Cellular Immunology 337 (2019) 54–61

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Dendritic cell line AP284 supports Th17 amplification

Pollyana Guimarães de Oliveira[a], Clayson Moura Gomes[b], Lucilla Ribeiro Ávila[a,c], Fatima Ribeiro-Dias[a], Pieter Johannes Maria Leenen[d], Milton Adriano Pelli de Oliveira[a,*]

[a] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235 S/N, Goiânia, Goiás 74605-050, Brazil
[b] Pontifícia Universidade Católica de Goiás, Av, Universitária 1069, Setor Universitário, Goiânia, Goiás 74605-010, Brazil
[c] UniCerrado – Centro Universitário de Goiatuba, Rodovia GO320, S/N – Jardim Santa Paula, Goiatuba, Goiás 75600-000, Brazil
[d] Department of Immunology, Erasmus University Medical Center, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands

ARTICLE INFO

Keywords:
Immortalized dendritic cell
IL-12p40
IL-23
Th17
Toll like receptor

ABSTRACT

Dendritic cells (DC) have the unique ability to capture microorganisms and activate naive T lymphocytes. Obtaining DC derived from progenitors demands high cost and prolonged cultivation. Different immortalized DC has been isolated but most of them have immature phenotype and depending on growing factors or other stimuli to be used. In this study we characterized the cell line AP284 as a DC. AP284 cells express high levels of CD11b, MHC class II, 33D1 and CD209b. They also express high amounts of CD80 costimulatory molecule and different toll like receptors (TLR). After stimuli with TLR agonist they produce surprising amount of IL-12p40 related to IL-23 formation but not IL-12p70. They are also able to produce IL-6 and favor amplification of a Th17 but not Th1 profile. This DC line may be useful for a better understanding of factors and cellular interactions responsible for the induction of IL-12p40, IL-23 and Th17 generation.

1. Introduction

Dendritic cells (DC) were initially described in 1973 by Steinman and Cohn as a novel cell type with unique morphological characteristics [1]. Currently, several DC populations have been described and separated based on different criteria, such as expression of surface markers, function and location [2]. DC have the unique ability to capture microorganisms, migrate to peripheral lymphoid organs and activate naive T lymphocytes [3]. To recognize microorganisms, DC present a variety of Pattern Recognition Receptor (PRR), of which, the Toll-like receptors (TLR) are studied most extensively [4,5].

When activated, DC produce different types of cytokines that stimulate differentiation of distinct $CD4^+$ T lymphocyte profiles [6]. For instance, IL-12 induces generation of Th1 lymphocytes, which are important to control intracellular microorganisms [7,8]. IL-4 induces generation of Th2 lymphocytes, which are important in helminthic infections, but have a pathogenic role in allergic diseases [9]. Production of IL-6 and TGF-β induces generation of the Th17 profile, important for the control of extracellular bacteria and fungi [10], while TGF-β can also lead to induction of T-regulatory cells (Treg), important for the control of the immune response [11].

IL-12 belongs to a family of cytokines that share subunits [12]. The IL-12p40 subunit links covalently to IL-12p35 to form IL-12p70 [13]. IL-12p40 can also combine with a IL-23p19, subunit forming IL-23 [12]. IL-23 is produced mainly by DC and is indispensable for the maintenance and amplification of Th17 cells, although it is not essential for their initial differentiation [14].

Due to the importance of DC in inducing differentiation of T lymphocytes, and their key role in regulating immune responses, DC are a potential target for vaccine generation and immunotherapy [15]. The main way to generate murine DC for research is by stimulating bone marrow progenitor cells with Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). However, the yield of DC derived from hematopoietic progenitors may be limiting for some experimental approaches. In addition, obtaining these cells *in vitro* demands high cost and prolonged cultivation [16]. To overcome these difficulties, immortalized DC have been isolated and are widely used in research [16].

The first immortalized murine DC generated was the CB1 cell line derived from DBA/2 mice followed by the D2SC/1 derived from BALB/c mice. They were immortalized by retroviral infection of spleen cells with a recombinant retrovirus encoding the v-myc oncogene of the avian MH2 virus fused to the env gene of the AKR leukemia virus [17,18]. Both the CB1 and the D2SC/1 line present a semi-mature phenotype with intermediate to high expression of MHC I, MHC II and CD80 molecules. Another murine immortalized DC generated is the fetal skin line, FS57 from C57BL/6 [19]. This line has an immature cell

* Corresponding author at: Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235 S/N, Goiânia, Goiás 74605-050, Brazil.
E-mail address: mapoliv@ufg.br (M.A.P. de Oliveira).

https://doi.org/10.1016/j.cellimm.2019.02.003
Received 17 October 2018; Received in revised form 22 January 2019; Accepted 8 February 2019
Available online 10 February 2019
0008-8749/

profile, and is therefore less effective in activation of T lymphocytes *in vitro*, but can be stimulated to mature in the presence of GM-CSF and IFN-γ [19]. The DC2.4 cell line was derived from bone marrow of C57BL/6 mice [20], and the cells exhibit a semi-mature phenotype in their resting state, but increase the expression of MHC molecules, costimulatory molecules, and the production of various proinflammatory cytokines following viral infection or stimulation with cytokines or TLR agonists [21–23]. The SVDC line (C57BL/6 origin) requires GM-CSF for its maintenance *in vitro*. This cell line grows at 33 °C, but stops to grow at temperature higher than 37 °C and dies at a 39 °C [24]. The SRDC line from CBA/J mice also has an immature phenotype and can become mature in the presence of LPS or *Toxoplasma gondii* antigen [25], similar to the D1 line isolated from the spleens of C57BL/6 mice that depend on LPS and GM-CSF to become mature [20,26,27]. Since most immortalized DC have immature phenotype and because AP284 cells was isolated from a spontaneous spleen tumor and was described as mature macrophage able to capture and present antigens to reactive T lymphocyte clones, inducing proliferation of the latter [28], we decided to investigate whether AP284 cells are more related to DC than a macrophage cell line. Here we showed that AP284 cells express DC markers, and have the ability to amplify an immune response *ex vivo* and skew T helper cell differentiation by producing IL-12 family cytokines after stimulation with different TLR agonists. We observed that AP284 cells are able to produce significant amounts of IL-12p40, IL-6 and IL-23. In agreement, these cells promote the maintenance of Th17 cells.

2. Material and methods

2.1. Mice

Male C57BL/6 mice were bred at the animal facilities of the Federal University of Goiás/IPTSP, Brazil. The mice used were 6–12 weeks old and were maintained in a clean conventional mouse facility with *ad libitum* access to water and food. All experimental procedures were conducted according to the guidelines of the Animal Research Ethical Committee (CETEA) of the Federal University of Goiás, approved under the protocol 038/2013.

