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(54) **Title:** A METHOD FOR PRODUCING LANGERHANS CELLS AND/OR DERMAL/INTERSTITIAL DENTRITIC CELLS FROM CD14+ MONOCYTES

(57) **Abstract:** The present invention relates to a method for preparing Langerhans cells (LC), or dermal/interstitial dendritic cells (IDC) or LCs and IDCs from CD 14+ monocytes stemming from peripheral circulatory blood of a living being, and notably of a human being, comprising differentiation of CD 14+ monocytes into LCs, or into IDCs, or into LCs and IDCs by putting the CD 14+ monocytes in the presence of a cell environment comprising epithelial cells, such as keratinocytes, and/or mesenchymatous cells such as dermal fibroblasts. The present invention also relates to cell or tissue models comprising at least LCs and/or IDCs, and optionally macrophages and endothelial cells, able to be obtained according to this method, and to their uses.

A method for producing Langerhans cells and/or dermal/interstitial dendritic cells from CD14+ monocytes

The invention relates to a method for producing dendritic cells, and notably Langerhans cells and/or interstitial dendritic cells/dermal dendritic cells, from CD14+ monocytes isolated from peripheral circulatory blood, notably by preparing tissue models, notably with the purpose of testing active ingredients and/or studying biological/biochemical phenomena involved at these models.

STATE OF THE ART

Dendritic cells (DC) are cells having antigens described as sentries of the immune system. Indeed, they have a quasi-ubiquitous localization, i.e., in the thymus, systemic circulation, secondary lymphoid organs and also peripheral tissues such as the skin and the mono-stratified or pluri-stratified mucosae. Although there are a very small number of them in the organism, the DCs are at the centre of the triggering of specific immune responses, by exerting a control on the specificity, the intensity and the nature of the immune response and are placed at the interface between innate and acquired immunity. In addition to their function for igniting the immune response, the DCs also play a role in inducing peripheral tolerance.

The precursors of DCs stem from the differentiation of CD34+ haematopoietic progenitors just as many populations of the immunitary system and blood cells. They are conveyed via the blood at the skin and the mucosae in order to differentiate and reside their as immature DCs. This immature state is expressed by a characteristic phenotype and a strong functional capability of capturing antigens. Two types of peripheral DCs are described according to their *in vivo* localization:

- The Langerhans cells (LC) are localized at the epithelia of the malpighian type (skin and mucosae) where they specifically express langerine which is a lectin of types C. Langerine is involved in the formation of an intracytoplasmic organite, Birbeck's granule which is the specific ultrastructural and reference label of the LCs. LCs also express characteristic labels such as CD1a and HLA-DR.
- Interstitial DCs (IDCs) as for them are found at the lamina of mucosae like at the dermis of the skin where they are also described as dermal DCs (DDCs). These cells share many similarities and common labels with the cell line of monocytes/macrophages. In the dermis of the skin, DCCs specifically express DC-SIGN but also characteristic labels such as HLA-DR, FXIIIa, MMR and CD1a. The description which follows generally refers to interstitial dendritic cells

(IDCs) independently of the fact that they are located at the lamina of the mucosae or at the dermis of the skin.

Following the capture of an antigen, the LCs and IDCs migrate towards the lymph nodes in order to present the antigenic information to the lymphocytes T. This migration which is correlated with activation of LCs and IDCs is expressed by phenotypical and functional changes. For example, the « activated » LCs and IDCs acquire the expression of CD80 and CD86 which are co-stimulation labels and acquire the expression of the CCR7 receptor which is absolutely necessary to skin migration of the cells. At the lymph nodes, the activated LCs and IDCs acquire a phenotype of « mature » or « interdigitated » DCs from their interaction with T lymphocytes. This mature condition is synonym with a characteristic phenotype such as for example the expression of CD83 and DC-LAMP and with a strong allostimulating capacity i.e. the capability of inducing proliferation of T lymphocytes. Because of their capability of migrating towards proximal nodes after having captured an exo-antigen, the LCs and IDCs are responsible for many skin pathologies such as contact allergy and are more recently described as the first targets of the human immunodeficiency virus (HIV).

An interesting use of LCs and IDCs, notably combined with epithelial cells or mesenchymatous cells of the fibroblast type, stemming from skin or human mucosae, consists of integrating them into three-dimensional organotypical cultures such as:

- Models of « reconstructed epidermises » and of « reconstructed mucosae », for example of the vaginal and oral type, exclusively integrating LCs (Régnier *et al.*, JID 1997; 109:510-2; Patent EP 0 789 074 of L'OREAL; Sivard et *al.*, Exp. Dermatol. 2003; 12:346-55),
- Models of « reconstructed dermis » and of « reconstructed chorion », integrating DCCs or IDCs (Guironnet *et al.*, JID 2001; 116:933-9 and Dumont et *al.*, AIDS Res. Hum. Retroviruses 2004; 20:383-97).

These uses are today extremely limited because: 1/ of the absence of a fast, simplified and not very expensive method on an industrial scale, for producing *in vitro* LCs and IDCs and, 2/ of the imperfection of the described 3D models which exclusively show the epithelial portion or the conjunctive portion of the reconstructed tissue and by no means both of the associated tissue compartments.

The articles of Régnier *et al.*, Sivard *et al.* and Dumont et *al.* deal with the use of CD34+ precursors stemming from umbilical chord blood which has the following major drawbacks: 1/ the number of isolated CD34+ progenitors is limited since they are extracted from umbilical chord blood which is a limited source, therefore difficult to use industrially; 2/ the prior culture (for 6 to 12 days) of the CD34+ progenitors before their integration into the 3D models and this in order to

induce their differentiation into LCs or IDCs; 3/ the described methods require the addition of exogenous cytokines in the media for growing reconstructed epidermises, reconstructed mucosae and reconstructed chorions integrating the LCs or some IDCs.

In the article of Guironnet *et al.*, the authors have generated DCCs from blood monocytes in order to integrate them into a reconstructed dermis model. As previously, two drawbacks should be underlined: 1/ the prior culture of monocytes (for 6 days) before their integration into the reconstructed dermis and this in order to induce their differentiation into DCCs; 2/ the addition of exogenous cytokines in the media for growing the reconstructed dermis integrating the DCCs. In this same article, the authors also show that monocytes directly integrated into the equivalent dermis and without adding any cytokines, do not differentiate into DCs.

L'Oréal's Patent US 6,130,482 describes the co-culture of keratinocytes and precursors of LC in an adequate nutritious medium in order to carry out differentiation of the precursors into LCs, notably with the purpose of integrating them into an epidermis model in order to assess the synthesizing irritating or allergenic potency of a product. However, this application describes the use of CD34+ haematopoietic progenitors stemming from umbilical chord blood with reference to the method described by Caux et al., Nature, 1992 Nov. 19, 360(6401):258-61. The method described in this article comprises the growing of CD34+ haematopoietic progenitors in the presence of exogenous cytokines. This patent generally defines the precursors of LCs as being able to express CD1a+, however only the use of CD34+ haematopoietic cells as CD34+ precursors of LCs is described therein. The CD34+ haematopoietic cells are however not very numerous in the peripheral blood and do not allow development of an industrially satisfactory differentiation method. The use of precursors stemming from umbilical chord blood is unsatisfactory in so far that this blood is not available in a large amount.

Recently, BASF Beauty Care Solutions France SAS (Coletica, French Patent FR 2 833 271B1, filed on December 10th 2001) describes the differentiation of CD14+ monocytes in order to obtain LCs, IDCs, some LCs and some DCIs simultaneously, which may then be grown in the presence of epithelial cells and/or mesenchymatous cells stemming from skin or human mucosae. Are notably described in this patent:

- Models of « reconstructed epidermis » and of « reconstructed mucosae » exclusively integrating LCs,
- Models of « reconstructed dermis » and of « reconstructed chorion » integrating DCCs or IDCs,
- Models of « reconstructed skins » and of « reconstructed mucosae » integrating LCs and IDCs simultaneously.

As previously, the drawback of the described method is the prior growing of monocytes (for 6 days) before integrating them into 3D models and this in order to induce their differentiation into LCs, or into IDCs, or into LCs and IDCs simultaneously, in the presence of exogenous cytokines.

To this day, only the method developed by Engelhard Lyon, which is the object of Patent FR 2 833 271 B1, describes the differentiation of monocytes into LCs and/or into IDCs with the purpose of providing LCs and/or IDCs for developing immunocompetent tissue models close to normal human skin. However, this method may still be notably improved by using a simple, not very costly and fast differentiation method.

