Review

Dendritic Cells *in vivo* and *in vitro*

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Dendritic cells (DC) are crucial cells of the immune system, and bridged the essential connection between innate and adaptive immunity. They reside in the periphery as sentinels where they take up antigens. Upon activation, they migrate to lymphoid organs and present there the processed antigens to T cells, thereby activating them and eliciting a potent immune response. Dendritic cells are bone marrow-derived cells, still big controversies exist about their *in vivo* development. *In vitro*, DC can be generated from multiple precursor cells, among them lymphoid and myeloid committed progenitors. Although it remains unknown how DC are generated *in vivo*, studying the functions of *in vitro* generated DC results in fundamental knowledge of the DC biology with promising applications for future medicine. Therefore, in this review, we present current protocols for the generation of DC from precursors *in vitro*. We will do this for the mouse system, where most research occurs and for the human system, where research concentrates on implementing DC biology in disease treatments. *Cellular & Molecular Immunology*. 2005;2(1):28-35.

Key Words: DC, differentiation, maturation, hematopoietic progenitor

Introduction

Dendritic cells (DCs) were initially described by Ralph Steinman in 1973 as dendritic appearing cells in lymphoid organs (1). Subsequently, DC have been described in more and more tissues. Nowadays, it is thought that most organs possess their own DC populations. DC belong to the mononuclear phagocyte system (MPS), in contrast to the polymorphonuclear neutrophils. Both of them show avid phagocytosis. Yet different from the other members of the MPS, the macrophages, phagocytosis is not DC's main task. Once DC have been activated, they will start to migrate to lymph nodes. During this migration, DC will process uptaken antigens and put them onto the cell surface complexes with MHC class I or class II molecules. They will also upregulate the expression of other cell surface molecules, so called costimulatory molecules, which will later be used to interact with T cells. The process of upregulating the expression of antigen-MHC class I or class II complexes and costimulatory molecules is called maturation. Maturation happens during migration and is essential for DC to perform their main function later: to activate T cells.

Once DC have arrived in draining lymph nodes, and push T cells into one of the direction followed by their interaction: polarize them toward a Th1 or a Th2 response, or induce T regulatory cells. The factors that dictate these directions are not exactly known so far and are subject to ongoing investigations. Nevertheless, what can be said is that the way DC are activated and the interactions DC are having during their migration into lymph nodes with other cells of the immune system such as macrophages, NK cells, NKT cells and B cells are shaping the activation state of DC. According to this state, DC will thereafter push T cells further or less far.

For T cells to get efficiently activated, they require three signals: (i) They need to recognize their antigen in the context of MHC class I (for $CD8^+$ T cells) or MHC class II (for $CD4^+$ T cells). Each T cell carries a unique T cell receptor and only those cells that recognize the antigens that the DC present will become activated. (ii) T cells need the costimulatory molecules that DC have upregulated during maturation. This is the reason why immature DC in the periphery cannot activate T cells efficiently. (iii) Next to these two signals, it has become clear during the last ten years that these two signals themselves are not enough to activate T cells and a third signal is required. This signal is most probably delivered in the form of soluble molecules, i.e., cytokines and chemokines, which are produced by DC and

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Abbreviations: HPC, hematopoietic progenitor cells; DC, dendritic cells; PBMC, peripheral blood mononuclear cells; PBMo, peripheral blood monocytes; PBDC, peripheral blood dendritic cells; TNF-α, tumor necrosis factor alpha.