2.2. Cell preparation and cultures

Bone marrow-derived dendritic cells (BMDC) were obtained based on the most common protocol used [29], stimulating bone marrow cells freshly isolated from the femurs and tibias of mice with GM-CSF. The cell suspension was cultured in 6-well culture dishes (TPP Switzerland) at 4×10^6 cells/mL for 6 days in RPMI 1640 medium (Sigma-Aldrich, St Louis, MD, USA) supplemented with 1.0 g/L sodium bicarbonate (Labsynth, São Paulo, Brazil), 2 mM L-glutamine 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 50 μM 2-mercaptoethanol, 1 M Hepes (all reagents were from Sigma-Aldrich), 10% fetal calf serum (SBF-Cripion, Andradina, São Paulo) inactivated (56 °C for 30 min) and GM-CSF at 10 ng/mL. The cultures were refreshed every 2 days by gently swirling the plates, aspirating the medium, and adding back fresh medium with GM-CSF at 10 ng/m. RAW264 cells (American Type Culture Collection) and AP284 cells [28] were cultured in 6-well plates in RPMI-supplemented medium as described above, without GM-CSF. Cultures were started at 2×10^5 cells/mL and expanded every 3 or 4 days.

2.3. Antibodies for cell markers

The following specific antibodies were obtained from hybridoma cultures: anti-CD11b (clone M1/70), anti-MHCII (clone ER-TR3), anti-CD209b SIGNR1 (clone ER-TR9), 33D1 (clone 33D1), anti-CD4 (clone GK1.5), anti-CD8 (clone 31M), anti-CD16/32 (clone 2.4G2), control antibodies IgG1 (clone GL113) and IgG2b (clone GL117). Commercial antibodies anti-CD107b (clone MAC3, Bioscience-BD, New Jersey, USA), anti-TLR2 FITC-labeled (clone T2.5, Abcam, Cambridge, UK), anti-TLR4/MD2 FITC-labeled (clone MTS510, Abcam), anti-TLR3 PE-labeled (Biolegend, San Diego, CA, USA), anti-TLR5 Alexa Fluor- labeled (Biolegend), anti-TLR7 FITC- labeled (Invitrogen, Waltham, USA), anti-TLR9 FITC-labeled (Molecular Probes, Eugene, USA), anti-CD40 (clone 1C10, Bioscience-BD), anti-CD80 (clone 1G10/B7, Bioscience-BD), anti-CD11c (clone HL3, eBioscience).

2.4. Flow cytometry

AP284, BMDC and RAW264 cells were collected from culture, centrifuged and suspended at 2×10^5 cells in 100 μL PBS containing 2% FBS, 5 mM EDTA and 0.02% sodium azide (FACS buffer). To label surface markers, the cells were incubated with specific antibody either or not conjugated with fluorescent probe, or with the respective control isotype for 20 min at room temperature. Cells incubated with unlabeled antibodies were washed with FACS buffer and suspended in FACS buffer containing FITC-conjugated goat anti-rat IgG antibody (eBioscience) and incubated again for 20 min. Finally, cells were washed and suspended in 100 μL FACS buffer. Twenty thousand events were acquired for each sample in a flow cytometer (BD Accuri™ C6 Cytometer - BD Biosciences). The percentage of labeled cells and the fluorescence increase were analyzed in FCS Express version 4.0 software (De Novo Software, Los Angeles, CA., USA).

To label intracellular markers the cells were suspended in 50 μL FACS buffer and incubated for 15 min with 4% paraformaldehyde (Merk, Darmstadt, Germany) in PBS. The cells were washed twice with FACS buffer and suspended in 0.02% saponin (Sigma-Aldrich) in PBS for 15 min. After washing the cells twice with FACs buffer cells were incubated with anti-TLR3, TLR7 and TLR9 antibodies.

2.5. Stimuli

AP284 and BMDC cells were treated for 24 h with the following TLR agonists: a TLR4 agonist lipopolysaccharide (LPS, *Escherichia coli* O111: B4; Sigma-Aldrich); a TLR2 agonist zymosan from *Saccharomyces cerevisiae* (Sigma-Aldrich); a TLR2/TLR6 agonist FSL-1 Syn from *Mycoplasma salivarium* (Pam2CGDPKHPKSF; InvivoGen, San Diego, Califórnia, EUA); a TLR7/TLR8 agonist resiquimod (1–4 Amino-2-ethoxymethylimidazo 4,5C, R848; Sigma-Aldrich); a TLR9 agonist CpG (ODN 2006 MACS; Miltenyi Biotec, Bergisch Gladbach, Germany); a TLR5 agonist Flagellin from *Salmonella typhimurium* (Sigma-Aldrich) or heat-killed *Escherichia coli* (ATCC® 11,229™). *E. coli* were cultured in thioglycollate medium (NIH Thioglycollate Broth, Himedia, India) at 37 °C for 48 h, washed twice in PBS and heated at 90 °C for 30 min prior to use. The number of bacteria was quantified by turbidity as previously described [30]. In some experiments, cells were pretreated with 0.2 ng/mL IFN-γ (R&D Systems, Minneapolis, USA) for 2 h prior to addition of the stimuli. AP284 and BMDC were also stimulated with gamma-irradiated Bacillus Calmette-Guérin (BCG, Statens Serum Institut, Artilleivej, Denmark).

2.6. Determination of nitric oxide (NO) production

AP284 and BMDC (5×10^5) were stimulated with LPS or *E. coli* bacteria and incubated for 24 h. After this time, culture supernatant was collected and NO production assessed by Griess method [31]. Briefly, culture supernatant (50 μL) was incubated with an equal volume of the Griess reagent (0.5% sulfanilamide, 0.05% N-1-naphthylethylenediamine dihydrochoride and 2.5% ortho-phosphoric acid) for 10 min at room temperature. The absorbance at 550 nm was determined in a microplate reader (Multiskan, Thermo Labsystems, Finland). The results were expressed as μM of nitrite based on a standard curve established with known concentrations of sodium nitrite ($NaNO_2$, Sigma-Aldrich) dissolved in culture medium with a detection limit of 1.5 μM.

