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Generation of Stable Th1/CTL-, Th2-, and Th17-Inducing Human Dendritic Cells

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Abstract

Dendritic cells (DC) are the most potent inducers and regulators of immune responses, responsible for communication within immune system. The ability of DC to act both as the inducers of immune responses and as regulatory/suppressive cells led to the interest in their immunotherapeutic use in different disease types, ranging from cancer to autoimmunity, and as a tool to prevent the rejection of transplanted tissues and organs. Over the last years, several groups including ours have demonstrated the feasibility of obtaining monocyte-derived DC with different functions, by modulating the conditions and the duration of DC maturation. The current chapter provides a detailed protocol of generating type-1-, type-2-, and type-17-polarized DC for testing the cytokine-producing abilities of these cells and their effectiveness in inducing Th1, Th2, and Th17 responses of $CD4^+$ T cells and CTL responses of naïve and memory $CD8^+$ T cells.

Keywords

Dendritic cells; Th cells; CTLs; vaccines; cancer

1. Introduction

Dendritic cells (DC) are the most potent inducers and regulators of immune responses, responsible for intercellular communication between other immune cells. They act as sentinel cells in the peripheral tissues, being key to the development of effective immune responses to the pathogens residing in different cellular compartments and susceptible to different immune mechanisms (1–8). In line with their central role in pathogen control, DC dysfunction has been implicated in the pathogenesis and progression of a wide range of disease conditions, ranging from autoimmunity to chronic infections and cancer, with multiple pathogens developing ways to interfere with DC functions as a mean to avoid eradication by the immune system (3,9–15).

Both the efficiency of DC as an effective element of immune system and their susceptibility to pathogen-induced dysfunction result from an enormous plasticity of the DC system (1,7,8). Distinct DC subsets or DC developing or maturing in different conditions show striking functional differences (1,2,5–8,16–19). One aspect of DC function that is a subject to strict regulation is their ability to induce such effector immune cells as Th1-, Th2-, or Th17-type $CD4^+$ Th cells or cytotoxic $CD8^+$ T cells (CTLs) (1,7,8) as opposed to regulatory T(reg) cells (20–25).

In contrast to the inhibitory Tregs, all the above effector T-cell types have been shown to provide essential elements of protection against different classes of pathogens and have been implicated in different forms of autoimmunity. Th1-type $CD4^+$ T cells (key producers of IFN-γ and lymphotoxin) and $CD8^+$ CTLs (main type of antigen-specific killer cells) are generally considered as the effector cells key to our ability to effectively fight intracellular bacteria and viruses, as well as to eliminate tumor cells. In addition, Th1 cells provide

support for the production of several immunoglobulin classes by B cells. Th2 cells, producing mainly IL-4 and IL-5, are an essential component of our defenses against intestinal parasites and contribute to the majority of antibody production. The more recently discovered IL-17-producing Th cells (Th17 cells) are required for the protection against certain bacteria. Moreover, Th17 cells have been implicated to play a role in the development and/or maintenance of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and colitis (26–40).

The ability of DC to act both as the inducers of immune responses and as regulatory/suppressive cells led to the interest in their immunotherapeutic use in different disease types, ranging from cancer to autoimmunity, and as a tool to prevent the rejection of transplanted tissues and organs (24,41–50). Taking into account the plasticity of DC and their ability to adopt different functions, it is important to match the desired type of the DC to the type of their clinical or laboratory application.

Over the last years, we and multiple other groups demonstrated the feasibility of obtaining monocyte-derived DC with different functions, by modulating the conditions of their early development (51,52), the conditions of their maturation (53–60), or the length of DC maturation period (54,61). The current chapter provides a detailed protocol of generating type-1-, type-2-, and type-17-polarized DC, the protocols used to test the cytokine-producing capacity of these cells, and their ability to induce Th1, Th2, and Th17 responses of $CD4^+$ Th cells as well as the CTL responses of naïve and memory $CD8^+$ T cells.