Table 1. Phenotype of different DCs subsets in human

	Phenotype of DC subsets					References
Thymus	Lin^- HLA-DR^{int} $CD11c^+$ $CD13^+$ IL-3Rα^{low} $CD45RA^{low}$ (Immature DC)	Lin^- HLA-DR^{int} $CD11c^-$ $CD13^-$ IL-3Rα^{high} $CD45RA^{high}$ (Plasmacytoid DC)	Lin^- HLA-DR^{high} $CD11c^+$ $CD13^+$ IL-3Rα^{low} $CD45RA^{low}$ (Mature DC)			6
Spleen	$CD11c^+$ $CD86^-$ $CD83^-$ $CD14^-$ $CD11b^-$	$CD11c^+$ $CD86^+$ $CD83^+$	$CD11c^+$ $CD86^-$ CD80- CD83- $CD14^{low}$ $CD11b^+$	HLA-DR^+ $CD11c^-$		7
Lymph nodes	$CD1a^+$ $CD86^{-/dim}$ $CD83^{-/dim}$	$CD1a^-$ $CD86^+$ $CD83^+$	$CD1a^{bright}$ $CD86^+$ $CD83^+$			8
Tonsils	HLA-DR^{hi} $CD11c^+$	HLA-DR^{mod} $CD11c^+$ $CD13^+$	HLA-DR^{mod} $CD11c^+$ $CD13^-$	HLA-DR^{mod} $CD11c^-$ $CD123^+$ (Plasmacytoid)	HLA-DR^{mod} $CD11c^-$ $CD123^-$	9
Peyer's patches	No available					-
Blood	Lin^- HLA-DR^+ $CD1b/c^+$	Lin^- HLA-DR^+ BDCA-3^+	Lin^- HLA-DR^+ $CD123^{hi}$ (plasmacytoid)	Lin^- HLA-DR^+ $CD16^+$	Lin^- HLA-DR^+ $CD34^+$	10
Skin (dermis)	$CD11c^+$ Factor $XIIIa^+$ $CD1a^+$ $CD14^-$	$CD11c^+$ Factor $XIIIa^+$ $CD1a^-$ $CD14^-$	$CD11c^+$ Factor $XIIIa^+$ $CD1a^-$ $CD14^+$	$CD11c^+$ Langerin $^+$		11

directly affect T cells in a paracrine fashion (2).

Due to the link function that DC perform between the periphery, where pathogens arise, and the lymph nodes, where immunity against them is induced, DC are at a key position of the immune system. Moreover, it has also been recognized recently that DC in a healthy body perform the crucial function to induce tolerance for the body's own antigens (3). Therefore, manipulating DC seems to be a potent way to treat persistent infectious diseases and cancers by enhancing DC functions, and to treat diseases of an overreacting immune system, such as autoimmune diseases and allergies, by enhancing DC functions to induce tolerance (4). Yet, in order to do so, DC have to be obtained, either by isolating them directly from the body or by culturing them from isolated precursors. In the following sections, we will discuss protocols to culture DC from human and from mouse precursors.

Heterogeneity of DC subsets

In the last two decades, the hybridoma technology has delivered us a wealth of monoclonal antibodies against multiple molecules on the surface of diverse cells types. By using these antibodies for immunochemistry or flow cytometry stainings, different DC subsets both in humans and in mice have been described. Thereby, both lymphoid organs (thymus and bone marrow as primary lymphoid organs; lymph nodes, spleen and gut associated lymphoid tissues such as appendix, tonsils, adenoids and Peyer's patches as secondary lymphoid organs) as well as non-lymphoid organs (blood, skin, other peripheral organs) have been investigated. Table 1 summarizes the current division of DC subpopulations in different organs in humans. For example, in the human thymus, three distinct DC subpopulations have been described, according to their different expression of HLA-DR (MHC class II), CD11c and CD13 (Figure 1A). In human lymph nodes, also three DC subsets are distinguished, whereas at least four DC subsets have been found in the spleen. And in human tonsils, five subpopulations have been identified (Figure 1B).

The situation in mice is as complex as the one in humans (Table 2). Two DC subsets were found in the murine thymus, distinguishable from each other by their CD8α expression level. In the mouse spleen, four DC subsets have been