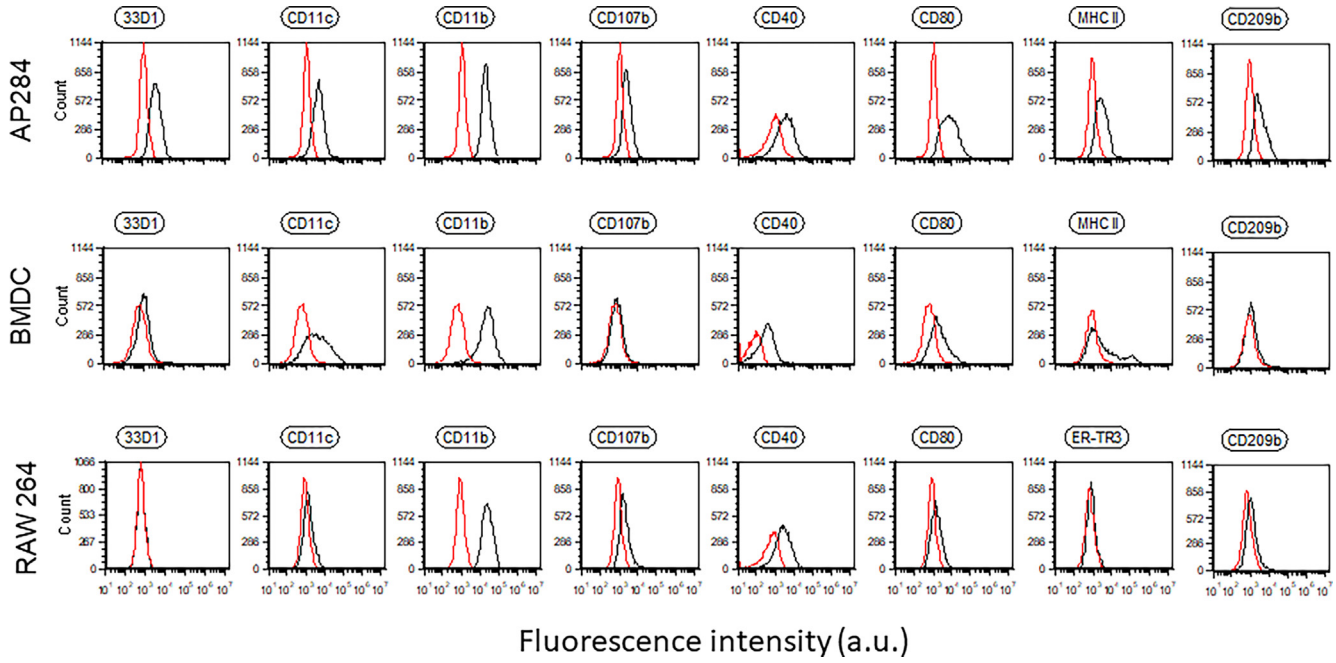

Fig. 1. Expression of cell surface markers on AP284, BMDC and RAW264 cells. AP284, BMDC, and RAW264 cells were incubated with antibodies for 33D1, CD11c, CD11b, CD107b, CD40, CD80, MHC class II and CD209b for analysis by flow cytometry. The histograms show the fluorescence intensity (arbitrary units) of a representative experiment from three independent experiments of the cells labeled with control isotype (red) and specific antibody (black).

2.7. Cytokine detection

Cytokine levels in the culture supernatants were measured by two-site sandwich ELISA. The following antibodies pairs were used, and the second cited was biotinylated: IL-12p40 (clones C17.8 and C15.6) and IL-12p70 (clones C18.2 and C17.15) were from hybridomas donated by Dr. Giorgio Trinchieri (National Cancer Institute, Frederick, MD, USA); IL-23, G23.8 (Biolegend) and C17.15; IFN-γ (clones XMG 1.2 and ASN-18) were from hybridomas donated by Dr. Ises Abrahamsohn (University of São Paulo, Brazil). Commercial kits were used for detection of IL-17 (R&D Systems), IL-10 (eBioscience), TNF-α (eBioscience), IL-6 (Invitrogen). Standard curves were obtained with recombinant mouse cytokines (Biolegend). The reaction was developed with peroxidase-conjugated streptavidin followed by the substrate TMB (Invitrogen) and the reaction was stopped with H_2SO_4. The supernatants were tested in serial dilutions, and the results expressed as the mean of duplicate determinations. The absorbance at 450 and 620 nm was determined in an ELISA reader (Multiskan).

2.8. Viability analysis

AP284 cells were stimulated with LPS and IFN-γ and incubated for 24 h with sterile MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich] [32] at 36 °C for 6 h. The reaction was stopped by the addition of 100 μL of aqueous solution containing 50% dimethyl sulfoxide and 10% sodium dodecyl sulfate to each well. The plate was incubated at 36 °C for 12 h, and the absorbance at 550 nm was determined in an ELISA reader (Multiskan). AP284 were killed by heat at 60 °C for 30 min to obtain the negative control.

2.9. Generation of BCG-specific lymphocytes and restimulation ex vivo DC

C57BL/6 mice were immunized with three doses of subcutaneous injections of emulsified Freund's Complete Adjuvant (CFA-Sigma-Aldrich) at 21 day intervals. After immunization, mice were euthanized for subsequent spleen extraction. Spleens were disrupted macerated and cells were washed in PBS and subsequently treated with red blood cells lysis solution (17 mM Tris-HCl + 0.144 M ammonium chloride pH 7.2) for 5 min. Cells were washed with PBS and cultured in 6-well plates with RPMI without serum at 36 °C for 2 h to allow macrophage adherence. Non-adherent cells, as source of lymphocytes, were collected and 1×10^6 cells were cultured in a 24-well plate together with AP284 (2×10^4) or BMDC (4×10^4) cells previously incubated for 24 h with 2×10^5 gamma-irradiated BCG. Non-adherent cells from spleens of non-immunized mice were used as controls. After 72 h of culture, the supernatant was collected to evaluate the production of IL-17 and IFN-γ.

2.10. Statistical analysis

Data are presented as mean ± standard deviation and compared for significance by Student's *t*-test or one-way ANOVA followed by Fisher's test using Graph-Pad Prism Software 6.0 (Inc. San Diego, CA, USA). The value of $p < 0.05$ is considered significant.

3. Results

3.1. AP284 cells share more characteristics with dendritic cells than macrophages

AP284 cells were initially characterized as mature macrophages able to present antigens and activate T lymphocytes [28]. As features of DC as exquisite antigen-presenting cells have been defined more extensively since then, we reanalyzed this cell line and found that they share more characteristics with DC than macrophages. We observed that, in culture, AP284 cells were weakly adherent and had long dendrites, in particular after stimulation with Zymosan or LPS (Fig. S1A-C). Comparing cell surface marker expression of mononuclear phagocytes present on the RAW264 cell line, representing macrophages, BMDC, representing conventional DC and AP284 showed that AP284 are closer to DC than macrophages. As observed in Fig. 1, AP284 cells express high levels of CD11c and MHC class II. In addition, the DC subpopulation markers 33D1 and CD209b was also observed in this cell line, which also expresses high amounts of CD80 costimulatory molecule. In contrast, RAW264 macrophages do not express significant levels of CD11c, 33D1 and MHC II molecules, but express common