2. Materials

2.1. Isolation of Peripheral Blood Monocytes and $CD45RA^+$ Naïve $CD4^+$ and $CD8^+$ T cells

1. Vacutainer blood collection tubes (sodium heparin; Becton-Dickinson, Franklin Lakes, NJ, USA).
2. 50-ml Polypropylene tubes.
3. 10-ml Polypropylene tubes.
4. Lymphocyte separation medium (CellGro/Mediatech, Manassas, VA, USA) (d = 1.077).
5. Percoll (Sigma) is aliquoted (30 ml) and stored at 4°C.
6. 10 X Concentrated “acidic” (pH 4.6, 1.051 g/ml) PBS: 13.5 g NaCl, 0.1 g Na_2HPO_4 (corresponding to 0.125 g of $Na_2HPO_4{\cdot}2H_2O$), 2.1 g KH_2PO_4, 200 ml distilled water. This PBS solution is sterilized by 0.22-μm filtration and stored at 4°C in 4-ml aliquots.
7. Medium for Percoll separation: IMDM (Gibco/Invitrogen, Grand Island, NY, USA) with 10% FCS (Hyclone, Logan, UT, USA) or serum-free media: AIM-V (Gibco) or Cell-Genix DC medium (CellGenix, Germany).
8. Medium for washing the cells: RPMI (Gibco/Invitrogen) with 2% FCS (Hyclone).
9. Isolation columns for human $CD4^+CD45RA^+$ naïve Th cells and $CD8^+CD45RA^+$ naïve Th cells and CTL precursors. We have been successfully using any of the three methods (a) CD4 (8)$^+$CD45R0$^-$ negative isolation columns from R&D, (b) customized StemSep system for the negative isolation of CD4 (8)$^+$CD45R0$^-$ cells (StemCell Technologies), or (c) positive selection using magnetic isolation columns from Miltenyi Biotech Gmbh. In this last method, naïve cells are isolated by the inclusion of additional CD45RO-depletion step applied prior to $CD4^+$ T-cell (or $CD8^+$ T cell) isolation.

2.2. Generation of Immature DC and Their Maturation in DC1, DC2, and DC17-Polarizing Conditions

1. Media for DC culture: (a) IMDM (BioWhittaker) with 10% FCS (Hyclone); (b) serum-free AIM-V medium (Gibco); (c) serum-free CellGenix DC medium (CellGenix, Germany).
2. Medium for washing the cells: 2% FCS/RPMI.
3. rhu GM-CSF (Schering-Plough; Kenilworth, NJ, USA).
4. rhuIL-4 (Strathmann Biotech/Miltenyi Gmbh, Germany).
5. rhuTNF-α (Strathmann/Miltenyi).
6. rhuIL-1β (Strathmann/Miltenyi).
7. IL-6 (Endogen, Woburn, MA, USA).
8. LPS (from *Escherichia coli* 011:B4; Sigma, St. Louis, MO, USA).
9. rhuIFN-γ (Strathmann/Miltenyi).
10. PGE_2 (Sigma, St. Louis, MO, USA).
11. Poly-I:C (Sigma).
12. IFN-α (IFN-α2b; Intron A; Schering-Plough).
13. Peptidoglycan (PGN; Invivogen, San Diego, CA, USA).

2.3. Analysis of Cytokine Production by Differentially Polarized DC

1. CD40L-transfected J558 cells were a kind gift from Dr Peter Lane (University of Birmingham, Birmingham, UK). They express high levels of mouse CD40L that binds both mouse and human CD40.
2. sCD40L (Alexis Biochemicals, San Diego, CA, USA).
3. Human $CD4^+$ Th cells (bulk population) used as IL-12 inducers are isolated as described in **Section 2.1, Item 9**.
4. SEB (Staphylococcal Enterotoxin B; Sigma or Toxin Technologies) is used as an Ag surrogate.

2.4. In Vitro Priming of $CD4^+CD45RA^+$ Naïve Th Cells with Polarized DC Subsets

1. SEB (Sigma or Toxin Technologies).
2. rhuIL-2 (10 U/ml; a gift of Cetus Corporation, Emeryville, CA, USA).
3. CD3 mAb (CLB-T3/3; CLB, Amsterdam, The Netherlands) plus CD28 mAb (CLB-CD28/1; CLB) or alternatively CD3/CD28 T-cell expander beads (Dynal AS, Oslo, Norway) were used to induce the cytokine production in differentially primed populations of Th cells.

2.5. In Vitro Priming of $CD8^+CD45RA^+$ CTL Precursors with Polarized DC Subsets

1. SEB (Sigma or Toxin Technologies).
2. rhuIL-2 (50 U/ml; a gift of Cetus Corporation, Emeryville, CA, USA).
3. IL-7 (10 ng/ml; PeproTech).