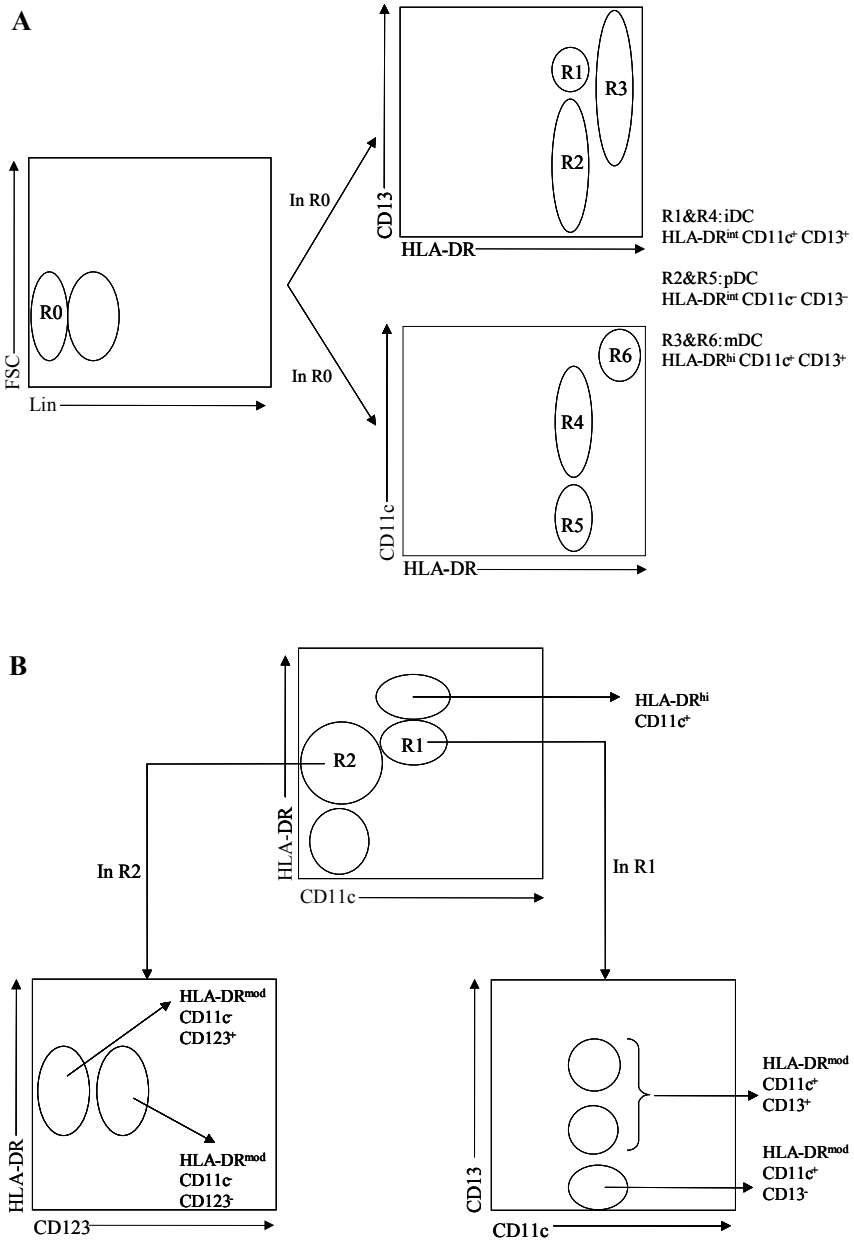

Figure 1. Human DC subsets identified by flow cytometry. (A) Human thymic DC subsets. Thymic single cell suspensions are gated according to their Lin^- (CD3, CD7, CD14, CD19 and CD56) phenotype (R0). Thereafter, three DC subpopulations can be identified according to their CD13 or CD11c and HLA-DR (MHC class II) expression: HLA-$DR^{int}CD11c^+$ $CD13^+$ iDC (R1 and R4), HLA-$DR^{int}CD11c^-CD13^-$ pDCp (R2 and R5) and HLA-$DR^{hi}CD11c^+CD13^+$ mDC (R3 and R6). (B) Human tonsil DC subsets. CD11c and HLA-DR define three tonsil DC subsets that are HLA-$DR^{hi}CD11c^+$, HLA-$DR^{mod}CD11c^+$ (R1), and HLA-$DR^{mod}CD11c^-$ (R2). Double negative cells are not DC. HLA-$DR^{mod}CD11c^+$ cells (R1) can be further divided into two subsets according to CD13 expression, and HLA-$DR^{mod}CD11c^-$cells (R2) can be divided into a $CD123^+$ and a $CD123^-$ subsets according to CD123 expression.