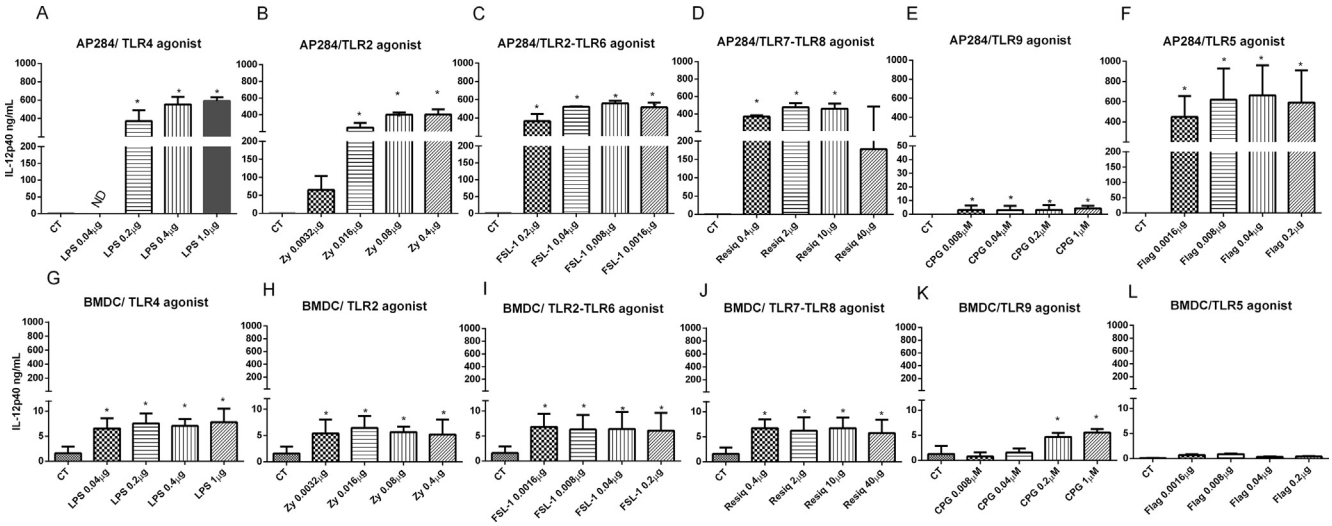

Fig. 2. AP284 cells produce high amount of IL-12p40 after stimulation with TLR agonists. AP284 cells (A-F) and BMDC (G-L) were stimulated with TLR agonists lipopolysaccharide (LPS); zymosan (Zy); Pam2CGDPKHPKSF (FSL-1); resiquimod (resiq) CpG-ODN (CPG) and flagellin (flag) at different concentrations for 24 h and production of IL-12p40 was evaluated by ELISA. The bars represent the mean ± SD of cytokine production from three independent experiments * indicates statistical difference between unstimulated (CT) and cells stimulated with TLR agonists by one-way ANOVA followed by Fisher's ($p < 0.05$).

markers of mononuclear phagocytes such as the CD11b and the CD40 costimulatory molecule, which are present on all cell types analyzed (Fig. 1). The Gr-1, CD8, CD4, NK1.1 and B220 markers that may be present in DC subpopulations were absent in cell populations tested (data not shown). Taken together, these data strongly suggest that AP284 cells share more characteristics with dendritic cells than macrophages, in particular by their spontaneous expression of MHC II and costimulatory molecules.

3.2. AP284 cells produce high amounts of IL-12p40 after stimulation with different TLR agonists

In addition to presenting specific antigens, DC must produce cytokines to induce polarization and differentiation of T lymphocytes. Initially we found that AP284 cells express significant levels of membrane TLR2 and TLR4 and intracellular TLR3, TLR7 and TLR9 (Fig. S2) what suggest that AP284 cells can respond to pathogen-associated molecular pattern PAMPs. To evaluate the cytokines produced by AP284 cells and BMDC, cells were incubated with different TLR agonists at different concentrations for 24 h. In Fig. 2 we observed that CpG (TLR9 agonist) was not able to induce significant production of IL-12p40 in our experiments. LPS (TLR4 agonist), Zymosan (TLR2 agonist), FSL-1 (TLR2/TLR6 agonist), resiquimod (TLR7/TLR8 agonist) and flagellin (TLR5 agonist) were able to induce a surprising production of IL- 12p40 (above 500 ng/mL). Except for flagellin, the same agonists also induced significant production of IL-12p40 in BMDC, but in lower amounts (below 10 ng/mL). The IL-12p40 subunit together with the IL-12p35 subunit forms the active IL-12p70 which is important for induction of a Th1 response profile [33]. Interestingly, AP284 cells were not able to produce IL-12p70 with any stimuli used. In contrast, BMDC produced IL-12p70 after stimulation with LPS, Zymozan, FSL-1 and Resiquimod (Fig. S3A-J). As IFN-γ induces IL-12p35 subunit expression and favors the production of IL-12p70 [33], AP284 and BMDC were primed for 2 h with IFN-γ and subsequently stimulated with LPS or *Escherichia coli*. The whole pathogen *E. coli* was used because it could bind to a higher number of PPRs and consequently increase the possibility for cytokine production. Surprisingly, the priming with IFN-γ followed by LPS or intact *E. coli* stimulation inhibited the IL-12p40 production by AP284 cells and was not able to favor the IL-12p70 production (Fig. S4A and B). In contrast, the priming of BMDC with IFN-γ stimulated the production of IL-12p40 and IL-12p70 (Fig. S4D and E). Inhibition of IL-12p40 production in AP284 cells by IFN-γ was not due to cell death (analyzed by MTT metabolism), high production of NO or TNF, since AP284 cells remained alive after stimulation with IFN-γ (Fig. S5); NO production was similar for AP284 and BMDC cells (Fig S6) and TNF production was also inhibited in AP284 after the addition of IFN-γ (Fig. S7).

3.3. AP284 cells produce IL-23 and IL-6 after stimulation with the different TLR agonists

The IL-23 is also a heterodimeric cytokine sharing the IL-12p40 subunit with IL-12p70. As AP284 cells can produce large amounts of IL-12p40 but not IL-12p70, we evaluated whether IL-12p40 produced after stimulation with different TLR agonists was related to IL-23 synthesis.

Similar to what was observed for IL-12p40, we observed that stimulation with LPS, zymozan, FSL-1 and resiquimod was able to induce significant production of IL-23 by AP284 cells (Fig. 3). FSL-1 was the more efficient TLR agonist to induce expression of IL-23 by AP284, reaching a production of around 10 ng/mL (Fig. 3C) and CpG did not induce the production of IL-23 (Fig. 3E). These data demonstrate that part of IL-12p40 produced by AP284 cells is being used for IL-23 production. Unlike AP284 cells, the IL-23 produced by BMDC did not reach statistical significance for any of stimuli used even after priming with IFN-γ (Fig. 3G-L and Fig. S4F). Similar to what was observed for the production of IL-12p40, the priming with IFN-γ also inhibited the IL-23 production in AP284 cells (Fig. S4A and C).

Since IL-23 is a cytokine related to the polarization and differentiation of Th17 cells, and AP284 cells are able to produce IL-23 but not IL-12p70, we investigated the ability of AP284 cells to produce IL-6, another key cytokine related to Th17 generation. Both AP284 and BMDC produced high levels of IL-6 after stimulation with all TLR agonists (Fig. 4A–K), and the amounts of IL-6 produced by both cell types are similar. These data suggest that AP284 cells might have more potential to generate Th17- rather than Th1 cells.

