Einwanderung von Langerhanszellvorläufern in die Epidermis: Entwicklung eines Modellsystems

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Migration of Langerhans cell precursors into the epidermis: development of an experimental model

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Contents

1.Introduction

Dendritic cells (DC), and thereby also Langerhans cells, are at the centre of a widespread interest in immunology today. Many research groups think of them as promising remedies in various fields, all connected with the misfunction, i.e. illness, of the immune system: allergy, autoimmunity, AIDS, tumour immunotherapy, transplantation and many others. Because of their unique features among the cells involved in an immune response scientists hope to be able to manipulate the immune system one day – with dendritic cells as tools – towards tolerance or a strong immune response, depending on the illness fought.

1.1 Historical aspects

Discovery

Dendritic cells were discovered in an attempt to understand and better define what were initially called "accessory cells". Clonal selection theory in the 70s stated, that antigens simply select and expand responsive lymphocytes – at that time it had not yet been discovered, that T cells recognize fragments of antigen presented on MHC molecules. Therefore, it was surprising that it was difficult to create immunity in tissue cultures: when attempts were made to stimulate purified lymphocytes with antigens and even mitogens, additional accessory cells were required. This phenomenon was studied in two main systems: the primary antibody response by mouse splenocytes (1) and the recall response to protein antigens by cells from primed guinea pigs (2,3) and humans (4,5).

The accessory cells were supposed to be macrophages. Macrophages, though, showed weak or absent functionality as accessory cells for the antibody response, and in cell biologic studies it could be shown that antigens they had taken up were thoroughly degraded (6,7). Therefore, an examination of adherent mouse splenocytes, the best source for accessory cells, was begun. One fraction of these cells was a cell that had not been described before, and it was termed "dendritic cell"(8,9).From today's point of view these dendritic cells must be considered as mature interdigitating DCs.

Spleen dendritic cells had unusual cell and nuclear shapes, and continually formed and retracted processes. Unlike macrophages, dendritic cells had scarce lysosomes, lacked Fc receptors, and were poorly endocytic *in vivo* and *in vitro* (9,10). They were bone marrow-derived, independent of T cells (present in nude mice), did not respond to either B- or T-cell mitogens, and had limited viability in culture (11).

Less than 1% of splenocytes were dendritic cells, but they could be enriched 10- to 20-fold by flotation of dense BSA (9,10,12). Non-phagocytic cells with a similar appearance to isolated dendritic cells were evident in sections of mouse spleen, primarily in the white pulp, rich in lymphocytes, rather than red pulp, rich in macrophages (8).

Dendritic cells were found to express high amounts of MHC class II, as shown by immunofluorescence and complement cytotoxicity with alloantisera and monoclonal antibodies (10,13). In quantitative binding studies this expression was much higher than on the prototype MHC class II-positive cells, B-lymphocytes (13).

In contrast, most macrophages in the peritoneal cavity and spleen of mice proved to be MHC class II negative (10). They only became MHC class II positive when exposed to lymphokines *in vitro* [interferon (IFN)-γ] (14,15) or when elicited during a cell-mediated immune response *in vivo* (16).
Finally, dendritic cells were proved to be the active accessory cells for the antibody response (17-20), whereas macrophages were inactive or suppressive (21).
The distinctive role of dendritic cells is to initiate immune responses of quiescent T lymphocytes. Once sensitised, the T lymphoblasts readily interact with other antigen-presenting cells, such as macrophages or B cells (20,22-24).
In summary these early studies showed that a novel cell type, distinct from macrophages, had been discovered.

1.2 Biology of dendritic cells – specific functional properties

There are three properties, that distinguish dendritic cells from all other cells involved in an immune response and that allow them to fulfil their task in such a "professional" way:

1. function as sentinel: acquisition of antigens in tissues
2. migratory function: migration to lymphoid organs
3. function as "nature's adjuvant": identification and activation of antigen-specific T cells.