described: two myeloid DC subsets, one lymphoid subset, and the plasmacytoid DC precursor subset (Table 2). The classification of DC subsets myeloid and lymphoid DC has been proposed, when it was found that T cell precursors, which are lymphoid committed cells, could form thymic DC as well (5). The expression of markers that are normally expressed by lymphocytes (i.e., CD8α) was taken as a hint that these cells *in vivo* indeed stem from lymphoid committed cells. In the meantime, it has been recognized that myeloid committed cells as well as lymphoid committed cells can give rise to both myeloid and lymphoid DC subsets. Moreover, until now it is still not clear whether CD8α expression on DC indeed means an independent DC development pathway, or if it is merely a DC activation

Table 2. Phenotype of different DCs subsets in mouse

	Phenotype of DC subsets						References
Thymus	DEC-205^{high} CD11b^{low} CD8α^{high}	DEC-205^{high} CD11b^{low} CD8α^{low}					12
Spleen	MHC II^{low} CD11c^{+} CD11b^{+} CD205^{-} CD8α^{-} CD4^{+} B220^{-} (Myeloid)	MHC II^{low} CD11c^{+} CD11b^{+} CD205^{-} CD8α^{-} CD4^{-} B220^{-} (Myeloid)	MHC II^{low} CD11c^{+} CD11b^{-} CD205^{+} CD8α^{+} B220^{-} (Lymphoid)	MHC II^{low} CD11c^{+} CD11b^{-} CD205^{-} CD8α$^{+/-}$ B220^{+} Gr1^{+} (Plasmacytoid)			12, 13, 16
Lymph nodes	MHC II^{low} CD11c^{+} CD11b^{+} CD205^{-} CD8α^{-} CD4^{+} B220^{-} (Myeloid)	MHC II^{low} CD11c^{+} CD11b^{+} CD205^{-} CD8α^{-} CD4^{-} B220^{-} (Myeloid)	MHC II^{low} CD11c^{+} CD11b^{-} CD205^{+} CD8α^{+} B220^{-} (Lymphoid)	MHC II^{low} CD11c^{+} CD11b^{-} CD205^{-} CD8α^{+}/CD4^{+} B220^{+} Gr1^{+} (Plasmacytoid)	MHC II^{high} CD11c^{+} CD11b^{+} CD205^{int} CD8α^{low} B220^{-} (Only in skin-draining LN and Mesenteric LN)	MHC II^{high} CD11c^{+} CD11b^{+} CD205^{high} CD8α^{int} B220^{-} (Only in skin-draining LN)	13
Peyer's patches	CD11c^{+} B220^{-} CD11b^{+} CD8α^{-} (Myeloid)	CD11c^{+} B220^{-} CD11b^{-} CD8α^{+} (Lymphoid)	CD11c^{+} B220^{-} CD11b^{-} CD8α^{-}	CD11c^{+} B220^{+} CD11b^{-} CD19^{-} (Plasmacytoid)			14, 18
Blood	CD11c^{low} CD11b^{-} CD45RA^{high} (Plasmacytoid)	CD11c^{+} CD11b^{+} CD45RA^{-}					15
Skin (dermis)	CD11c^{+} Langerin^{-}	CD11c^{+} Langerin^{+}					17

marker. Langerhans cells, the DC subset of the epidermis (see also below), have been shown to upregulate CD8α expression upon maturation. Nevertheless, the terms myeloid and lymphoid DC subsets are still used in connection to their expression of myeloid *vs.* lymphoid markers (CD11b *vs.* CD8α) (19). Plasmacytoid DC precursors have been described quite a long time ago as plasmacytoid cells (thus cells with a large cytoplasm) in T cell areas of lymphoid organs. They were recognized not be classical plasma cell, but their true function remained unknown. As a consequence, they were called plasmacytoid monocytes or plasmacytoid T cells. Recently, it has been discovered that plasmacytoid DC precursors produce massive amounts of type I interferons (e.g. IFN-α and IFN-β) upon activation, Moreover, once activated, these cells develop into genuine DC that can potentially stimulate T cells. Therefore, they were renamed plasmacytoid DC precursors. About these cells, there are convincing hints that they may *in vivo* originate from lymphoid precursors: they show D-J recombinations in their heavy chain loci, express the CD3ε mRNA, and their MHC class II expression is regulated the same way as for B cells but different from other DC (20).