3. Methods

3.1. Isolation of Peripheral Blood Monocytes and $CD4^+CD45RA^+$ Naïve Th Cells

3.1.1. Collection of Peripheral Blood

1. Collect blood in heparinized tubes and dilute 1:1 with RPMI.

3.1.2. Isolation of PBMC

1. Overlay 30 ml of diluted blood over 15 ml of lymphocyte isolation medium in each 50-ml tube.
2. Centrifuge at 1,000 × *g* for 30 min, at room temperature (RT; 21°C). Acceleration: 1–1,000 × *g* should take 60 s. Deceleration: 5 min. Wash the cells twice at RT.

3.1.3. Isolation of the Light Fraction of PBMC on Percoll Gradient

1. Prepare standard isotonic Percoll solution (SIP) by mixing nine parts of Percoll with one part of 10X concentrated "acidic" PBS.
2. Prepare three dilutions of SIP (v/v) in 10% FCS/IMDM (*see* Notes 1–4):
   a. 60% SIP (9 ml)$^+$ 40% FCS/IMDM (6 ml)
   b. 48% SIP (9.6 ml)$^+$ 52% FCS/IMDM (10.4 ml)
   c. 34% SIP (3.4 ml)$^+$ 66% FCS/IMDM (6.6 ml)
3. Suspend PBMC (maximum 3×10^7 cells per ml) in 60% SIP. Layer 2–2.5 ml of cell suspension at the bottom of each of the 15-ml tube (maximum 7.5×10^7 cells/tube), overlay with 48% SIP (5 ml), and next with 34% SIP (2 ml).
4. Centrifugation: 2,400 × *g*, 45 min, at RT (21°C). Acceleration: 60 s. Deceleration: 5 min.
5. Harvest monocytes from the upper interphase (the inter-phase corresponding to 48% (or 45%) SIP and 34% SIP) and lymphocytes from the lower interphase (60% SIP and 48% (or 45%) SIP).
6. Wash the monocyte fraction three times and count the cells (*see* Note 5).

3.1.4. Adherence and Depletion of Non-adherent Cells

1. Seed the cells at 0.5×10^6 per ml per well in 24-well plate (or 2×10^6 in 4 ml in 6-well plate) and let them adhere for 45 min, 37°C, 5% CO_2 (*see* Note 6).
2. Remove non-adherent cells by washing the wells —two to three times with a gentle stream of medium. This step requires eye-control of the washing to assure high purity of monocytes and to prevent an excessive loss of the attached cells. Use washing medium at room temperature.

3.1.5. Isolation of $CD4^+CD45RA^+$ Naïve Th and $CD8^+$ $CD45RA^+$ Naïve T cells from Peripheral Blood

1. Harvest the lymphocytes from the heavy fraction of PBMC (*see* **Section 3.1.3**) and wash two times.
2. Isolate naïve Th cells ($CD4^+CD45RA^+$ cells) or naïve CTL precursors ($CD8^+CD45RA^+$ cells), by one of the negative selection systems (*see* **Section 2.1**) according to the manufacturers' instructions. Although rare subsets of pathogen-specific $CD8^+CD45RA^+$ T cells can contain effector cells, the overall polyclonal

population of "bulk" peripheral blood $CD8^+CD45RA^+$ T cells displays a uniform $CD62L^{high}/CCR7^+$ phenotype and functions characteristic of naïve $CD8^+$ T cells (58,62). Please note that the optimal generation of Th17 cells benefits from the use of "bulk" $CD4^+$ T cells or memory-enriched $CD4^+CD45R0^+$ T cells as the starting population (27) (*see* Note 24). The use of memory T-cell fraction or the use of bulk, unseparated $CD4^+$ or $CD8^+$ T cells is also recommended when inducing antigen-specific responses (60,63,64), since Ag-specific precursor cells are enriched in the memory cell population.