OBJECT OF THE INVENTION

With the invention, it is possible for the first time to solve each of the technical problems set forth earlier, in a safe and reliable, reproducible way and which may be used on an industrial and commercial scale and notably on an agro-feeding and/or medical and/or pharmaceutical and/or cosmetic industrial scale.

The invention mainly consists of generating LCs, or IDCs or LCs and IDCs simultaneously, or IDCs and macrophages and endothelial cells simultaneously or LCs and IDCs and macrophages and endothelial cells simultaneously, from a unique living precursor stemming from peripheral circulatory blood.

The invention notably consists of providing epidermis, epithelium, dermis, chorion, skin or mucosa models containing the aforementioned cells (LC, IDC/DCC, etc.), said models being of the best possible quality in order to reproduce an epidermis, an epithelium, a dermis, a chorion, a skin or a mucosa of a living being, in particular of a mammal, and notably of a human being. The object of the present invention further is to provide epithelial and/or conjunctive sheets as well as immunocompetent equivalents of skin or of mucosae.

The present invention notably consists of providing a method for differentiating monocytes stemming from peripheral circulatory blood in order to obtain the aforementioned cells (LC, IDC/DCC, etc.)

The present invention also consists of providing models described above as alternative methods to animal experimentation, notably for testing the irritating and/or sensitizing potency of a cosmetic ingredient.

The object of the present invention is also to provide models as described above for testing active ingredients notably in cosmetics, in dermo-pharmacy, and in pharmacy, notably for assessing their activity and/or their toxicity or pharmacotoxicity.

The object of the present invention is also to provide models as described above for testing molecules or chemicals notably for assessing their toxicity.

The present invention also consists of providing models as described above for investigating biological/biochemical phenomena at intercellular and intracellular level.

The main object of the invention is to provide a model/tool for pharmacotoxicological investigation, for example with the purpose of conducting tests *in vitro* for predicting the allergizing/irritating/sensitizing power of external agents. The object of the present invention is still to provide a model/tool for investigating substances having immunomodulating properties.

The invention also relates to the use of the models as described above in tissue or cell engineering, notably for repairing at least one portion of the tissues of a living being.

SUMMARY OF THE INVENTION

Within the scope of the invention, when discussing « cells », these are always « living cells », unless stated otherwise.

By « peripheral circulatory blood », is meant according to the invention the blood of any living being having a blood system in which the blood accomplishes a circuit, notably at the periphery, in particular in animals, in mammals, preferably in a human being.

By « active ingredient », is meant any substance, product or composition potentially capable of having an interesting activity in industry, in particular in the agro-feeding, food, dermo-pharmaceutical, pharmaceutical, cosmetics industry, etc.

By « addition of exogenous cytokine» is meant addition of at least one cytokine in addition to the cytokines synthesized by the cells present in the relevant cell medium. The invention therefore relates to differentiation of CD14+ monocytes, in the presence of epithelial cells and/or mesenchymatous cells, for example of the fibroblast type, into:

- LCs,
- IDCs,
- LCs and IDCs simultaneously,
- IDCs and macrophages and/or endothelial cells simultaneously,
- LCs and IDCs and macrophages and/or endothelial cells simultaneously.

The invention notably relates to differentiation of CD14+ monocytes into LCs and/or IDCs (without prior growing under conditions promoting their differentiation, and notably without prior growing with exogenous cytokines) in the presence of epithelial cells and/or mesenchymatous cells, for example of the fibroblast type, in order to obtain a cell model comprising LCs and/or IDCs, their

culture being preferably performed essentially without significant addition of exogenous cytokines, i.e. without adding any cytokine in addition to the endogenous cytokines synthesized by the cells present in the medium.

The invention notably relates to a method for differentiating CD14+ monocytes into LCs and/or IDCs, comprising the following steps:

- collecting CD14+ monocytes from circulatory blood, notably of a human being;

- maintaining CD14+ monocytes under conditions which do not promote their differentiation into DCs;

- placing CD14+ monocytes into contact in a cell environment comprising epithelial cells, for example keratinocytes, and/or mesenchymatous cells, for example fibroblasts, under conditions which do not promote differentiation of CD14+ monocytes into LCs, or IDCs, or LCs and IDCs.

The step for maintaining CD14+ monocytes under conditions which do not promote their differentiation into CDs is preferably limited in time in order to notably carry out sowing of the CD14+ monocytes shortly or even directly after they have been collected from the circulatory blood.

Advantageously, conditions which do not promote differentiation of CD14+ monocytes into DCs are obtained under culture conditions which do not comprise any exogenous cytokine. The purpose here is notably that the CD14+ monocytes do not engage in DC differentiation paths.

With the invention, it is notably possible to obtain a gain in the number of precursors/cells as it avoids prior growing of CD14+ monocytes which is accompanied by a certain level of cell mortality.

DCs generated *in vitro* are very sensitive and fragile cells. The first parameter to be checked is the yield in the obtained cells. It is obvious, but not quantifiable, that the loss and/or cell mortality of DCs is significant when the latter are sown in any cell culture model. The use of CD14+ monocytes according to the present invention, as precursors of DCs, remedies this problem and thus allows a better yield in cells.

With the invention, it is notably possible to obtain a gain in time as the differentiation of monocytes into LCs or IDCs requires 6 days of culture.

With the invention it is notably possible to obtain a gain in reagents as preferably no exogenous cytokine is used.

With the invention it is notably possible to obtain a gain in the quality of the generated cells. *In vitro* generation of DCs from monocytes is related to a cell differentiation phase under the effect of immune soluble mediators which are cytokines. Recently in an article (Nicolas BECHETOILLE *et al.*, *Journal of Leukocytes Biology*: Mixed Langerhans cell and interstitial/dermal dendritic cell

subsets emanating from monocytes in Th2-mediated inflammatory conditions respond differently to pro-inflammatory stimuli) it was described that DCs of the LC and DDC type derived from monocytes are not « synchronous » in their cell differentiation process. In other words the CD14+ monocytes which have initiated their differentiation generate LCs and DCCs which do not all have the same differentiation stage. This imperfection may be dispensed with by avoiding this differentiation step. The monocytes grown in the present invention are influenced by the cell, cytokine and matrix environment of the cell reconstructions, which forms a more physiological environment for differentiation of DCs.

It is well known that DCs generated *in vitro* have the capability of « maturating » spontaneously, which is a major problem as mature DCs may no longer be activated and stimulated. Although the inventors control the immature conditions of these cells *in vitro*, the risk of spontaneous maturity *in vitro* may be dispensed with by directly using monocytes as precursors of LCs and DCCs. Also, the advantageous use of cell or tissue culture models according to the invention as a system for differentiation of CD14+ monocytes into DCs is more physiological as it better reproduces the natural conditions for differentiation of DCs in the skin and in the mucosae and immature DCs comparable to their homologs *in vivo* may thereby be obtained.

In this same context, by using CD14+ monocytes as precursors of DCs, as opposed to the use of DCs generated beforehand, it is possible to generate in the present models for growing phenotypically more immature and functionally more sensitive LCs and DCCs. Indeed, and in this context, the inventors have recently demonstrated that DCs derived from CD34+ progenitors and then grown in a reconstructed chorion in the presence of exogenous cytokines has a more satisfactory immaturity condition, in terms of replication of the HIV virus, than its homologs grown in the presence of exogenous cytokines (Sandy DUMONT *et al.*, *When integrated in a sub-epithelial mucosal layer equivalent, dendritic cells keep their immature stage and their ability to replicate type R5 HIV-1 strains in absence of T cell subsets*. AIDS Res. and Hum. Retroviruses 20: 383-397, 2004). The obtained DCs are very different from those described previously and notably in French Patent FR 2 833 271B1, filled by Coletica on December 10th 2001.

Further, direct use of CD14+ monocytes as precursors of skin DCs is a more physiological process as compared with the process described earlier. Indeed, *in vivo* in the organism, blood monocytes colonize the skin, where the cell, cytokine and matrix environment controls their differentiation. Integration of DCs differentiated beforehand in the present culture models was an interesting alternative which today is less satisfactory than directly using their precursors which are the CD14+ monocytes. This improvement of the system is immunohistologically expressed by a

more homogenous cell distribution of LCs and DCCs in the present three-dimensional culture models.

With the invention, it is notably possible to freeze freshly isolated monocytes before their use in the models.

With the present invention it is possible to generate cells having substantially the same phenotype and the same functions as their *in vivo* homologs.

The present invention notably relates to a unique cell precursor which, when it is co-cultivated:

- In an epithelial environment, differentiates phenotypically and/or functionally into LCs,
- In a conjunctive environment differentiates phenotypically and/or functionally into IDCs, macrophages and endothelial cells.