Murine lymph nodes (LN) have been shown to harbor the same four DC subsets as the spleen does. Moreover, lymph nodes possess additional DC subsets: The mesenteric LN, which belong to the intestine-draining LN, harbors one additional DC subsets, whereas skin draining lymph nodes harbor two additional DC subsets, one derived from the epidermis and the other one from the dermis (see also below).

In non-lymphoid organs, human blood has been shown to contain five different subsets of DC, whereas mice possess two. Another organ that is under intense investigation due to its easy accessibility is the skin. Skin is a vast organ, and except its physical barrier function, it also plays an important role in immunity. The skin is made up of three layers: epidermis, dermis and subcutaneous tissue. Both in humans and mice, Langerhans cells as DC of the epidermis have been recognized. These cells can now easily been stained with antibodies that recognize Langerin, a Langerhans cell-specific

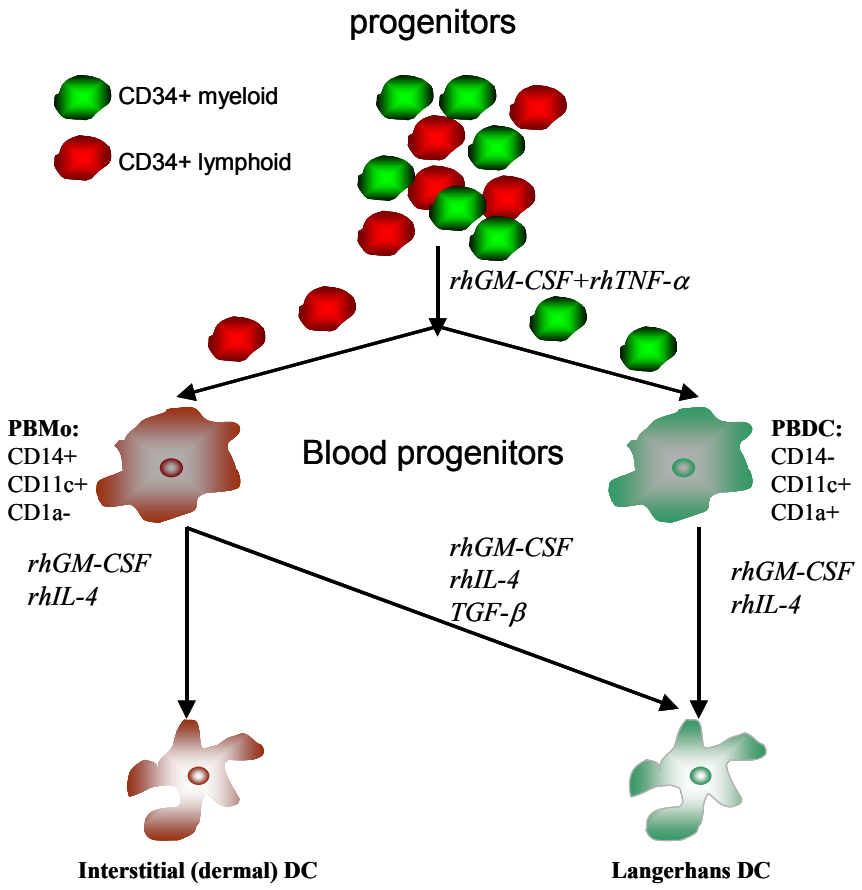

Figure 2. *In vitro* human DC generation. Culturing $CD34^+$ hematopoietic stem cells with rhGM-CSF and rhTNF-α for 5 to 6 days leads to the generation of two subsets that are either $CD1a^-CD14^+CD11c^+$ or $CD1a^+CD14^-CD11c^+$. The first subset can further develop into interstitial DC if cultured with rhGM-CSF and rhTNF-α, or develop into Langerhans DCs if cultured with rhGM-CSF, rhTNF-α and TGF-β. The $CD1a^+CD14^-CD11c^+$ cells will develop further into Langerhans DCs when cultured with rhGM-CSF and rhTNF-α.

molecule. The murine dermis consists of another dermal DC population. Langerhans cells that are migrating through the dermis can be found there as well. The human dermis has been shown to harbor three dermal DC subpopulations, all of which express the coagulation factor XIIIa (FXIIIa). DC subpopulations in the subcutaneous tissue have not yet been described so far.