3. Freeze the isolated T cells until use.

3.2. DC Culture and Maturation

1. After the last wash of the monocytes, add fresh culture medium (IMDM/FCS; CellGenix or AIM-V), containing at least 500 U/ml GM-CSF and 250 U/ml IL-4 (1 ml per well; currently we use 1,000 U/ml of each of these cytokines).
2. On day 3 of the culture, remove 1/2 of medium and add the same amount of fresh medium with the double-concentrated growth factors. At this time-point, a portion of the cells are already non-adherent, so it is necessary to let them sediment for 10 min, resting the plate at a certain angle, supported at one side. Gently, to avoid taking up the cells, take up 0.5 ml of medium with a 1-ml pipette, from the lower side of each well. Add the new medium with double-concentrated GM-CSF and IL-4 (pre-warmed to room temperature) at the same spot, releasing the volume gently to reduce stirring up the cultures.
3. At day 6 (*see* Note 7), take out 1/2 of the spent medium and add new medium containing GM-CSF, double-concentrated maturation-inducing factors without or with a polarizing factor (see below). Within 2 days the expression of CD80, CD86, and CCR7, will increase, and the cells will lose the ability to re-adhere, after moving to another well (*see* Note 9). At the very early stage of maturation (6–12 h) the cells become $CD83^+$ and lose the expression of CD115.
    a. DC1-inducing cocktail applicable for serum-supplemented media (57):

       LPS (final conc. 250 ng/ml) plus IFN-γ (final conc. 1,000 U/ml).

       Maturation time: 42–48 h.

    b. αDC1-inducing cocktail (clinical-grade DC1-inducing cocktail effective both in serum-supplemented media and in serum-free CellGenix DC medium and in AIM-V) (60):

       Poly-I:C (final conc. 20 μg/ml), TNF-α (final conc. 50 ng/ml), IL-1β (final conc. 25 ng/ml), IFN-α (final conc. 3,000 U/ml), and IFN-γ (final conc. 1,000 U/ml).

       Maturation time: 42–48 h.

    c. DC2/standard(s) DC-inducing cytokine cocktail (clinical grade; all media) (60,65):

       TNF-α (final conc. 50 ng/ml), IL-1β (final conc. 25 ng/ml), IL-6 (final conc. 1,000 U/ml, and PGE_2(final conc. 1 μM).

    d. DC17-inducing conditions (26):

       PGN (final conc. 10 μg/ml).

For the optimal induction of Th17 cells, DC should be matured *only for 16 h*, rather than 42–48 h (26).

3.3. Analysis of Cytokine Production by Differentially Polarized DC

1. Harvest DC to polypropylene tubes and wash thoroughly to remove all the cytokines.
2. Plate the cells at 2×10^4 cells/well in flat-bottomed 96-well plates.
3. Add the cytokine-inducing stimulus. We normally use three types of CD40L-based stimuli: J558-CD40L (5×10^4 cells/well), soluble CD40L, and cross-linking kit (Alexis Biochemicals, San Diego, CA, USA), either alone or in combination with rhuIFN-γ (1,000 U/ml) or $CD4^+$ T cells (1×10^5 cells/well) in the presence of superantigen (SEB; 1 ng/ml). The induction of cytokine production is routinely performed in a final volume of 200 μl/well (*see* Note 10).
4. Following either of the first two modes of stimulation, we harvest 24-h supernatants, while the T-cell-dependent IL-12p70 induction requires a longer, 48 h stimulation (to allow T cells to elevate CD40L expression).

3.4. In Vitro Priming of $CD4^+$CD45RA Naïve Th Cells with Polarized DC Subsets

1. Harvest DC to polypropylene tubes and wash thoroughly to remove all the cytokines.
2. Plate the DCs in flat-bottomed 96-well or 48-well plates. Add SEB (1 ng/ml) and (after 1 h) T cells at 10:1 ratio (e.g., 2×10^3 DC and 2×10^4 T cells in 200 μl or 5×10^4 DC and 5×10^5 T cells in 500 μl). For the optimal induction of Th17 cells, the concentration of SEB may be reduced to 100 pg/ml (26).
3. At day 5, add rhuIL-2 (final conc. 10 U/ml).
4. Starting from this point onward the cells proliferate rapidly over the period of next 4–6 days. The next day after the IL-2 addition, the cells usually need to be transferred to 1 ml wells. Subsequently, every 1–3 days, each well needs to be divided into —two to three wells. At this point, the optimal culture density for the expansion of Th cells is $1.5–3 \times 10^6$ cells per well (1 ml). The cultures reach quiescence about days 9–12 and need to be restimulated (*see* Note 11).
5. At 10–14 days after priming, induce the cytokine production in Th cells by their restimulation for 24 h with CD3 mAb (1 μg/ml; CLB-T3/3; CLB) plus CD28 mAb (1 μg/ml; CLB-CD28/1; CLB), or CD3/28-coated T-cell-activating beads. The levels of IFN-γ, IL-4, and IL-5 in 24 h supernatants can be then analyzed by specific ELISAs. Alternatively, the differentially primed Th cells can be restimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 6 h, the last 4 h in the presence of Brefeldin A (10 μg/ml) and the intracellular expression of IFN-γ, IL-4, and IL-17 is determined following cell permeabilization using saponin and cytokine-specific staining using αIL-17 abs (R&D), αIFN-γ Abs (Pharmingen), and αIL-4 Abs (Pharmingen).