DESCRIPTION OF THE INVENTION

Thus, the present invention according to a first aspect, relates to a method for preparing LCs, or IDCs, or LCs and IDCs, from CD14+ monocytes stemming from peripheral circulatory blood of a living being, and notably of a human being, comprising differentiation of CD14+ monocytes into LCs or into IDCs or into LCs and IDCs by putting CD14+ monocytes in presence with a cell environment comprising epithelial cells, such as keratinocytes, and/or mesenchymatous cells, such as dermal fibroblasts.

Advantageously, differentiation of CD14+ monocytes allows to obtain IDCs and macrophages and endothelial cells, or LCs, IDCs, macrophages and endothelial cells. Advantageously, the distribution of the cell population of LCs and IDCs is a function of the cell type which is jointly grown with monocytes. The use of keratinocytes promotes differentiation into LCs and the use of fibroblasts promotes differentiation into IDCs.

Advantageously, the differentiation of the monocytes is carried out without significantly adding any exogenous cytokine. According to one embodiment, no exogenous cytokine is added.

Advantageously, growing monocytes with an epithelial cell environment, such as keratinocytes, promotes differentiation of the monocytes into immature and functional LCs. By « immature », the inventors notably mean: that they do not or only very slightly express activation labels (CD80, CD86, CCR7) and maturation labels (CD83, DC-LAMP). On the other hand, immature LCs express CCR6.

By « functional », the inventors notably mean: provided with antigene internalization capacities (immature condition), cell migration capacities (immature and activated condition), and antigenic presentation capacities (mature condition).

According to one embodiment, the culture of monocytes with a mesenchymatous cell environment, such as dermal fibroblasts, promotes differentiation of the monocytes into immature and functional IDCs. Reference is made to the definition above, but immature IDCs do not express CCR6.

Advantageously, culture of monocytes with a cell environment comprising epithelial cells, such as keratinocytes, and mesenchymatous cells, such as dermal fibroblasts, promotes differentiation of the monocytes into typical LCs and IDCs. By « typical », the inventors mean: close to their homologs *in vivo* in terms of immature phenotype and functionality, i.e., of their capabilities of reacting after stimulation, stress, etc.

The proportion of epithelial cells and/or mesenchymatous cells, relatively to the CD14+ monocytes, used for the culture (for the differentiation) depends on the cell distribution between LCs and/or IDCs and epithelial cells and/or mesenchymatous cells, which one wishes to obtain.

The invention according to a second aspect relates to a method for growing CD14+ monocytes, the growing method comprising the integration into a cell or tissue model of CD14+ monocytes stemming from peripheral circulatory blood of a living being, and notably of a human being, said cell or tissue model comprising epithelial cells, such as keratinocytes and/or mesenchymatous cells, such as dermal fibroblasts, in order to obtain differentiation of CD14+ monocytes within the model into LCs, or IDCs, or into LCs and IDCs, by growing CD14+ monocytes in the presence of epithelial cells, such as keratinocytes and/or mesenchymatous cells, such as dermal fibroblasts.

Advantageously, the growing of monocytes is carried out without adding any exogenous cytokine.

Advantageously, the cell or tissue model is selected from the group consisting in an epidermis model, an epithelium model, a dermis model, a chorion model, a skin model or a model of a mucosa, in particular of a gingival or vaginal mucosa.

Advantageously, the three-dimensional culture model comprises a dermal or chorion matrix support selected from the group consisting of:

- a collagen- or fibrin-based gel or film comprising mesenchymatous cells, in particular fibroblasts,

- a porous matrix which is made from collagen which may contain one or more glycosaminoglycans and/or optionally chitosan, these porous matrices either integrating mesenchymatous cells, in particular fibroblasts, or not,
- an inert support selected from the group consisting of a semi-permeable synthetic membrane, in particular a semi-permeable nitrocellulose membrane, a semi-permeable nylon membrane, a polytetrafluoroethylene (Teflon®, PTFE), a membrane or sponge, a semi-permeable polycarbonate or polyethylene terephthalate (PET) membrane, an inorganic membrane with a capillary porous structure of aluminum oxide (semi-permeable Anopore membrane), a semi-permeable polyester membrane, said inert support either containing mesenchymatous cells, in particular fibroblasts, or not.
- an inert support treated for polycarbonate- or polystyrene-based culture, said inert support either containing mesenchymatous cells, in particular fibroblasts, or not.

Advantageously, the tissue model used comprises said dermal or chorion support on which epithelial cells, in particular keratinocytes, have been deposited at the surface.

Advantageously, the cell or tissue model comprises at least one additional cell type, for example nerve cells and/or endothelial cells and/or melanocytes and/or lymphocytes and/or adipous cells and/or cutaneous annexes, such as bristles, hairs, sebaceous glands.

Advantageously, a portion of the CD14+ monocytes differentiates into endothelial cells and macrophages, in particular when they are put into a cell or tissue model comprising at least mesenchymatous cells.

Advantageously, the method mainly comprises LCs, or IDCs, or a mixture of LCs and IDCs, or a mixture of LCs, IDCs, endothelial cells and macrophages, or a mixture of IDCs, endothelial cells and macrophages.

Advantageously, the cell or tissue model comprises an epithelial portion and a conjunctive matrix and is characterized in that the LCs are essentially localized in the epithelial portion, and in that the IDCs, the macrophages and the endothelial cells are essentially localized in the conjunctive matrix.

The invention according to a third aspect, relates to a cell model comprising at least one of said LC and/or IDC populations, and further optionally comprising a population of macrophages and/or endothelial cells, capable of being obtained according to a method as defined earlier.

Said model is characterized in that the obtained DCs are different from those obtained according to the methods described earlier, the main differences being concerned with synchronization of the cell differentiation steps for LCs and IDCs.

The invention according to the fourth aspect, relates to a tissue model, comprising at least one of said LC and/or IDC populations, and further optionally comprising a population of macrophages and/or endothelial cells, capable of being obtained according to a method as defined earlier, said tissue model being selected from the group consisting of an epidermis model, an epithelium model, a dermis model, a chorion model, a skin model, or a model of a mucosa, in particular of a gingival or vaginal mucosa.

Advantageously, the cell or tissue model described above is immunocompetent.

Advantageously, the tissue model comprises an epithelial portion comprising epithelial cells, such as keratinocytes, and a conjunctive matrix comprising mesenchymatous cells, such as dermal or chorion fibroblasts, said model being characterized in that the LCs are essentially localized in the epithelial portion, and in that the present IDCs and macrophages and/or endothelial cells are essentially localized in the conjunctive matrix.

Said model is characterized in that the obtained DCs are different from those obtained according to the method described earlier, the main differences being concerned with the more homogenous cell distribution of LCs and DCCs in the present three-dimensional culture models.

The invention according to a fifth aspect relates to frozen CD14+ monocytes, isolated from the peripheral circulatory blood of a living being, in particular of a human being.

In particular with the invention, it is possible to generate populations of different DCs, the different functionalities of which may account for the whole of the phenomena involved in the organism's defense/infection processes, such as irritation, allergenicity and sensitization phenomena.

Thus, the invention according to sixth aspect, relates to the use of at least one cell or tissue model as defined earlier as an investigation model in the field of cosmetics, dermo-pharmacy or pharmacy, and/or for active ingredient selection.

Another interesting use of LCs and/or IDCs consists of using them for assessing the irritating versus the sensitizing potency of new molecules. European directive 2003/15/EC, which prohibits since 2005 the use of animals for assessing the toxicity of a cosmetic finished product, strongly urges public and industrial laboratories to develop *in vitro* or *in silico* predictive methods for predicting the sensitizing potency of new molecules. In this context, because of their key role, in triggering contact allergy, the use of cutaneous DCs as an alternative method to animal experimentation, is today an axis of development.

The invention according to a seventh aspect relates to the use of at least one cell or tissue model as defined earlier, for investigating phenomena occurring in the organism's defense/infection processes, and activity, in particular the immuno-stimulating or immunosuppressive activity, of an

active ingredient or for assessing or for inducing immunomodulation (immunotolerance or immunoactivation) by said active ingredient, or for conducting *in vitro* tests for predicting the allergizing/irritating/sensitizing potency of external agents or for investigating toxicity of molecules or chemicals.

The invention according to an eighth aspect relates to the use of at least one cell or tissue model as defined earlier, for investigating the physiopathology of epithelial barriers; irritation of the skin and mucosae; aggressions of a biological nature such as for example viruses, retroviruses, such as HIV, bacteria, fungi, micro-organisms, particle antigens; phototoxicity; photoprotection; the effect of active ingredients, in particular of cosmetic or pharmaceutical ingredients; the effect of finished products, in particular of cosmetic or pharmaceutical products: the effect of molecules or chemicals; the mechanisms for infection by a pathogenic agent.