3.4. AP284 cells restimulate Th17 cells ex vivo but not a Th1 cells.

In order to evaluate which T cell profile the AP284 cells are able to enforce, C57BL/6 mice were immunized repeatedly with CFA. After 21 days the spleen was removed and non-adherent cells, representing

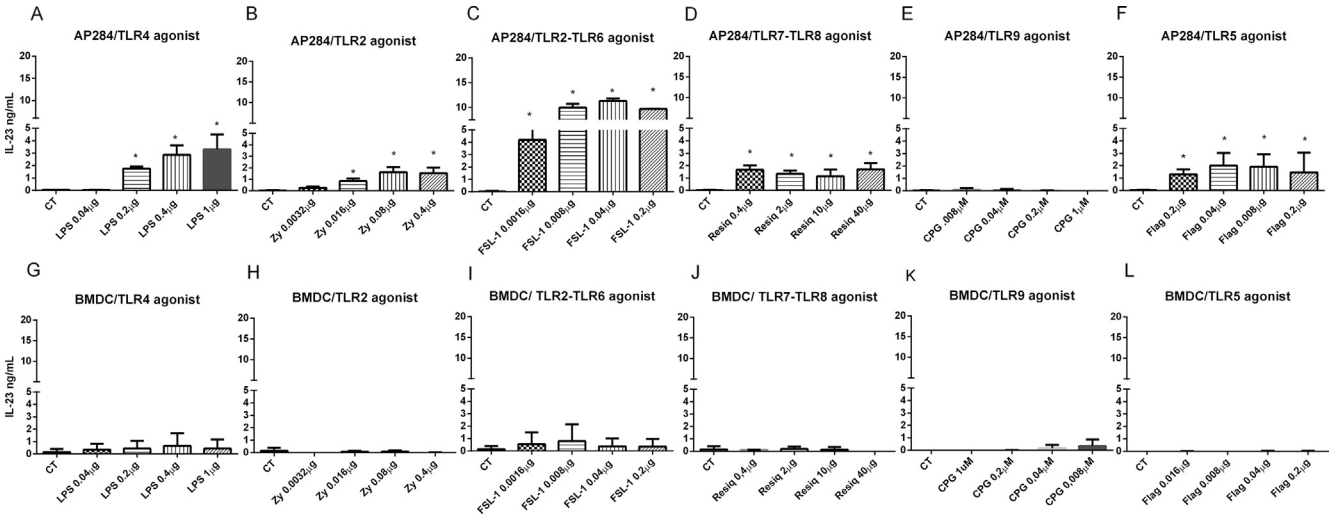

Fig. 3. AP284 cells produce IL-23 after stimulation with TLR agonists. AP284 cells (A-F) and BMDC (G-L) were stimulated with TLRs agonists lipopolysaccharide (LPS); zymosan (Zy); Pam2CGDPKHPKSF (FSL-1); resiquimod (resiq) CpG-ODN (CPG) and flagellin (flag) at different concentrations for 24 h and production of IL-23 was evaluated by ELISA. The bars represent the mean ± SD of cytokine production from three independent experiments. * indicates statistical difference between unstimulated (CT) and cells stimulated with TLR agonists by one-way ANOVA followed by Fisher's ($p < 0.05$).

primarily lymphocytes, were cultured with AP284 or BMDC in presence or absence of BCG for 72 h, and the cytokines IL-17, IFN-γ and IL-10 were assayed by ELISA (Fig. 5). Both AP284 and BMDC were able to increase the production of IL-17 by non-adherent spleen cells from CFA-immunized mice when BCG was added in culture (Fig. 5A and D). In contrast, only BMDC were able to increase the production of IFN-γ by CFA-immunized non-adherent spleen cells, while AP284 cells significantly inhibited IFN-γ production (Fig. 5B and E). In addition, both AP284 cells and BMDC failed to increase IL-10 production by immunized spleen cells above the spontaneous level of production (Fig. 5C and F).

4. Discussion

In this study we identified the cell line AP284, formerly characterized as macrophage [28], as a dendritic cell line with unique antigen-presenting features. Our new data show that AP284 cells share more phenotypic characteristics with myeloid DC than with macrophages. It was also previously described that AP284 cells were able to capture antigens and present them to T lymphocyte clones, inducing their proliferation, in accordance with the notion that this is a dendritic cell line [28]. Activation of naive $CD4^+$ T lymphocytes depends on the expression of MHC class II molecules and co-stimulatory molecules by mature DC such as CD40, CD80 and CD86 [3], and we show that AP284 cells express MHC class II, CD40 and CD80 molecules, indicating that they have characteristics of mature DC. This contrasts with the phenotype of the RAW264 macrophage cell line, which only showed CD40 expression.

The origin of this cell line from mouse spleen can be the reason for the expression of the DC markers 33D1 and CD11c. The 33D1 marker was reported in a large population of myeloid DC from spleen that are $33D1^+CD11b^{int}$ [34] and preferentially stimulate $CD4^+$ T cells based upon their unique migratory profile in the T cell zone of the white pulp [35].

To recognize and present pathogen antigens, DC must express PRRs that bind to PAMPs [36], which is crucial for activation and maturation

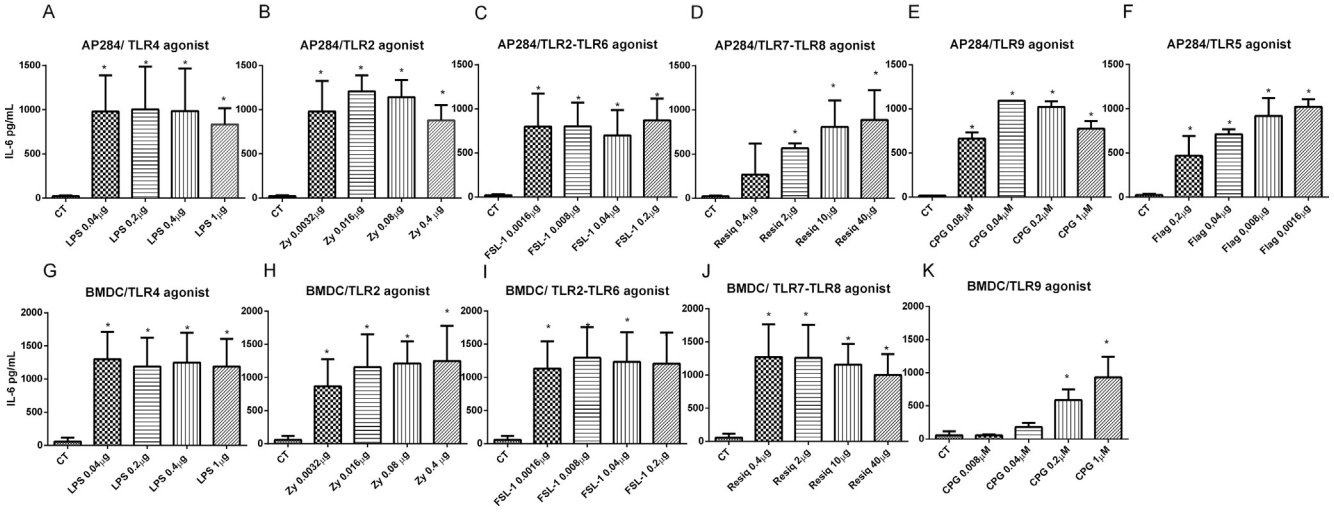

Fig. 4. AP284 cells produce IL-6 after stimulation with TLRs agonists. AP284 cells (A-F) and BMDC (G-K) were stimulated with TLRs agonists lipopolysaccharide (LPS); zymosan (Zy); Pam2CGDPKHPKSF (FSL-1); resiquimod (resiq) CpG-ODN (CPG) and flagellin (flag) at different concentrations for 24 h and production of IL-6 was evaluated by ELISA. The bars represent mean ± SD of three independent experiments. * indicates statistical difference between unstimulated (CT) and cells stimulated with TLR agonists by one-way ANOVA followed by Fisher's ($p < 0.05$).