These 3 functions are corresponding to different periods during the life cycle of a dendritic cell and happen in separate tissues in the body, as portrayed in simplified terms in table 1.
Only the main, basic functions and properties of dendritic cells are shown, necessary for a first understanding of the dendritic cell system. Other fields of dendritic cell activity, such as the stimulation of peripheral memory T cells or the induction of tolerance in the periphery or the thymus, are not mentioned.

Table 1: Functions of dendritic cells in correlation with their maturation stage, properties and localisation in the body (simplified). Legend: DC...dendritic cell, Φ...macrophage.

Maturation stage	Function	Properties	Localisation
immature DC	sentinel	intermediate antigen uptake as compared to Φ, high ability for antigen processing	stratified squamous epithelia, interstitium (exception: brain)
maturing DC	migration	upregulation of (peptide-loaded) MHC I+II and costimulators (CD40, CD54, CD58, CD80, CD86), migration to T-cell areas of the draining lymph nodes or spleen	afferent lymph (so called "veiled cells"), blood
fully mature DC	"nature's adjuvant"	optimal presentation of antigenic peptides and interaction with antigen-specific T cells for induction of an immune response	T-cell area of lymph node or spleen (so called "interdigitating DC")

Today the details of this course and its regulation are a quickly developing field of investigation.

1. Function as sentinel

Dendritic cells, and thereby Langerhans cells, arise from progenitors in the bone marrow (11,25-27). During their ontogeny, described in more detail in chapter 1.3, they migrate to non-lymphoid tissues where they reside as immature dendritic cells.
Immature dendritic cells can be found in all stratified squamous epithelia such as the skin, where they are named Langerhans cells (28), and – as cells similar in morphology to Langerhans cells – in epithelium of anus, pharynx, oesophagus, vagina (29-31) and cervix (32,33). They are also found in the epithelium of the airways of the lung (34-41), the intestine (42,43), and the iris and ciliary body (44,45). There, even if laying within the epithelium, they maintain contact with the subepithelial space.
Dendritic cells can also be found in the interstitial spaces of most organs, like for example heart, liver or kidney (46-48). Only the brain makes an exception (46). Dendritic cells resident in the dermis are also considered as interstitial dendritic cells (49). Furthermore in spleen and lymph nodes a sub-population of immature dendritic cells can be found.
This tissue distribution maximises the possibility of antigen capture and thereby the development of an immune response.

There is little information about the turn-over rates of dendritic cells in these different compartments: Langerhans cells in the epidermis have a half-life of at least one month (25), in the conducting airway epithelium a very short half-life of 2 to 3 days (50).
As to the phenotype dendritic cells in non-lymphoid tissues, a well studied example being Langerhans cells, are immature and still express some monocyte/macrophage-markers (Fc-receptor/CD32$^+$, complement-receptor/CD11b$^+$, F4/80$^+$, membrane bound ATPase$^+$, unspecific esterase$^+$, endogenous peroxidase$^+$, receptor for M-CSF/CD115$^+$) (51-53). MHC class I and II are expressed constiutively at high levels (intracellularly, not on the surface membrane), that are further upregulated and translocated to the cell surface (54) upon induction of maturation (55-59). Costimulatory molecules can be detected only at low levels if at all (52,60).
Also mechanisms for antigen uptake are active constitutively, specially macropinocytosis (61). Other mechanisms used are receptor-mediated molecule uptake (61-65) and phagocytosis of particles or micro-organisms, like latex (66), yeasts (67) or leishmania (68).
Even if dendritic cells are not so potent in phagocytosis as macrophages they are highly specialised in processing the antigen for a short period of time (69). Only fresh isolates from epidermis (70,71) and spleen (72) present native proteins. The specialisation for antigen processing is reflected in the presence of a large amount of suitable organelles in immature dendritic cells freshly isolated or cultured form skin, spleen, blood and bone marrow: endosomal compartments in which peptides derived from exogenous antigens likely gain access to newly synthesised MHC class II (54,73-76).
The constitutively active macropinocytosis and the effective antigen processing supply the basis for eliciting an immune response even with very low concentrations of antigen. So, while residing in non-lymphoid tissues dendritic cells are "actively awaiting" the moment of antigen contact and inflammation.