Advantageously, this invention relates to investigating toxicity of active ingredients or other substances. Notably this investigation is carried out by studying cell labels, notably including those of DCs.

The invention according to a ninth aspect relates to the use of at least one cell or tissue model as defined earlier, for investigating mechanisms involved in the phenomena of viral infection, replication and transmission of viruses, including retroviruses like HIV, or for investigating and developing alternative therapeutic methods, including the administration of vaccines or drugs.

The invention according to a tenth aspect relates to the use of at least one cell or tissue model as defined earlier for detecting the presence of a pathogenic agent such as for example viruses, retroviruses, such as HIV, bacteria, fungi, micro-organisms, particle antigens.

The invention according to an eleventh aspect relates to the use of at least one cell or tissue model as defined earlier, for a medical, biomedical, or cosmetic application, in particular for modulating the immune or tolerance response *in vitro* or *in vivo*, as a result of an environmental aggression, in particular of the physical type, such as UV irradiation, of the chemical type, such as irritating/allergizing/sensitizing agents, of the biological type, in particular with a preventive or curative therapeutic purpose.

The invention according to a twelfth aspect relates to the use of at least one cell or tissue model as defined earlier, for cell or tissue engineering applications, for medical or biomedical applications, for example in anti-cancer cell therapy, for example by a DCs injection capable of stimulating the immune response; for example in cell therapy in the case of an auto-immune disease, for example by creating an immunotolerance stimulation, for example by producing anergic

T cells; for example in gene therapy of diseases affecting the immunitary system; or for developing and making vaccines.

The invention according to a thirteenth aspect relates to a method for making a tissue model comprising:

- isolation of CD14+ monocytes from peripheral circulatory blood of a living being,
- sowing skin or mucosal cells on a support and growing them in a nutritious medium in order to obtain reconstructed tissue,
- sowing in the reconstructed tissue CD14+ monocytes either simultaneously or not with skin or mucosal cells,
- growing the reconstructed tissue comprising CD14+ monocytes and skin or mucosal cells under conditions allowing differentiation of CD14+ monocytes into LCs, into a mixture of IDCs, endothelial cells and macrophages, into a mixture of LCs, IDCs, endothelial cells and macrophages, the skin cells being epidermal keratinocytes when the reconstructed tissue is an epidermis model, the skin cells being dermal fibroblasts when the reconstructed tissue is a dermis model, the mucosal cells being epithelial mucosal cells when the reconstructed tissue is an epithelium model, the mucosal cells being mucosal fibroblasts when the reconstructed tissue is a chorion model.

The epithelial cells are for example isolated from at least one skin tissue.

Sowing of the skin or mucosal cells may be carried out before sowing or after having sown CD14+ monocytes.

Preferably, sowing of CD14+ monocytes is carried out simultaneously with the sowing of skin or mucosal cells.

The invention according to a fourteenth aspect relates to a method for making a reconstructed skin or a reconstructed mucosa comprising an epithelial portion comprising keratinocytes or epithelial mucosal cells, optionally in the presence of Merkel cells and/or melanocytes, and a conjunctive matrix comprising dermal or mucosal fibroblasts, said method comprising:

- sowing and growing in a nutritious medium, keratinocytes or epithelial mucosal cells, possibly in the presence of Merkel cells and/or melanocytes, at the surface of a dermis or chorion model, comprising IDCs and optionally endothelial cells and macrophages, said dermis or chorion model being able to be obtained by a method for making a dermis or chorion model as defined above,
- sowing and growing either simultaneously with keratinocytes or epithelial mucosal cells or not, CD14+ monocytes isolated from peripheral circulatory blood of a living being in the presence of keratinocytes or epithelial mucosal cells under conditions allowing differentiation of CD14+ monocytes into LCs.

Advantageously, the cells are cells of a human being. Preferably, these methods for making tissue models do not comprise any addition of exogenous cytokine.

Other objects, features and advantages of the invention will become clearly apparent to one skilled in the art after reading the explanatory description which refers to examples which are only given as an illustration and which may by no means limit the scope of the invention.

The examples are an integral part of the presence invention and any feature which appears to be novel relatively to any prior state of the art, from the description taken as a whole, including the examples, is an integral part of the invention in its function and in its generality.

Thus, each example has a general scope.

On the other hand, in the examples all the percentages are given by weight, unless stated otherwise, and the temperature is expressed in degrees Celsius, unless stated otherwise, and the pressure is atmospheric pressure, unless stated otherwise.

EXAMPLES

Example 1: Methods for separating monocytes from peripheral circulatory blood

The peripheral circulatory blood was harvested by sampling venous blood on one or more human donors, preferably in vacutainers or bags supplemented with usual anti-coagulant products such as lithium heparin.

Separation of monocytes from circulatory blood may advantageously be performed according to the following protocols:

1/ After centrifuging blood on a lymphocyte separation medium, the mononucleated cells are recovered, and then:

- Either labeled with a cocktail of antibodies, such as for example anti-CD3, anti-CD7, anti-CD16, anti-CD19, anti-CD56, anti-CD123 antibodies, anti-glycophorin A coupled with magnetic beads. After passing over a magnetized column, only the non-labeled monocytes are eluted and recovered,
- Or labeled with a specific antibody of the monocytes, such as an anti-CD14 antibody, coupled with magnetic beads; after passing over a magnetic column, only the labeled monocytes are retained in the column. After elution from the column, the labeled monocytes are recovered.
- Or labeled with a specific antibody of the monocytes, such as an anti-CD16 antibody, coupled with a fluorochrome, such as phycoerythrin. After cell sorting in flux cytometry, only the labeled monocytes are recovered.

2/ The monocytes are recovered by proceeding with any physical separation method well-known to one skilled in the art and notably by sedimentation or centrifugation and they are eluted as such for subsequent cultures.

3/ For 100 mL of sample blood, up to about 150 million (± 20 million) mononucleated cells are extracted and up to 40 million monocytes are purified.

Example 2: Method for freezing monocytes isolated from peripheral circulatory blood

The monocytes, as obtained in Example 1, are suspended in a nutritious medium, for example RPMI medium, supplemented with serum and a cryoprotective agent, such as DMSO (*dimethyl sulfoxide*), and then frozen.

When thawing out monocytes, cell mortality is less than 30%.

For 100 mL of sample peripheral circulatory blood, up to 80.10^6 monocytes are frozen, and up to 76.10^6 monocytes are recovered after thawing them out.

Example 3: A pluricellular monolayer model of keratinocytes and LCs in a co-culture

Obtaining the monocytes: see Example 1 or 2.

1 to 2.10^6 human keratinocytes and 1 to 2.10^6 human monocytes (obtained according to Example 1 or 2) are jointly grown in a nutritious medium, for example of the K-SFM type, in culture dishes, for example of the 6-well plate type. The joint culture is maintained for 6 days in a nutritious medium, for example of the K-SFM type, without adding any exogenous cytokine.

The cells are then recovered by an enzymatic method well-known to one skilled in the art and notably by trypsination. 2.10^5 cells of the mixed cell suspension consisting of keratinocytes and monocytes are incubated with a monoclonal anti-Langerine antibody, and then analyzed in flux cytometry. We observe up to 40% of Langerine+ LCs.

Example 4: Pluricellular monolayer model of fibroblasts and DDC in a co-culture

Obtaining the monocytes: see Example 1 or 2.

1 to 2.10^6 human fibroblasts and 1 to 2.10^6 human monocytes (obtained according to Example 1 or 2) are jointly grown in a nutritious medium, for example of the FBM type, in culture dishes for example of the 6-well plate type. The joint culture is maintained for 6 days in a nutritious medium, for example of the FBM type, without adding any exogenous cytokine.

The cells are then recovered by an enzymatic method well-known to one skilled in the art and notably by trypsination. 2.10^5 cells of the mixed cell suspension consisting of fibroblasts and

monocytes are incubated with a monoclonal anti-DC-SIGN antibody, and then analyzed in flux cytometry. We observe up to 60% of DC-SIGN+ DCCs.

Example 5: The use of monolayer pluricellular models described in Examples 3 and 4 for investigating the profile of cytokines secreted under the effect of an active ingredient

In order to assess the irritating, sensitizing, allergizing potency, and to estimate a possible pro- or anti-inflammatory activity of an active ingredient intended for human skin, we quantify in the culture supernatants, secretion of cytokines, for example, IL-1, IL-6, IL-8, IL-10, TNF-□ INF□, according to the following protocol:

- The model is made according to the protocol described in Example 3 or 4.
- Retinol is then added into the culture medium at a final concentration of 0.05% for 3 days.
- The culture supernatants are then recovered and analyzed.