Knowledge of different DC subsets in different organs is essential for studying their origins and their differentiation pathways. Only with this knowledge, we can correlate *in vitro* generated DC with their *in vivo* counterparts and draw conclusions about the functions of *in vitro* generated DC that are used for *in vivo* vaccinations.

In vitro generation of human DC

The earliest DC precursors can be found in the bone marrow, where it has been shown that hematopoietic stem cells can give rise to DC. Sufficient numbers of hematopoietic stem cells can also been obtained from human umbilical cord blood. Additionally, blood monocytes have been shown to be DC precursors, and DC can be generated efficiently by culturing monocytes. DC populations can also be isolated directly from peripheral blood, lymphoid organs or peripheral tissues (e.g. Langerhans cells from human epidermis). In the following sections, we will describe the generally used protocols to generate human DC from precursors.

Culture of myeloid DC using $CD34^+$ hematopoietic stem cells isolated from human cord blood (21, 22)

Hematopoietic stem cells (HSC) can be collected from umbilical cord blood through positive selection using CD34 monoclonal Abs (mAbs) and goat anti-mouse IgG-coated microbeads. $CD34^+$ HSC are then cultured in RPMI medium, supplemented with 10% FCS, rhGM-CSF and rhTNF-α for 5 days. Optimal conditions are maintained by splitting these cultures at day 4 with medium containing fresh GM-CSF and TNF-α, the most efficient cell concentration used has been determined to be 1 to 3×10^5 cells/ml. After 5 to 6 days of culture, cells are separated into $CD14^+CD1a^-$ or $CD14^-CD1a^+$