3.5. In Vitro Induction of Peptide-Specific CTLs

1. Harvest DCs (e.g., αDC1 or sDC) to polypropylene tubes (to limit adherence) and wash thoroughly to remove all the cytokines.
2. Plate DC at 5×10^4 cells/well in flat-bottomed 48-well plates in 10% HS/IMDM. Add SEB (1 ng/ml) or antigenic peptide(s) (at 1–10 μM), $CD8^+$ T cells (5×10^5/well; *see* **Section 3.1** for the isolation procedure; depending on application naïve or

bulk CD8+ T cells may be used). As an option, 3000R-irradiated J558-CD40L cells (5×10^4/well, as a surrogate of CD40L-expressing Th cells *may* be added; *see* **Comment 23**). The addition of CD40L was originally used in our protocols (60) to assure that the differences in the magnitude and quality of the CTL responses induced by polarized DC1 and non-polarizing sDC cannot be overcome by the presence of Th cell-related signals. However, our recent studies demonstrated that similar differences can be observed in the absence or presence of CD40L (62,64).

3. At days 3–4, add rhuIL-2 (final conc. 50 U/ml) and IL-7 (10 ng/ml).

4. The proliferation of cells is significantly less pronounced than in the SEB model ($CD4^+$ T cells). The cells usually need to be fed with 50% of fresh IL-2-containing medium every 3 days and transferred to 1-ml wells at about day 7. The cultures reach quiescence about days 12–14 and need to be restimulated.

5. At days 12–14 after priming, the cells are restimulated with peptide-pulsed autologous PBMC (at 1:1 ratio) or with peptide-loaded Th2 cells (at 2:1 ratio) *(peptide-pulsing is important at this stage: do not add peptide directly to CTL cultures to prevent CTL fratricide)* and expanded for another 12–14 days. This restimulation step allows to demonstrate the stability of the DC-induced differences in CTL activity and facilitates ELISPOT analysis of Ag-specific responses, by reducing the non-specific background (LAK activity; significant in $CD8^+$ T cells recently stimulated by αDC1s; especially in the presence of CD40L). At days 24–28 (10–14 days after secondary stimulation), the frequency of Ag-specific T cells is analyzed by IFN-γ ELISPOT. This secondary stimulation step can be omitted, allowing to compare the CTL induction already at days 12–14.

4. Notes

Our serum-supplemented conditions of DC culture involve FCS, rather than human serum, since DC obtained in the presence of human serum do not express CD1a and show a relative resistance to maturation. FCS/IMDM-based media allow the generation of type-1-polarized DC (DC1), using a combination of TNF-α and IFN-γ (or LPS and IFN-γ). In contrast, the generation of fully mature DC1 in serum-free media (such as AIM-V or CellGenix) requires the addition of IFN-α and poly-I:C (αDC1, Ref. (60).

The SEB-based model of naïve Th cell priming was first described in Ref. (51). It is based on the ability of SEB to activate a substantial proportion of naïve T cells (66,67). This allows to use it as a substitute of the TCR-transgenic models that are not available in the human system. In contrast, the traditional allogeneic MLR model does not allow to induce any detectable amounts of IL-12 within the first 3 days of DC–Th cell interaction, most likely due to 100–1,000-fold lower frequency of responsive T cells. The possible applications and the typical results obtained with use of the described protocols can be found in our previous publications (51,53,54,57–[59],62,64,68–72).

Based on the past experiences on introducing the described protocol in other labs, we would like to draw your attention to the following issues critical for its outcome.

1. Monocytes isolated from fresh blood give better results than monocytes isolated from buffy coats that often yield a lower percentage of $CD1a^+$ cells. In addition, DC generated from buffy coat-isolated monocytes frequently show signs of partial maturation (loss of CD115) and tend to produce lower amounts of IL-12p70. They are also less susceptible to polarization. The reason(s) for these differences is not completely clear to us, but the quality of DC appears to inversely correlate with the level of platelet contamination that is substantially higher in case of the monocytes isolated from buffy coat, compared to fresh blood.