It is observed that retinol causes stimulation of secretion of pro-inflammatory cytokines.

Example 6: The use of pluricellular monolayer models described in Examples 3 to 4 for investigating the immunoactivating or immunosuppressive activity of an active ingredient

In order to assess the capacity of the LCs and DCCs of inducing immune and/or tolerogenic responses towards an active ingredient or not, we studied their phenotype profile by flux cytometry, according to the following protocol:

- The model is made according to the protocol described in Example 3 or 4.
- The active ingredient is then added into the culture medium at various concentrations and for 3 days.
- The cells are then recovered by trypsination. 2.10^5 cells of the mixed cell suspension consisting of keratinocytes and LCs or of fibroblasts and DCCs are incubated in an antibody battery: anti-CCR7, anti-HLA-DR, anti-CD80, anti-CD83, anti-CD86, anti-DC-LAMP.

With cell phenotyping, the immunoactivating (induction and/or increase of the expression of labeled molecules) or immunosuppressive profile (inhibition and/or suppression of the expression of labeled molecules) of the tested active ingredients may be established.

Example 7: The use of pluricellular monolayer models described in Examples 3 to 4 for investigating the immunomodulating activity of an active ingredient

The immunomodulating effect of an active ingredient after an irritating, sensitizing or allergizing stress is investigated according to the following protocol:

- The model is made according to the protocol described in Example 3 or 4.

- 300 µl of TNP (*2,4,6-trinitrobenzene-sulfonic acid*) are then added at a concentration of 5 mM for 30 min at 37°C.
- After this stimulation, the active ingredients tested at various concentrations are added into the culture medium for 2 days.
- The culture media comprising the cells are then recovered in order to investigate the secretion of immune cytokines, for example IL-12; the cells are recovered and then incubated either with an anti-Langerine antibody or with an anti-DC-SIGN antibody in order to sort the LCs or the DCCs in flux cytometry, respectively.
- The sorted LCs or DCCs are then sown in migration chambers of the Boyden type (porosity of the membrane from 5 to 8 µm either covered with MATRIGEL™ or not) and then incubated for up to 72 hrs at 37°C.
- The number of LCs or DCCs having migrated is quantified, for example by counting in optical microscopy.

The results of migration and/or IL-12 synthesis and secretion tests enable the immunomodulating profile of the active ingredients tested to be established.

Example 8: Three-dimensional pluricellular reconstructed epidermis model containing LCs

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the following protocol:

- 0.5 to 1.10^6 keratinocytes of normal human skin are sown in inserts of the Boyden chamber type (membrane with porosity 0.4 µm) and then grown in a nutritious medium, for example of the DMEM-Glutamax type supplemented with calf serum, ascorbic acid and preferably a final concentration of 1 mM of EGF (*epidermal growth factor*) and preferably at a final concentration of 10 ng/mL, hydrocortisone and preferably at a final concentration of 0.4 µg/mL, umulin and preferably at a final concentration of 0.12 IU/mL, isuprel and preferably at a final concentration of 0.4 µg/mL, triiodothyronine and preferably at a final concentration of 2.10^{-9} M, adenine and preferably at a final concentration of 24.3 µg/mL, normocin and preferably at a final concentration of 100 µg/mL, for 2 days.
- Next 1 to 5.10^4 monocytes are sown at the surface of the epithelium equivalent, which is grown for a further period of 2 days.
- The cultures are then placed at the air-liquid interface for a further period of 10 days in the same medium used for the immersion culture, except for calf serum, hydrocortisone, umulin, isuprel and triiodothyronine.

- The cultures are then embedded in an amorphous resin, such as Tissue-Teck®, and directly frozen in liquid nitrogen.
- Immunohistochemical investigations are then conducted on histological sections in order to characterize the cell types by means of specific antibodies.
- The performed labelings show the presence of LCs (Langerine+ cells).

Conclusion

The monocytes integrated into the reconstructed epidermis model differentiate into DCs of the LC type as demonstrated by observation of the lectin, Langerine.

Example 9: Three-dimensional pluricellular pigmented and/or nervous reconstructed epidermis model containing LCs

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the protocol described in Example 8, by simultaneously sowing together 0.5 à 1.10^5 melanocytes and/or Merkel cells stemming from normal human skin with keratinocytes.

In addition to the labelings described in Example 8, labeling of the melanocytes (HMB45) and a DOPA reaction are carried out in order to detect melanin as well as labeling with an anti-keratin 20 antibody for identifying Merkel cells.

Conclusion

The monocytes integrated into the pigmented and/or nervous reconstructed epidermis model differentiate into DCs of the LC type as demonstrated by observation of the lectin, Langerine.

Example 10: Three-dimensional pluricellular epithelium model of gingival and vaginal reconstructed mucosae containing LCs

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the protocol described in Example 8 with the following changes:

- Normal human keratinocytes are replaced with epithelial cells of normal human gingival and vaginal mucosae.
- The percentage of serum in the culture medium is 1%.
- Growing is totally performed with immersion in the medium.
- The achieved labelings show the presence of LCs (Langerine+ cells).

Conclusion

The monocytes integrated into the epithelium model of gingival and vaginal reconstructed mucosae differentiate into DCs or the LC type as demonstrated by observation of the lectin Langerine.

Example 11: Three-dimensional pluricellular reconstructed skin model

This model is the association of a reconstructed dermis culture on which an additional culture of a reconstructed epidermis is then performed.

The reconstructed dermis model is made according to the following protocol:

- 0.5 to 1.10^6 fibroblasts of normal human skin are sown on a matrix substrate based on cross-linked collagen with diphenyl-phosphoryl azide, and then grown in a nutritious medium, for example DMEM-Glutamax supplemented with 10% calf serum, ascorbic acid and preferably at a final concentration of 1 mM, EGF (*epidermal growth factor*) and preferably at a final concentration of 10 ng/mL, normocin and preferably at a final concentration of 100 µg/mL, for 14 days.

The reconstructed skin model is made according to the following protocol:

- 0.5 to 1.10^6 normal human keratinocytes are sown on the dermal equivalent, and then grown in a nutritious medium, for example DMEM-Glutamax/Ham F-12 (ratio 3/1 v/v) supplemented with calf serum, ascorbic acid and preferably at a final concentration of 1 mM, EGF (*epidermal growth factor*) and preferably at a final concentration of 10 ng/mL, hydrocortisone and preferably at a final concentration of 0.4 µg/mL, umulin and preferably at a final concentration of 0.12 IU/mL, isuprel and preferably at a final concentration of 0.4 µg/mL, triiodothyronine and preferably at a final concentration of 2.10^{-9} M, adenine and preferably at a final concentration of 24.3 µg/mL, normocin and preferably at a final concentration of 100 µg/mL. Growing continues for 7 days in the immersed condition.

The cultures are then placed at the air-liquid interface for 14 additional days in the same medium as the immersion culture, except for calf serum, hydrocortisone, isuprel, triiodothyronine and umulin.

Conclusion

We are capable of reconstructing *in vitro* both cell compartments of the skin which are the epidermis and the dermis.

Example 12: Three-dimensional pluricellular reconstructed dermis model containing DCCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

Growing the reconstructed dermis is performed according to Example 11.

The model is made according to the following protocol:

- 1 to 5.10^4 monocytes are sown at the surface of the dermal equivalent, which is grown for a further period of 7 days,
- The cultures are then embedded in an amorphous resin, such as Tissue-Teck®, and then directly frozen in liquid nitrogen.
- Immunohistochemical investigations are carried out on histological sections in order to characterize the cell types present by means of specific antibodies.
- The performed labelings show the simultaneous presence of DDC (DC-SIGN+ cells), macrophages (CD68+ cells) and endothelial cells (CD31+ and CD36+ cells).

Conclusion

The monocytes integrated and then grown in our reconstructed dermis model are capable of simultaneously differentiating into DCs of the DDC type, into macrophages and endothelial cells.

Example 13: Three-dimensional pluricellular reconstructed chorion model containing IDCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the protocol described in Example 12 with the following changes:

- The fibroblasts are fibroblasts stemming from normal human gingival and vaginal mucosae.
- The performed labelings show the simultaneous presence of IDCs (DC-SIGN+ cells), macrophages (CD68+ cells) and endothelial cells (CD31+ and CD36+ cells).

Conclusion

The monocytes integrated and then grown in our reconstructed chorion model of the gingival and vaginal type are capable of simultaneously differentiating into DCs of the IDC type, into macrophages and endothelial cells.