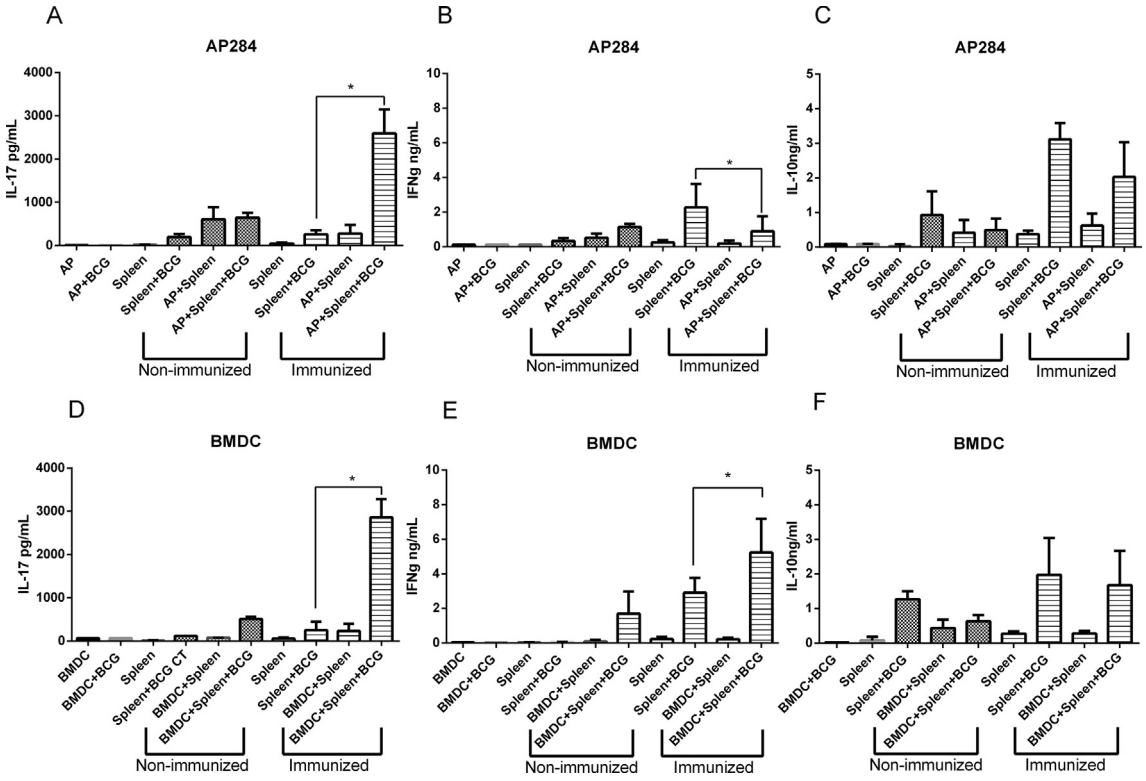

Fig. 5. AP284 cells increase production of IL-17 by CFA-immunized spleen cells. Spleen cells from C57BL/6 mice immunized with CFA or spleen cells from un-immunized mice were stimulated *in vitro* with gamma-irradiated BCG for 72 h in presence or absence of AP284 or BMDC. Production of IL-17, IFN-γ and IL-10 was evaluated in culture supernatant by ELISA. The bars represent mean ± SD of three independent experiments. * indicates statistical difference between unstimulated (CT) and cells stimulated one-way ANOVA followed by Fisher's ($p < 0.05$).

of DC [37]. AP284 cells express high levels of extracellular TLR2 and TLR4 and intracellular TLR3, TLR7 and TLR9 showing that they can recognize different group of pathogens from extracellular fungi and bacteria to intracellular virus. The ability of AP284 cells to respond to PAMPs was confirmed by the cytokines secreted when TLR agonists were used. The profile of cytokines produced by DC is crucial to define the fate of activated T lymphocytes. Among these, the group of cytokines belonging to the IL-12 family deserves special attention due to their action in the skewing and maintenance of major profiles of Th cells [12]. IL-12p70 and IL-23 are members of the IL-12 family sharing the IL-12p40 subunit, and are secreted primarily by activated DC. [12]. It was shown before that murine BMDC are able to produce significant amounts of IL-12p40 when stimulated with LPS, resiquimod, CpG, zymosan and FSL-1, a finding that we could confirm here [38–41]. IL-12p40 is generally produced in excess when compared to the IL-12p70 heterodimer, and free IL-12p40 can be produced 10 to 1000 times more than IL-12p35 [42,43]. Interestingly, AP284 cells produce 10–100 times more IL-12p40 than BMDC do, but were unable to produce IL-12p70, even after IFN-γ stimulation. It is important to emphasize that some biological functions have been identified for the IL-12p40 subunit, such as the induction of NO production by mouse microglial cells [31] and inhibition of the expression of FOXP3 in murine spleen T cells, indicating a role in Treg-mediated suppression [32]. However, we did not observe alterations in the production of NO by stimulated AP284 cells or IL-10 when AP284 cells were used to stimulate BCG-immunized spleen cells. The explanation for this remarkably strong production of IL-12p40 by AP284 cells is a matter for further study and several mechanisms can be involved. This regulation may involve proteins such as p38 or microRNA such as miR-128 that modulate the expression of IL-12p40 [44].

We were unable to explain the absence of IL-12p70 production by AP284 cells. It was suggested before that a second stimulus, such as IFN-γ, is necessary for IL-12p70 production by DC [45]. IFN-γ participates in transcriptional activation of the IL-12p35 subunit and favors IL-12p70 production [46]. In the present study we found that BMDC produce IL-12p70 independent of IFN-γ, as was also demonstrated by others [40,41,44]. Additionally, IFN-γ increased IL-12p70 production by BMDC, but not by AP284 cells. Our data suggest that the excessive production of IL-12p40 may be associated with the production of the p19 subunit, forming active IL-23. Alternatively, IL-12p40 may associate in a homodimer form to generate IL-12p80 that binds to the IL-12 receptor, but has an antagonistic function [47].

While the production of IL-12p70 by DC is important for the generation of a Th1 profile, the cytokines TGF-β and IL-6 are required for differentiation of Th17 cells. IL-23 is critical for stabilization and maintenance of this response [10]. Except for CPG, all TLR stimuli used were able to induce significant production of IL-6 and IL-23 by AP284. Together, these results indicate that AP284 cells are DC involved in amplification of a Th17 profile.

