TME}

Co-cultures of BM and spleen cells were established at a ratio of 1:7. Non-adherent BMSP cells were collected for phenotypic analysis after 10 days. Cell groups were stained with specific antibodies (open histograms) or isotype-matched negative control antibodies (filled histograms). (A) Forward (FSC) vs. side scatter (SSC) analysis of DC^{TME} compared to control cells comprising BMDC and spleen cells. Cells were initially gated to determine the percentage of DC with CD11c surface markers. (B) Percentage representation of MHCII and costimulatory molecules CD40, CD80, and CD86 in DC^{TME} in comparison to BMDC and spleen cells. (C) Expression of lineage surface markers in DC^{TME} was also determined by flow cytometry in comparison to BMDC. FACS analysis of DC^{TME} cells was performed in three separate experiments.

Fig. 4. Cytokine production of DC^{TME} cells after stimulation with LPS for 24h
 (A) IL-10 level, measured by ELISA in supernatants of DC^{TME} cells, was significantly higher than that of BMDCs ($p = 0.018$). (B) & (C) IL-12p70 and IL-23 were measured by ELISA in supernatants of DC^{TME} cells; there were no significant differences between DC^{TME} and BMDCs ($p > 0.05$). Data are from two independent experiments. Bars are SEM. *: $p < 0.05$.

Fig. 5. DC^{TME} can phagocytize soluble antigens (FITC-OVA) and also present MOG to CD4⁺ T cells in a similar way as BMDCs

Phagocytic effect of DCs^{TME} (A) is similar to that of BMDCs (B) ($p > 0.05$). Figures C and D are control groups of DCs^{TME} and BMDCs at 4°C, respectively (E) Thymidine uptake was compared between DC^{TME} cells and BMDCs, which showed no significant difference ($p > 0.05$). Data are from two independent experiments. Bars are SEM.

Fig. 6. Combinational ratio of BM and spleen cells from co-culture cells

Co-cultures of spleen cells derived from E-GFP C57BL/6-Tg mice and BM cells originated from C57BL/6 mice under light microscope (A) and fluorescent microscope (B) (40 ×) on day 9 showed that 43% of co-cultured cells had been produced from spleen cells.

Table 1Production of highly purified DCs^{TME} from cocultured BMSP

Origin	Non-adherent Cells	CD11c(%)	calculation	Absolute No. of DCs
BMSP	11×10 ⁷	90.2 *	11×10 ⁷ × (90.2/100) =	99×10 ⁶
BM	60×10 ⁶	77.2	60×10 ⁶ × (77.2/100) =	46×10 ⁶

Abbreviations: BMSP: bone marrow spleen cell, BM: bone marrow, No.: number.

*
p< 0.05.