Example 14: Three-dimensional pluricellular reconstructed skin model containing LCs

Obtaining the monocytes: see Example 1 or 2.

The culture of the reconstructed skin model is performed according to Example 11.

The model is made according to the following protocol:

- 1 to 5.10^4 monocytes are jointly sown with keratinocytes on the dermal equivalent.
- The performed labelings show the presence of LCs (Langerine+ cells and Birbeck+ granules) in the epidermal compartment of the cultures.

Conclusion

The monocytes grown with keratinocytes in our reconstructed skin model differentiate into DCs of the LC type.

Example 15: Three-dimensional pluricellular pigmented and/or nervous reconstructed skin model containing LCs

Obtaining the monocytes: see Example 1 or 2.

Growing the reconstructed skin model containing LCs is performed according to Example 14.

The model is made according to the following protocol:

- 1 to 5.10^4 melanocytes and/or Merkel cells stemming from normal human skin are jointly sown with keratinocytes and monocytes on the reconstructed dermis.
- In addition to the labelings described in Example 14, labeling of the melanocytes (HMB45) and a DOPA reaction are carried out in order to detect melanin, as well as labeling with an anti-keratin 20 antibody for identifying Merkel cells.

Conclusion

The monocytes grown with keratinocytes, melanocytes and/or Merkel cells in our reconstructed skin model differentiate into DCs of the LC type.

Example 16: Three-dimensional pluricellular reconstructed mucosa model containing LCs

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the protocol described in Example 14 with the following changes:

- Keratinocytes are replaced with epithelial cells of normal human gingival and vaginal mucosae, and the fibroblasts are fibroblasts stemming from normal human gingival and vaginal mucosae.
- The percentage of calf serum in the culture medium for the epithelialization step is 1%.
- Growing is totally accomplished in immersion in the medium
- The performed labelings show the presence of LCs (Langerine+ cells and Birbeck+ granules) in the epithelial compartment of the cultures.

Conclusion

The monocytes grown with epithelial cells of the gingival or vaginal type in our reconstructed mucosa model differentiate into DCs of the LC type.

Example 17: Three-dimensional pluricellular reconstructed skin model containing DCCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

Growing the reconstructed skin model is achieved according to Example 11.

The model is made according to the following protocol:

- 1 to 5.10^4 monocytes are sown at the surface of the dermal equivalent which is grown for a further period of 7 days.
- The performed labelings show the simultaneous presence of DCCs (DC-SIGN+ cells), macrophages (CD68+ cells) and endothelial cells (CD31+ and CD36+ cells).

Conclusion

The monocytes integrated into the dermal compartment of the reconstructed skin model and grown therein differentiate into DCs of the DDC type, into macrophages and endothelial cells, simultaneously.

Example 18: Three-dimensional pluricellular pigmented and/or nervous reconstructed skin model containing DCCs, macrophages and endothelial cells

The model is made according to the protocol described in Example 17, by simultaneously sowing together 1 to 5.10^4 melanocytes and/or Merkel cells stemming from normal human skin with keratinocytes.

In addition to the labelings described in Example 17, labeling of melanocytes (HMB45) and a DOPA reaction are carried out in order to detect melanin, as well as a labeling with anti-keratin 20 antibody for observing Merkel cells.

Conclusion

The monocytes integrated into the dermal compartment of the pigmented and/or nervous reconstructed skin model and grown therein differentiate into DCs of the DDC type, into macrophages and endothelial cells simultaneously.

Example 19: Three-dimensional pluricellular model of reconstructed mucosae containing IDCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the protocol described in Example 17 with the following changes:

- The keratinocytes are replaced with epithelial cells of normal human gingival and vaginal mucosae, and the fibroblasts are fibroblasts stemming from normal human gingival and vaginal mucosae.
- The percentage of calf serum in the culture medium for the epithelialization step is 1%.
- Growing is totally performed in immersion in the medium.
- The performed labelings show the simultaneous presence of IDCs (DC-SIGN+ cells), macrophages (CD68+ cells) and endothelial cells (CD31+ cells and CD36+).

Conclusion

The monocytes integrated into the conjunctive compartment of the reconstructed mucosa model of the gingival and vaginal type and grown therein differentiate into DCs of the IDC type, into macrophages and into endothelial cells, simultaneously.

Example 20: Three-dimensional pluricellular reconstructed skin model containing LCs, DCCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the following protocol:

- The reconstructed dermis model containing DCCs, macrophages and endothelial cells is made according to Example 12.
- The reconstructed skin model containing LCs is made according to Example 14.
- The performed labelings showing the simultaneous presence of LCs (Langerine+ cells and Birbeck+ granules) in the epidermal compartment of the cultures, and of DCCs (DC-SIGN+

cells), macrophages (CD68+ cells) and endothelial cells (CD31+ and CD36+ cells) in the dermal compartment of the cultures.

Conclusion

The monocytes successively grown in the dermal compartment and then with keratinocytes in the reconstructed skin model differentiate into DCs of the LC type in the epidermal compartment and into DCs of the DDC type, into macrophages and endothelial cells, in the dermal compartment, simultaneously.

Example 21: Three-dimensional pluricellular pigmented and/or nervous reconstructed skin model containing LCs, DCCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the following protocol:

- The reconstructed dermis model containing DCCs, macrophages and endothelial cells is made according to Example 12.
- The pigmented and/or nervous reconstructed skin model containing LCls is made according to Example 15.
- The performed labelings detect simultaneous presence of LCs (Langerine+ cells and Birbeck granules) in the epidermal compartment of the cultures, and of DCCs (DC-SIGN+ cells), macrophages (CD68+ cells) and endothelial cells (CD31+ and CD36+ cells) in the dermal compartment of the cultures.

Conclusion

Te monocytes successively grown in the dermal compartment and then simultaneously with keratinocytes, melanocytes and/or Merkel cells in the pigmented and/or nervous reconstructed skin model differentiate into DCs of the LC type in the epidermal compartment and into DCs of the DDC type, into macrophages and into endothelial cells in the dermal compartment simultaneously.

Example 22: Three-dimensional pluricellular reconstructed mucosa model containing LCs, IDCs, macrophages and endothelial cells

Obtaining the monocytes: see Example 1 or 2.

The model is made according to the following protocol:

- The reconstructed chorion model containing IDCs, macrophages and endothelial cells is made according to Example 13.
- The reconstructed mucosa model containing LCs is made according to Example 16.
- The performed labelings show the simultaneous presence of LCs (Langerine+ cells and Birbeck+ granules) in the epithelial compartment, and the presence of IDCs (DC-SIGN+ cells), macrophages (CD68+ cells) and endothelial cells (CD31+ and CD36+ cells) in the conjunctive compartment of the cultures.

Conclusion

The monocytes successively cultivated in the conjunctive compartment and then with epithelial cells in the reconstructed mucosa model of the gingival of vaginal type differentiate into DCs of the LC type in the epithelial compartment and into DCs of the IDC type, into macrophages and endothelial cells in the conjunctive compartment simultaneously.

Example 23: Use of the three-dimensional pluricellular reconstructed skin model described in Example 20 for investigating the influence of solar UV radiation

In order to investigate the influence of various environmental factors and in particular of solar UV radiation, we assessed the migration and the phenotype profile of LCs and DCCs in the reconstructed skin model by immuno-histochemical investigations, according to the following protocol:

- The model is made according to protocol described in Example 20
- On the 42nd day of culture, the reconstructed skins are irradiated, for example at a single dose of 526 kJ/cm^2 of solar energy corresponding to 2 J/cm^2 of UVA and 0.5 J/cm^2 of UVB (solar irradiator Suntest CPS+, ATLAS). Growing is continued for a further period of 2 days.
- The immuno-histochemical investigations are then made with an antibody battery (anti-CD1a, anti-CD80, anti-CD83, anti-CD86, anti-CCR7, anti-DC-LAMP, anti-DC-SIGN, anti-Langerine, anti-HLA-DR) in order to observe migration of the LCs and DCCs and to characterize their phenotype condition.

After solar UV irradiation, migration of the LCs is observed in the dermal compartment of the cultures, as well as acquisition of the expression of the CCR7 receptor on the Cls and DCCs. Further, only the LCs which have migrated into the dermal compartment, express the maturation label DC- LAMP.

Conclusion

Our pluricellular model is therefore predictive of UV stress by the targeting of LCs and DCCs which have migration, activation and maturation capability.