2. Isolation of monocytes should be performed at room temperature. Rapid changes of temperature increase the risk of monocyte activation and clumping. We advise the use of polypropylene tubes to reduce cell attachment.

3. We also recommend the use of heparin as anticoagulant to avoid activation of monocytes in the course of decalcification/recalcification. Use Ca^{++}/Mg^{++}-containing media at all stages of the monocyte isolation.

4. 48% layer of SIP is designed for freshly drawn blood. A lower-density layer of SIP (45%) should be used for the isolation of monocytes from buffy coats.

5. At this stage, the monocytes should be 80–90% pure (judged by CD14 expression). Higher contamination with $CD14^{-}$ cells indicates the need to reduce the concentration of SIP in the middle layer.

6. Do not exceed the starting cell density of 0.5×10^6 cells per ml of culture medium. Consider reducing it to 0.4×10^6 if the CD1a expression is poor. Generally, the lower the starting density of the monocytes, the higher the purity of resulting $CD1a^{+}$ DC, although very low-density cultures result in a poor recovery of DC (as a percentage of the plated monocytes).

7. Relevant for FCS-supplemented cultures: At day 6, the cultures contain up to 90% $CD1a^{+}CD115^{+}$ immature DC. They are expressing low-to-intermediate levels of CD80 and CD86 and lack CD83 expression. Poor CD1a expression may indicate (a) too high initial density of monocytes at the onset of cultures; (b) poor batch of serum/medium (*see* Notes 12–14); (c) poor mAb (in our hands, OKT6 proved superior to several other CD1a mAbs). It may also suggest poor activity of the IL-4 used and the need to increase its concentration.

8. Optimal type-1 polarization of DC requires complete DC maturation and is impaired in DC that do not undergo full CD83/CCR7 conversion. IFN-γ and the maturation-inducing factor should be administered simultaneously. Pre-treatment of DC, with either of the factors alone, reduces the ability of DC to produce IL-12p70 after subsequent stimulation.

9. While our standard protocol of generation of polarized effector DC involves a 48 h maturation stage, a shorter maturation/polarization time may be considered depending on the DC application.

10. Bacterial products, such as LPS or SAC (alone or in combination with IFN-γ), are effective inducers of IL-12p70 production in immature ($CD83^{-}$) DC, but not in mature DC. CD40L stimulation remains effective in mature DC, although mature DC show impaired responsiveness to the IL-12p70 enhancing action of IFN-γ (54).

11. The proliferation of Th cells is very susceptible to the temperature changes, especially within the first 5 days of culture. To optimize the yield of the differentially primed Th cells, try to minimize the length of time when the cells are outside the incubator and use pre-warmed medium to dilute the cultures.

12. A batch of FCS is important. We observed strong differences between several different batches of FCS in their ability to support the DC1.

13. The source of medium can make a difference as well.

14. We advocate using disposable plastic tubes, media flasks, and pipettes to reduce the chance of endotoxin contamination at the onset of cultures.

15. Although difficult to avoid for some applications, gamma irradiation impairs the ability of DC to produce IL-12. Typically, the IL-12p70 production by 2500R-irradiated DC is only 15–25% compared to non-irradiated DC.

16. We routinely observe that the addition of even 0.5% of human serum or plasma, particularly not only from cancer patients but also banked human AB serum, inhibits DC maturation with negative effects on the expression of CCR7, migratory properties and the ability to produce IL-12p70.

17. We advocate a thorough testing of the applicability of each media, rather than assuming that the suggested concentrations of cytokines will be optimal for any media. For example, our collaborators observed that cultures performed in X-VIVO medium may require up to 10,000 U of IFN-α (instead of our 3,000 U) and up to 100 ng/ml of TNF-α for the optimal activity of αDC1s. We are not sure if these differences reflect the differential impact of medium or different specific activity of the cytokines used in the “alpha-type-1” maturation cocktail. *Excessive cell adherence* seems to be the most sensitive indicator of an incomplete DC maturation. Our recent back-to-back comparison of different serum-free media demonstrated that the cells generated in CellGro DC medium from CellGenix yield DC1s with the highest quality and yield (64).