Studies by Wang and He [48] showed that immunization of mice with CFA for three weeks induced a mixed Th17- and Th1 profile with IL-17 and IFN-γ production by spleen cells. Because BCG-stimulated AP284 cells produce IL-12p40 and IL-23 *in vitro* (data not shown), we decide to evaluate which profile of T lymphocytes were preferentially restimulated when CFA-primed spleen cells are cultured in presence of AP284 cells. We used non-adherent spleen cells from CFA-immunized mice as responders to BCG processed and presented by AP284 or BMDC. While BMDC supported both IFN-γ and IL-17 production, AP284 cells were able to induce IL-17 production, but not IFN-γ production under these same conditions. This is in agreement with our previous data, which showed that AP284 cells are not able to produce IL-12p70, which is the cytokine critical for the generation of the Th1 response. In this model, both AP284 cells and BMDC were not able to increase IL-10 production beyond the level produced spontaneously by non-adherent

splenocytes from immunized mice.

In our experiments, we observed a similar production of IL-17 by CFA-primed spleen cells cultured with AP284 or BMDC. Beyond IL-23, IL-6, TGF-β and IL-1 are important cytokines able to stimulate the production of IL-17 and generate Th17 cells. We did not detect significant IL-23 production by *in vitro* stimulated BMDC. However, BMDC and AP284 produce similar amount of IL-6. Additionally, production of IL-1 and TGF- β was not evaluated. Differences in the production of IL-1 and TGF- β would justify the similar production of IL-17 in CFA-primed spleen cells.

Here, we did not evaluate whether IL17 and IFN-γ came from different lymphocytes or from a Th17.1 population. However, it is possible that BMDC may stimulate Th17.1, while AP284 only stimulate Th17 cells that might even become more suppressive than pro-inflammatory [49].

Taken together, our data support the idea that AP284 cells are a new DC line associated with the Th17 rather than Th1 type response. Additionally this cell line can be an important tool to improve the knowledge about factors and cellular interactions responsible for the induction of IL-12p40 and IL-23 and could be used to clarify the molecular pathways involved in the production of these cytokines. We also suggest that AP284 cells may be useful for the testing of new inhibitory or stimulatory drugs of IL-12p40 and IL-23.

5. Conclusion

In summary, our data provide evidence that AP284 cells constitute a newly identified DC line harboring several TLRs and producing surprising amount of IL-12p40. The IL-12p40 production is related to IL-23 formation but not IL-12p70. AP284 cells are also able to produce IL-6 and favor development of a Th17 profile. We believe that this new DC line may be useful for a better understanding of factors and cellular interactions responsible for the induction of IL-12p40 and IL-23 and test new drugs that may potentially inhibit or stimulate these cytokines.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

We are grateful to Dr. Daniel Silva Goulard, Ms Marco Vitor Silva de Melo and Iraci Maria Cardoso for animal care.

Funding

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de Goiás (FAPEG). Milton Adriano Pelli de Oliveira and Fátima Ribeiro Dias are fellows from (CNPq) and Instituto Nacional de Ciência e Tecnologia da Interação Patógeno Hospedeiro (INCT-IPH)/Brazil.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.02.003.

References

[1] R.M. Steinman, Z.A. Cohn, Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution, J. Exp. Med. 137 (1973) 1142–1162.
[2] M. Chopin, R.S. Allan, G.T. Belz, Transcriptional regulation of dendritic cell diversity, Front. Immunol. 3 (2012) 26.
[3] K. Shortman, S.H. Naik, Steady-state and inflammatory dendritic-cell development, Nat. Rev. Immunol. 7 (2007) 19–30.
[4] S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, Nat. Immunol. 2 (2001) 675–680.
[5] H. Hemmi, T. Kaisho, K. Takeda, S. Akira, The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets, J. Immunol. 170 (2003) 3059–3064.
[6] R.N. Germain, E.A. Robey, M.D. Cahalan, A decade of imaging cellular motility and interaction dynamics in the immune system, Science 336 (2012) 1676–1681.
[7] R.V. Luckheeram, R. Zhou, A.D. Verma, B. Xia, CD4(+)T cells: differentiation and functions, Clin. Dev. Immunol. 2012 (2012) 925135.
[8] H. Yamane, W.E. Paul, Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets, Immunol. Rev. 252 (2013) 12–23.
[9] R. Goswami, M.H. Kaplan, A brief history of IL-9, J. Immunol. 186 (2011) 3283–3288.
[10] E. Bettelli, Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, V.K. Kuchroo, Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, Nature 441 (2006) 235–238.
[11] S. Sakaguchi, T. Yamaguchi, T. Nomura, M. Ono, Regulatory T cells and immune tolerance, Cell 133 (2008) 775–787.
[12] D.A. Vignali, V.K. Kuchroo, IL-12 family cytokines: immunological playmakers, Nat. Immunol. 13 (2012) 722–728.
[13] G. Trinchieri, Cancer and inflammation: an old intuition with rapidly evolving new concepts, Annu. Rev. Immunol. 30 (2012) 677–706.
[14] C.L. Langrish, B.S. McKenzie, N.J. Wilson, R. de Waal Malefyt, R.A. Kastelein, D.J. Cua, IL-12 and IL- 23: master regulators of innate and adaptive immunity, Immunol. Rev. 202 (2004) 96–105.
[15] A.D. Garg, P.G. Coulie, B.J. Van den Eynde, P. Agostinis, Integrating next-generation dendritic cell vaccines into the current cancer immunotherapy landscape, Trends Immunol. 38 (2017) 577–593.
[16] K.M. Hargadon, Murine and human model systems for the study of dendritic cell immunobiology, Int. Rev. Immunol. 35 (2016) 85–115.
[17] P. Paglia, G. Girolomoni, F. Robbiati, F. Granucci, P. Ricciardi-Castagnoli, Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses in vivo, J. Exp. Med. 178 (1993) 1893–1901.
[18] F. Granucci, G. Girolomoni, M.B. Lutz, M. Foti, G. Marconi, P. Gnocchi, L. Nolli, P. Ricciardi-Castagnoli, Modulation of cytokine expression in mouse dendritic cell clones, Eur. J. Immunol. 24 (1994) 2522–2526.
[19] G. Girolomoni, M.B. Lutz, S. Pastore, C.U. Assmann, A. Cavani, P. Ricciardi-Castagnoli, Establishment of a cell line with features of early dendritic cell precursors from fetal mouse skin, Eur. J. Immunol. 25 (1995) 2163–2169.
[20] Z. Shen, G. Reznikoff, G. Dranoff, K.L. Rock, Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules, J. Immunol. 158 (1997) 2723–2730.
[21] K.M. Hargadon, Tumor-altered dendritic cell function: implications for anti-tumor immunity, Front. Immunol. 4 (2013) 192.
[22] M. Brown, Y. Zhang, S. Dermine, E.A. de Wynter, C. Hart, H. Kitchener, P.L. Stern, M.A. Skinner, S.N. Stacey, Dendritic cells infected with recombinant fowlpox virus vectors are potent and long-acting stimulators of transgene-specific class I restricted T lymphocyte activity, Gene Ther. 7 (2000) 1680–1689.
[23] A. Rhule, B. Rase, J.R. Smith, D.M. Shepherd, Toll-like receptor ligand-induced activation of murine DC2.4 cells is attenuated by Panax notoginseng, J. Ethnopharmacol. 116 (2008) 179–186.
[24] S. Ebihara, S. Endo, K. Ito, Y. Ito, K. Akiyama, M. Obinata, T. Takai, Immortalized dendritic cell line with efficient cross-priming ability established from transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene, J. Biochem. 136 (2004) 321–328.
[25] S. Ruiz, C. Beauvillain, M.N. Mevelec, P. Roingeard, P. Breton, D. Bout, I. Dimier-Poisson, A novel CD4-CD8alpha + CD205 + CD11b- murine spleen dendritic cell line: establishment, characterization and functional analysis in a model of vaccination to toxoplasmosis, Cell. Microbiol. 7 (2005) 1659–1671.
[26] A. Mortellaro, M. Urbano, S. Citterio, M. Foti, F. Granucci, P. Ricciardi-Castagnoli, Generation of murine growth factor-dependent long-term dendritic cell lines to investigate host-parasite interactions, Methods Mol. Biol. 531 (2009) 17–27.
[27] C. Winzler, P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V.S. Zimmermann, J. Davoust, P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures, J. Exp. Med. 185 (1997) 317–328.
[28] I.S. Klasen, J.P. de Jong, J.S. Voerman, R.M. Ladestein, P.J. Leenen, R. Benner, Murine macrophage cell line AP284 presents antigen to cloned MT4 +, Lyt-2- T cells in vitro and in vivo, Immunobiology 178 (1988) 261–274.
[29] K. Inaba, M. Inaba, M. Naito, R.M. Steinman, Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo, J. Exp. Med. 178 (1993) 479–488.
[30] E. Neumann, M.A. Oliveira, C.M. Cabral, L.N. Moura, J.R. Nicoli, E.C. Vieira, D.C. Cara, G.I. Podoprigora, L.Q. Vieira, Monoassociation with Lactobacillus acidophilus UFV-H2b20 stimulates the immune defense mechanisms of germfree mice, Braz. J. Med. Biol. Res. 31 (1998) 1565–1573.
[31] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[32] A. Dutta, S. Bandyopadhyay, C. Mandal, M. Chatterjee, Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis, Parasitol. Int. 54 (2005) 119–122.
[33] G. Trinchieri, Interleukin-12 and the regulation of innate resistance and adaptive immunity, Nat. Rev. Immunol. 3 (2003) 133–146.