Example 24: The use of the three-dimensional pluricellular reconstructed skin model described in Example 20 for investigating the efficiency of active ingredients

In order to assess the anti-inflammatory potency of active ingredients intended for human skin, we quantify in the culture supernatants, the secretion of pro-inflammatory cytokines, for example IL-1, IL-6, IL-8, TNF-alpha and INF-gamma, according to the following protocol:

- The model is made according to the protocol described in Example 20.
- On the 42nd day of culture, the reconstructed skins are irradiated, for example with a unique dose of 526 kJ/cm^2 of solar energy corresponding to 2 J/cm^2 of UVA and 0.5 J/cm^2 of UVB (solar irradiator Suntest CPS+, ATLAS).
- Subsequently, 8 µl of a cosmetic formulation either containing 3% anti-oxidant active for example Flavagrum® (Hesperitine Laurate, ENGELHARD) and Flavenger® (Quercitine Caprylate, ENGELHARD) or not, are applied on the reconstructed skins for 10 days.
- At the end of the treatment, the reconstructed skins are grown for a further period of 48 hours in the immersion medium and then the efficiency of the anti-oxidant treatment is assessed by: 1/ analysis of cell viability (*methylthiazoletetrazolium* - MTT test), 2/ flux cytometry dosage (CBA – *Cytometric Bead Array*) of the secretion of cytokines in the culture supernatants.

	Reconstructed skin Control	Reconstructed skin UV irradiated	Reconstructed skin UV irradiated + Flavagrum	Reconstructed skin UV irradiated + Flavenger
MTT (viability	100%	76%	88%	92%
IL1β (pg/mL)	76	179	125	97
IL6 (pg/mL)	379	649	452	426
IL8 (pg/mL)	275	395	312	294
TNFβ (pg/mL	53	152	105	89

With the inventive methods, it is possible to see that solar UV stress induces a reduction in the cell viability as well as an increase in the synthesis of pro-inflammatory interleukins. It is therefore of interest to limit this synthesis of pro-inflammatory molecules as well as cell mortality by using properly selected active ingredients. Among the screened active ingredients, two of them,

Flavagrum and Flavenger, have demonstrated efficiency with a possible tendency to restore the reference level for both of these parameters.

Example 25: Use of the three-dimensional pluricellular reconstructed skin model described in Examples 14, 17 and 20 for investigating the immunostimulating or immunosupressive activity of an active ingredient

In order to assess whether LCs and/or DCCs are capable of inducing immune and/or tolerogenic responses towards an active ingredient or not, we investigated the functionality of the cells to stimulate proliferation of allogenic naive T lymphocytes, according to the following protocol:

- The model is made according to the protocol described in Examples 14, 17 and 20.
- On the 42nd day of culture, either the active ingredient is directly applied on the reconstructed skins by means of a swab (topical application) or the active ingredient is introduced into the culture medium (systemic application) for 10 days.
- At the end of the treatment, the cultures are successively digested by 2 hr enzymatic treatments at 37°C with trypsin (1 mg/mL) and with collagenase (1 mg/mL) in order to recover the LCs and/or DCCs.
- The LCs and/or DCCs are grown for 3 days with allogenic naive T lymphocytes in an RPMI culture medium supplemented with 10% human AB serum. A range of LCs and/or DCs between 125 and 8,000 cells is achieved and grown with 10^5 naive T lymphocytes. On the 3rd day of the mixed lymphocyte culture, 20 µl of 5 mCi tritiated thymidine are added for a period of 18 hrs.
- The results are expressed on a graph with the number of LCs and/or DCCs in abscissae and in ordinates the incorporation of tritiated thymidine into allogenic naive T lymphocytes expressed in cpm (*counts per minute*).

Number of LCs or DCCs	CPM (untreated)	CPM (active)
125	500	500
250	800	1500
500	1000	10000
1000	1500	22500
2000	2300	39850
4000	2800	47250
6000	3000	52500
8000	3800	55000

After treatment with our active X, the LCs and/or DCCs strongly stimulate proliferation of T lymphocytes (between 5.10^4 and 7.10^4 cpm) as compared with the untreated LCs and/or DCCs which only induce weak proliferation of naive T lymphocytes (between 1.10^3 and 4.10^3 cpm).

Example 26: Use of three-dimensional pluricellular models for performing screening of active ingredients capable of modulating allergic reactions

The immunomodulating effect of an active ingredient after inducing an irritating, sensitizing and allergizing stress is investigated according to the following protocol:

- The model is made according to the protocol described in Example 12.
- After 21 days of culture, the irritant (*sodium dodecyl sulphate*) preferably at the final concentration of 2% and the sensitizer DNCB (*1,4-chloro-dinitrobenzene*) preferably at a final concentration of 0.25% are introduced into the culture medium for 24 hrs.
- Next, 100 µl of a cosmetic formulation either containing a soothing active at the final concentration of 3% or not, are introduced into the culture medium of equivalent dermises for 3 days.
- At the end of the treatment, the culture supernatants are recovered for dosing IL-10 and IL-12 for example, and an immunohistochemical study is developed with an antibody battery: anti-CD80, anti-CD83, anti-CD86, anti-CCR7, anti-DC-LAMP, anti-HLA-DR.

With the results of the IL-10 and IL-12 secretion and of the phenotype study of the DCCs by immunohistochemistry, the immunomodulating profile of the tested active ingredients may be established.

Conclusion

Our pluricellular model is therefore a predictive tool with which it is possible to assess the potential immmunomodulating effect of an active ingredient by screening DCCs which have cytokine secretion, activation and maturation capabilities.

CLAIMS

1. A method for preparing Langerhans cells (LC), or interstitial/dermal dendritic cells (IDC), or LCs and IDCs from CD14+ monocytes stemming from peripheral circulatory blood of a living being, and notably of a human being, said method comprising placing the CD14+ monocytes in the presence of a cell environment comprising epithelial cells, such as keratinocytes, and/or mesenchymatous cells, such as dermal fibroblasts, for differentiating the CD14+ monocytes into LCs or into IDCs, or into LCs and IDCs.

2. The method according to claim 1, characterized in that the method comprises placing the CD14+ monocytes in the presence of a cell environment comprising epithelial cells, such as keratinocytes, and/or mesenchymatous cells, such as dermal fibroblasts, for differentiating CD14+ monocytes into IDCs, and macrophages and/or endothelial cells; or LCs, IDCs, macrophages and/or endothelial cells.

3. The method according to any of the preceding claims, characterized in that differentiation of CD14+ monocytes is carried out without any significant addition of exogenous cytokine.

4. The method according to any of the preceding claims, characterized in that the method comprises placing monocytes in the presence of a cell environment comprising epithelial cells such as keratinocytes, for promoting the differentiation of monocytes into immature and functional LCs.

5. The method according to any of the preceding claims, characterized in that the method comprises placing monocytes in the presence of a cell environment comprising mesenchymatous cells, such as dermal fibroblasts, for promoting the differentiation of the monocytes into immature and functional IDCs.

6. The method according to any of the preceding claims, characterized in that the method comprises placing monocytes in the presence of a cell environment comprising epithelial cells, such as keratinocytes, and mesenchymatous cells, such as dermal fibroblasts, for promoting the differentiation of the monocytes into typical LCs and IDCs.

7. A method for growing CD14+ monocytes, the growing method comprising the integration in a cell or tissue model, of CD14+ monocytes stemming from peripheral circulatory blood of a living

being, and notably of a human being, said cell or tissue model comprising epithelial cells, such as keratinocytes, and/or mesenchymatous cells, such as dermal fibroblasts, in order to obtain differentiation of the CD14+ monocytes within the model into LCs, or IDCs, or LCs and IDCs, by growing the CD14+ monocytes in the presence of epithelial cells, such as keratinocytes, and/or mesenchymatous cells, such as dermal fibroblasts.

8. The method according to claim 7, characterized in that growing of CD14+ monocytes is performed without any significant addition of exogenous cytokine.

9. The method according to claim 7 or 8, characterized in that the cell or tissue model is selected from the group consisting of an epidermis model, an epithelium model, a dermis model, a chorion model, a skin model and a mucosa model.

10. The method according to any of claims 7 to 9, characterized in that the cell or tissue culture model comprises a dermal or chorion matrix support selected from the group consisting of:

- a collagen- or fibrin-based gel or film comprising mesenchymatous cells, in particular fibroblasts,
- a porous matrix which is made from collagen, which may contain one or more glycosaminoglycans and/or optionally chitosan, these porous matrices either integrating mesenchymatous cells, in particular fibroblasts, or not,
- an inert support selected from the group consisting of a semi-permeable synthetic membrane, in particular a semi-permeable nitrocellulose membrane, a semi-permeable nylon membrane, a polytetrafluoroethylene (Teflon®, PTFE) membrane or sponge, a semi-permeable polycarbonate or polyethylene terephthalate (PET) membrane, an inorganic membrane with a capillary porous structure of aluminum oxide (semi-permeable Anopore membrane), of cellulose acetate or ester (HATF), a semi-permeable Biopore-CM membrane, a semi-permeable polyester membrane, said inert support either containing mesenchymatous cells, in particular fibroblasts or not,
- an inert support treated for polycarbonate- or polystyrene-based culture, said inert support either containing mesenchymatous cells, in particular fibroblasts, or not.