18. In any of the functional assays or for the preparation of the vaccine, we *do not harvest the adherent cells (whenever present*). These macrophage-like cells are not stimulatory and may be suppressive. We have seen that scraping the cell or using Ca/Mg-free medium to wash the cells reduces their IL-12-producing capacity. EDTA is even worse.

19. In a limited number of experiments using our LPS/IFN-γ-based protocol of inducing DC1s, we have attempted to obtain polarized DC1 in Teflon bags. These attempts were met with a limited success, raising the possibility that cell adherence may be important for the generation of DC1s. While this issue needs to be readdressed using currently available culture bags, our clinically applied αDC1s are currently grown in T25 and T75 culture flasks.

20. Although αDC1 can be frozen without any significant reduction of their viability (compared to standard, PGE2-matured DC; sDC), freezing reduces their subsequent ability of both cell types to produce IL-12 by about 60–70%. Although freezing of αDC1 and sDC preserves the ratio of their IL-12-producing capacities, if you have a choice between freezing patients’ monocytes (and vaccinating with freshly-generated DCs) or freezing the ready to use vaccine, the first option (less convenient) may allow to fully benefit from DC1 biology. We cannot say at this moment if freezing makes any difference for the final performance of DCs, but we will use fresh DCs in our first protocol.

21. Although we currently generate DC, using 1,000 U/ml of both GM-CSF and IL-4, it is possible to reduce the levels of these cytokines to at least 500 U/ml (GM-CSF) and 250 U/ml (IL-4). The cells need to be monitored for the signs of decreased yield (insufficient GM-CSF) and excessive cell adherence and persistence of CD14 (signs of insufficient IL-4). The exact minimum level of IL-4 needed highly depends on the amount of activation of monocytes during their isolation (quality of reagents, de/re-calcification, duration of cell adherence) and the cell density (affecting the concentration of endogenous monocyte-derived factors affecting their differentiation, such as prostanoids or CSF-1).

22. Please note that DC1 produces only limited amounts of IL-12 spontaneously after removing them from the maturation cultures (low pg concentrations can be

detected) but produces a “second wave” of IL-12p70 following interaction with T cells, particularly not only $CD4^+$ T cells, but also $CD8^+$ T cells. While our early work with isolated $CD8^+$ T cells involved CD40L-transduced J58 cells as a surrogate of Th cells (60), we have recently observed, using the systems of in vitro CTL induction using polyclonal stimuli (SEB) and Ag-specific stimulation of $CD8^+$ T cells that the inclusion of CD40L in these assays (during the DC-mediated sensitization of tumor-specific $CD8^+$ T cells) may be counterproductive and induce LAK activity in $CD8^+$ T cells (increasing non-specific background in ELISPOT observed after 2 weeks of priming). No CD40L has been used in our recent work demonstrating the advantage of using αDC1s in inducing tumor-specific CTLs (62,64).

23. Recently, it was suggested that type-1 DC polarization is suboptimal in X-VIVO medium suggested (73). In our experience *(see* Note 17) the maturation of alpha-DC1s cultured in some batches of X-VIVO medium is associated with excessive cell adherence and low cell recovery, but we did not see such effects with all batches of that medium, so such deficit may be batch-dependent. While we occasionally observe differences in the DC1 generation in different batches of the same medium, the CellGenix DC is our current medium of choice. The comparison of different media was performed in our recent paper (64).

24. In the mouse system, the development of Th17 cells from naïve precursors is well-documented: the activation of naïve $CD4^+$ Th cells in the presence of IL-6 and TGF-β will readily induce the development of high numbers of RORγT expressing Th17 cells (29,35,38–40,74,75). In contrast, it is less clear as to how effective is the direct pathway of development of Th17 from human naïve precursors. In contrast, human Th17 cells can be efficiently and reproducibly induced from the population of $CD45RO^+$ memory Th cells by the DC activated by bacteria or by peptidoglycan (PGN) (26).

25. In addition to their superior ability to induce Th1 and CTL responses αDC1 shows also preferential ability to attract with these T cell types (76), which may contribute to their previously documented elevated activity in promoting tumor-specific Th1 and CTL responses (60,63,64). In contrast to αDC1s which mainly produce Th1- and CTL-attracting CXCR3-ligands and CCR5 ligands (MIG, IP10, RANTES and similar chemokines), standard(s) DC mainly produce Treg-attracting CCL22 (76). As a result, DC1 attracts overall higher numbers of T cells, but significantly lower numbers of Tregs, compared to sDC (76).

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