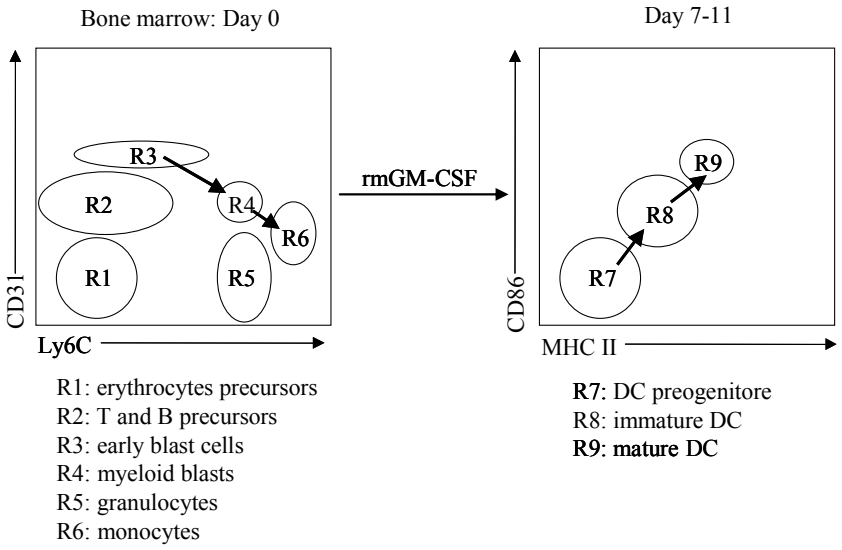

Figure 3. *In vitro* murine bone marrow derived DC generation. The freshly isolated murine bone marrow contains six populations according to CD31 and Ly6C expression. They are $CD31^{-}Ly6C^{-}$ erythrocytes precursors (R1), $CD31^{low}Ly6C^{-}$ lymphoid precursors (R2), $CD31^{+}Ly6C^{-}$ early blast cells (R3), $CD31^{low}Ly6C^{+}$ myeloid blasts (R4), $CD31^{-}Ly6C^{+}$ granulocytes (R5), and $CD31^{low}Ly6C^{high}$ monocytes (R6). During culturing with rmGM-CSF, $CD31^{+}Ly6C^{-}$ early blast cells will differentiate into $CD31^{low}Ly6C^{+}$ myeloid blasts that will further differentiate into $CD31^{low}Ly6C^{high}$ monocytes. After 7 to 11 days culture with rmGM-CSF, bone marrow precursors will differentiate from MHC class $II^{-}CD86^{-}$ progenitors (R7) into MHC class II^{+} $CD86^{+}$ immature DC (R8) and further on into MHC class II^{high} $CD86^{high}$ mature DC (R9).

subpopulations, either of the two subpopulations can be cultured further on for 6 to 7 additional days in the presence of GM-CSF plus TNF, with a last medium refreshing step being performed at day 10. Routinely between day 11 and day 14, two DC populations can be collected suitable for further DC experiments (Figure 2).

Culture of myeloid DC using peripheral blood mononuclear cells (PBMC) (23-26)

PBMC are isolated from whole blood by Ficoll-Hypaque gradient centrifugation. By selecting for CD14, $CD14^{+}$ and $CD14^{-}$ precursors can be obtained and subsequently cultured separately for 6-7 days in 10% FCS, rhGM-CSF and rhIL-4 supplemented RPMI-medium, with or without rhTGF-β. Whereas a lack of rhTGF-β gives rise to typical interstitial (dermal) DC, the addition of rhTGF-β skews them into a more Langerhans cells-like phenotype. Both DC subsets show typical phenotypic and functional profile of immature DC. Up to 8×10^{6} DC have been obtained from 40 ml of blood. Moreover, other cytokine combinations can be used to obtain different DC subpopulations (Figure 2).

Culture of plasmacytoid DC precursors (27)

Plasmacytoid DC precursors (pDCp) can be cultured from peripheral blood hematopoietic stem cells *in vitro* (30). Alternatively, they can be purified from human blood, tonsils or other lymphoid organs by their specific expression of BDCA-2 (a pDCp specific C-type lectin), BDCA-4 (neurophilin-1) or CD123 (IL-3Rα). Whereas selecting pDCp with BDCA-2 mAbs has been shown to inhibit type I interferon production of pDCp, BDCA-4 mAbs did not have any effect on these cells. pDCp cannot be sustained with GM-CSF, but they proliferate when cultured with IL-3 and they can be mobilized *in vivo* by Flt-3L treatment. For *in vitro* culturing, in brief, tonsils are cut into small pieces and digested for 10-15 min with collagenase IV and deoxyribonuclease I, after two rounds of tissue digestion, cells are centrifuged over 50% Percoll for 20 min at 400 g. Then deplete $CD3^{+}$ T cells, $CD14^{+}$ monocytes, $CD19^{+}$ and $CD20^{+}$ B cells, and $CD56^{+}$ NK cells, $CD4^{+}CD3^{-}CD11c^{-}Lin^{-}$ cells are isolated by cell sorting, and in the end put into culture with IL-3 supplemented RPMI-10% FCS medium for 3-6 days.

There is no popular method to culture lymphoid DC *in vitro*, but claims exist that lymphoid DC can be derived from bone marrow and thymus (28, 29), Galy et al. identified a subset of progenitor cells defined by the phenotype $CD34^{+}$ $CD38^{+}Thy\text{-}1^{-}CD10^{+}$. When cultured under appropriate condition, they were capable of giving rise to T, B, NK and DC but not to myeloid cell types. CD10 was suggested to distinguish myeloid and lymphoid origins. Similarly, a $CD34^{+}$ $Thy\text{-}1^{-}$ but $CD38^{dim}$ foetal thymic precursor gave rise to T, NK and DC but not myeloid lineages. One important question is that this population can still be plasmacytoid DC, which are also lymphoid committed cells, so further identification need to be done.