11. The method according to claim 10, characterized in that the cell or tissue module used comprises said dermal or chorion support on which epithelial cells in particular keratinocytes, have been placed at the surface.

12. The method according to any of claims 10 and 11, characterized in that the cell or tissue model comprises at least one additional cell type, for example nerve cells and/or Merkel cells, and/or endothelial cells and/or macrophages, and/or melanocytes and/or lymphocytes and/or adipous cells, and/or cutaneous annexes, like bristles, hairs, sebaceous glands.

13. The method according to any of claims 7 to 12, characterized in that the method comprises growing CD14+ monocytes in a cell or tissue model comprising at least mesenchymatous cells so that a portion of the monocytes differentiates into endothelial cells and/or into macrophages.

14. The method according to any of claims 7 to 12, characterized in that the cell or tissue model comprises an epithelial portion and a conjunctive matrix, and in that the LCs are essentially localized in the epithelial portion, and in that the IDCs, macrophages and/or endothelial cells are essentially localized in the conjunctive matrix.

15. A method for differentiating CD14+ monocytes into LCs and/or IDCs, comprising the following steps:

- collecting CD14+ monocytes from circulatory blood;
- maintaining CD14+ monocytes under conditions which do not promote their differentiation into DCs;
- placing the CD14+ monocytes in the presence of a cell environment comprising epithelial cells, for example keratinocytes, and/or mesenchymatous cells, for example fibroblasts, under conditions promoting differentiation of the CD14+ monocytes into LCs, or IDCs, or LCs and IDCs.

16. A cell model comprising at least one of said LC and/or IDC populations, and further optionally comprising a population of macrophages and/or endothelial cells, capable of being obtained according to a method as defined in any of claims 1 to 15.

17. A tissue model comprising at least one of said LC and/or IDC populations and further optionally comprising a population of macrophages and/or endothelial cells, capable of being obtained according to a method as defined in any of claims 1 to 15, said tissue model being selected from the group consisting of an epidermis model, an epithelium model, a dermis model, a chorion model, a skin model and a mucosa model.

18. The tissue model according to claim 17, characterized in that it comprises an epithelial portion comprising epithelial cells, such as keratinocytes, and a conjunctive matrix comprising mesenchymatous cells, such as dermal or chorion fibroblasts, and characterized in that the LCs are essentially localized in the epithelial portion and in that the present IDCs and macrophages and/or endothelial cells are essentially localized in the conjunctive matrix.

19. Frozen CD14+ monocytes, isolated from peripheral circulatory blood of a living being, in particular of a human being.

20. The use of at least one cell model as defined in claim 16 or of at least one tissue model as defined in claim 17 or 18, as an investigation model in the field of cosmetics, dermo-pharmacy, pharmacy, and/or for active ingredient selection.

21. The use of at least one cell model as defined in claim 16 or of at least one tissue model as defined in claim 17 or 18, for investigating phenomena occurring in the organism's defense/infection processes and activity, and in particular the immunostimulating or immunosuppressive activity, of an active ingredient or for assessing or inducing immunomodulation by said active ingredient, or for performing *in vitro* tests for predicting the allergizing, irritating, sensitizing potency of external agents, or for conducting investigations, notably toxicity investigations, of molecules or chemicals.

22. The use of at least one cell model as defined in claim 16 and of at least one tissue model as defined in claim 17 or 18, for investigating physiopathology of epithelial barriers; the irritation of the skin and mucosae; the aggressions of a biological nature such as for example viruses, retroviruses, such as HIV, bacteria, fungi, micro-organisms, particle antigens; photo-toxicity; photo-protection; as well as the effect of active ingredients, in particular cosmetic or pharmaceutical active ingredients; the effect of finished products, in particular of cosmetic or pharmaceutical products; investigating the mechanisms for infection by a pathogenic agent.

23. The use of at least one cell model as defined in claim 16 and or at least one tissue model as defined 17 or 18, for investigating mechanisms involved in the infection, replication and transmission phenomena of viruses, including retroviruses such as HIV, or for searching or developing therapeutic alternative methods, including administration of vaccines or drugs.

24. The use of at least one cell model as defined in claim 16, or of at least one tissue model as defined in claim 17 or 18, for detecting the presence of a pathogenic agent such as for example viruses, retroviruses, such as HIV, bacteria, fungi, micro-organisms, particle antigens.

25. The use of at least one cell model as defined in claim 16, or of at least one tissue model as defined in claim 17 or 18, for a medical, biomedical or cosmetic application, in particular for modulating the immune or tolerance response *in vitro* or *in vivo*, as a result of an environmental aggression, in particular of the physical type, such as UV irradiation, of the chemical type, such as irritating/allergizing/sensitizing agents, of the biological type, in particular with a preventive or curative therapeutic purpose.

26. The use of at least one cell model as defined in claim 16, or of at least one tissue model as defined in claim 17 or 18, for tissue and cell engineering applications, for medical or biomedical applications, for example in anti-cancer cell therapy, for example by a DCs injection capable of stimulating the immune response; for example in cell therapy, in the case of an auto-immune disease, for example by creating an immunotolerance stimulation, for example by producing anergic T cells; for example in gene therapy of a disease affecting the immunitary system; or for developing and making vaccines.

27. A method for making a tissue model comprising:

- isolating CD14+ monocytes from peripheral circulatory blood of a living being,
- sowing skin or mucosal cells on a support and growing them in a nutritious medium in order to obtain a reconstructed tissue,
- sowing in the reconstructed tissue, CD14+ monocytes either simultaneously or not with skin or mucosal cells,
- growing the reconstructed tissue comprising the CD14+ monocytes and the skin or mucosal cells under conditions allowing differentiation of the CD14+ monocytes into LCs, into a mixture of IDCs, endothelial cells and/or macrophages, into a mixture of LCs, IDCs, endothelial cells, and/or macrophages, the skin cells being epidermal keratinocytes when the reconstructed tissue is an epidermis model, the skin cells being dermal fibroblasts when the reconstructed tissue is a dermis model, the mucosal cells being epithelial mucosal cells when the reconstructed tissue is an epithelium model, the mucosal cells being mucosal fibroblasts when the reconstructed tissue is a chorion model.

28. A method for making a reconstructed skin or a reconstructed mucosa comprising an epithelial portion comprising keratinocytes or epithelial mucosal cells, optionally Merkel cells and/or melanocytes, and a conjunctive matrix comprising dermis or chorion fibroblasts, said method comprising:

- sowing and growing in a nutritious medium, keratinocytes or epithelial mucosal cells, optionally in the presence of Merkel cells and/or melanocytes, at the surface of a dermis or chorion model, comprising IDCs and optionally endothelial cells and/or macrophages, said dermis or chorion model being able to be obtained by a method for making a dermis or chorion model as defined in claim 27,
- sowing and growing, either simultaneously or not, with keratinocytes or epithelial mucosal cells, CD14+ monocytes isolated from peripheral circulatory blood of a living being in the presence of the keratinocyes or epithelial mucosal cells under conditions allowing differentiation of CD14+ monocytes into LCs.

29. The method according to claim 27 or 28, characterized in that the cells are human cells.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/059165

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 688 483 A (KIRIN BREWERY [JP]) 9 August 2006 (2006-08-09) column 62; claims 1-28	1-28
X	GUIRONNET G ET AL: "Phenotypic and functional outcome of human monocytes or monocyte-derived dendritic cells in a dermal equivalent" JOURNAL OF INVESTIGATIVE DERMATOLOGY, NEW YORK, NY, US, vol. 116, no. 6, June 2001 (2001-06), pages 933-939, XP002211124 ISSN: 0022-202X the whole document	1-18

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 2007	20/11/2007

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/059165

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	EP 0 789 074 A1 (OREAL [FR]) 13 August 1997 (1997-08-13) the whole document	16-28
X	BAZIL V ET AL: "SHEDDING AS A MECHANISM OF DOWN-MODULATION OF CD14 ON STIMULATED HUMAN MONOCYTES" JOURNAL OF IMMUNOLOGY, vol. 147, no. 5, 1991, pages 1567-1574, XP002455997 ISSN: 0022-1767 figure 7	19

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