[34] J.M. Stolley, D.J. Campbell, A 33D1+ dendritic cell/autoreactive CD4+ T cell circuit maintains IL-2-dependent regulatory T Cells in the spleen, J. Immunol. 197 (2016) 2635–2645.
[35] S. Calabro, D. Liu, A. Gallman, M.S. Nascimento, Z. Yu, T.T. Zhang, P. Chen, B. Zhang, L. Xu, U. Gowthaman, J.K. Krishnaswamy, A.M. Haberman, A. Williams, S.C. Eisenbarth, Differential intrasplenic migration of dendritic cell subsets tailors adaptive immunity, Cell Rep. 16 (2016) 2472–2485.
[36] M. Matsumoto, Y. Takeda, M. Tatematsu, T. Seya, Toll-like receptor 3 signal in dendritic cells benefits cancer immunotherapy, Front. Immunol. 8 (2017) 1897.
[37] M.J. Jimenez-Dalmaroni, M.E. Gerswhin, I.E. Adamopoulos, The critical role of toll-like receptors–from microbial recognition to autoimmunity: a comprehensive review, Autoimmun. Rev. 15 (2016) 1–8.
[38] C. Ma, M. Muranyi, C.H. Chu, J. Zhang, W.M. Chu, Involvement of DNA-PKcs in the IL-6 and IL-12 response to CpG-ODN is mediated by its interaction with TRAF6 in dendritic cells, PLoS One 8 (2013) e58072.
[39] E.C. Slack, M.J. Robinson, P. Hernanz-Falcon, G.D. Brown, D.L. Williams, E. Schweighoffer, V.L. Tybulewicz, C., Reis e Sousa, Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan, Eur. J. Immunol. 37 (2007) 1600–1612.
[40] K. Kiura, H. Kataoka, T. Nakata, T. Into, M. Yasuda, S. Akira, N. Inoue, K. Shibata, The synthetic analogue of mycoplasmal lipoprotein FSL-1 induces dendritic cell maturation through Toll-like receptor 2, FEMS Immunol. Med. Microbiol. 46 (2006) 78–84.
[41] S. Kasahara, E.A. Clark, Dendritic cell-associated lectin 2 (DCAL2) defines a distinct CD8alpha- dendritic cell subset, J. Leukoc. Biol. 91 (2012) 437–448.
[42] G. Trinchieri, S. Pflanz, R.A. Kastelein, The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses, Immunity 19 (2003) 641–644.
[43] G. Trinchieri, Interleukin-12: a cytokine at the interface of inflammation and immunity, Adv. Immunol. 70 (1998) 83–243.
[44] X. Liang, W. Shangguan, M. Zhang, S. Mei, L. Wang, R. Yang, miR-128 enhances dendritic cell-mediated anti-tumor immunity via targeting of p38, Mol. Med. Rep. 16 (2017) 1307–1313.
[45] R.E. Roses, S. Xu, M. Xu, U. Koldovsky, G. Koski, B.J. Czerniecki, Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists, J. Immunol. 181 (2008) 5120–5127.
[46] J. Liu, X. Guan, T. Tamura, K. Ozato, X. Ma, Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein, J. Biol. Chem. 279 (2004) 55609–55617.
[47] C. Holscher, R.A. Atkinson, B. Arendse, N. Brown, E. Myburgh, G. Alber, F. Brombacher, A protective and agonistic function of IL-12p40 in mycobacterial infection, J. Immunol. 167 (2001) 6957–6966.
[48] H.P. Wang, Z.G. He, Treatment with incomplete Freund's adjuvant and Listeria monocytogenes delays diabetes via an interleukin-17-secretion-independent pathway, Exp. Ther. Med. 9 (2015) 1934–1938.
[49] N. Gagliani, M.C. Amezcua Vesely, A. Iseppon, L. Brockmann, H. Xu, N.W. Palm, M.R. de Zoete, P. Licona-Limon, R.S. Paiva, T. Ching, C. Weaver, X. Zi, X. Pan, R. Fan, L.X. Garmire, M.J. Cotton, Y. Drier, B. Bernstein, J. Geginat, B. Stockinger, E. Esplugues, S. Huber, R.A. Flavell, Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation, Nature 523 (2015) 221–225.