In vitro generation of murine DC

In the mouse system it is inconvenient to isolate DC

subpopulations or precursors directly from peripheral blood or tissues, due to the small sample size (with maybe the skin as an exception). Therefore, DC are mostly generated from bone marrow precursors. Bone marrow contains various stem cells that can be used *in vitro* to generate B cells, polymorphonuclear neutrophiles, macrophages and DC by using different cytokines and growth factors. As in the human situation, GM-CSF is essential for the generation of DC from bone marrow precursors, whereas IL-4 can be omitted.

The standard protocol for generating large quantities of highly pure dendritic cells from whole bone marrow precursors has been described by Lutz et al. in 1999 (31). Thereby fresh whole bone marrow cells are cultured with 20 ng/ml rmGM-CSF for six days with refreshment of the whole medium at day 3 and supplementation with another volume of new medium at day 5. At day 6 an activation stimulus (LPS or IFN-γ) is added for one day. The differentiation and maturation state of cultured DC can be followed by their MHC class II and CD86 expression: $MHCII^{-}CD86^{-}$ precursors develop into MHC $II^{mid}CD86^{mid}$ immature DC (iDC). When activated, those iDC mature further on into MHC $II^{high}CD86^{high}$ mature DC (mDCs) (Figure 3). Alternatively, DC precursors can be isolated from bone marrow and subsequently cultured. Bone marrow DC precursors have been identified to show different expression levels of CD31 and Ly6C (Figure 3). $CD31^{high}Ly6C^{-}$ early blast cells develop into $CD31^{+}Ly6C^{+}$ myeloid blasts that further develop into $CD31^{-}Ly6C^{high}$ bone marrow monocytes (32). All of these three subsets can be cultured to generate DC, earlier stage cells need more time to do so than later stage cells, on the other hand they show a higher proliferation. During experiments, it is recommended to leave as many factors unchanged as possible. One factor that may influence the outcome of DC cultures is the age of the bone marrow donor mice. To check if it is feasible to freeze bone marrow cells or immature DC to increase experiment reliability, an experiment was peroformed to compared the yield and phenotype of mature DC that have either been obtained (i) by culturing fresh bone marrow cells for five days and stimulating them for one day, (ii) by culturing frozen bone marrow cells for five days and stimulating them for one day, or (iii) by stimulating frozen five day immature DC that have been isolated over their $CD86^{low}$ expression for one day. No differences were found concerning phenotype (CD86, CD80, MHC II, CD11c, CD40 and F4/80 expression) and cytokine production (IL-10, IL-12p70, and TNF-α) before and after stimulation (not published), but a remarkable difference was found in cells yield in the third method where most of the immature DC died during the freezing and thawing process, the DC yield from fresh and frozen bone marrow were comparable. Similar findings have been published before (33). Therefore freezing stocks of bone marrow precursors from mice that have the same age is a feasible step in enhancing reliability of DC cultures.

Murine plasmacytoid DC precursors can be generated similarly to human cells by culturing bone marrow cells with Flt3-ligand, which resulted in the generation of 40% pure $CD11c^{+}CD11b^{-}B220^{+}Gr\text{-}1^{+}$ plasmacytoid DC precursors. By contrast, GM-CSF or TNF-α, which promote the generation of $CD11c^{+}CD11b^{+}B220^{-}$ myeloid DC, blocked completely the development of plasmacytoid DC precursors (34). These results suggest again that the two lineages - myeloid DC and plasmacytoid DC precursors - are less related.

Conclusion

Progress in understanding the molecular regulation of DC development from hematopoietic stem cells has led to the development of *in vitro* culture systems for the generation of large numbers of myeloid DCs from $CD34^{+}$ hematopoetic stem cells with GM-CSF and TNF-α or peripheral blood monocytes with GM-CSF and IL-4. Also in the mouse, the bone marrow–derived DC can now be generated in a regular culture method. The ready culture systems not only permit detailed studies of DC biology, but also hold promises for DC to become widespread used in medical practice such as immunotherapy in cancer and autoimmune diseases.

Decades after discovering DC, the DC founding father Steinman RM once said, "The field is only just beginning."

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