2. Migratory function

Antigens usually enter the body through its surfaces. The vast majority of T cells though is contained in peripheral lymphoid organs. To obtain a successful primary immune response the distance between body surface and T-cell area – for example of the draining lymph node – must be covered. Dendritic cells are specialised to fulfil this task and build a system that occupies discrete portions of nonlymphoid and lymphoid organs, interconnected by defined pathways of movement: via the blood to the T cell areas of spleen or via the afferent lymph to T cell areas of lymph nodes.
Langerhans cells are a well studied example for migration through the afferent lymphatics. After antigen encounter *in vivo* they begin to mature and to migrate, when appropriate inflammatory stimuli like GM-CSF, TNF-α or IL-1β are present (77-81). The cytokine requirements for maturation are likely to be complex and are still under investigation.
The maturing Langerhans cells begin to enter the afferent lymph, forming cords of "veiled cells" in dermal lymphatics (82-87). The importance of this pathway is underlined by the finding, that in certain primary responses, like sensitisation to skin transplants (88) and contact allergens (89) afferent lymphatics need to be intact.

Several chemokines are discussed to play a role in the attraction towards the lymphatic vessels. Some examples are RANTES, MCP-1, TNF-α, SLC and MIP-3β (90-93). Molecules, that apparently play a role in emigration of Langerhans cells from the epidermis, are E-cadherin, the mediator for adhesion to keratinocytes, that is down-regulated during maturation (94,95), CD44 (96), p-glycoprotein (MDR-1) (97), and alpha 6 integrins (98). Already this "simple" detachment from the tissue where dendritic cells reside and the movement towards the peripheral lymphoid organs requires high specialisation and the mechanisms involved are not yet fully elucidated.
Another pathway for migration is the blood. On this route dendritic cells gain access to the spleen. Evidence for this second pathway was obtained in heart transplant models, in which donor derived dendritic cells were found in the blood stream and spleen of the recipient (99,100). Alternatively, dendritic cells found in the blood stream could be migrating from the bone marrow to nonlymphoid tissues This point will be discussed later (1.3 Ontogeny of Langerhans cells).
Another interesting property of dendritic cells is that they thoroughly change their phenotype during migration: they mature (52,86,87,101-103). In vitro MHC class II molecules loaded with processed antigen are quickly upregulated and retained at the cell surface with high stability (104). This guarantees that dendritic cells remain immunogenic until they reach the peripheral lymphoid tissue and encounter T cells. To effectively cluster T cells and to activate them co-stimulatory molecules (CD40, CD54, CD58, CD80, CD86) are expressed at high levels. At the same time dendritic cells undergoing maturation lose the abilities they needed as immature cells: antigen capture and MHC biosynthesis are downregulated (104).
The development of dendritic cells summarised with "migratory function" describes a far-reaching change of phenotype, skills and position in the body. This surely makes these cells unique among all others and again shows their great extent of specialisation.

3. Function as nature's adjuvant

Once dendritic cells reach the peripheral lymphoid organs they are fully mature. Only afferent, but not efferent, lymph contains "veiled" cells (105-111). Nevertheless dendritic cells do not accumulate in the lymph node and probably quickly die of apoptosis if no fitting T cell clone could be bound (59,112,113). *In vivo* antigen-pulsed dendritic cells injected without any adjuvant cluster large numbers of antigen-specific $CD4^+$ T cells around them in the T-cell areas of peripheral lymphoid organs (114).
In vitro mature dendritic cells are unusually potent stimulators in the primary mixed leukocyte reaction (MLR): small numbers of dendritic cells pulsed with low doses of antigen stimulate strong T-cell responses (115,116). Enriched dendritic cells could be used in roughly 100 times smaller numbers than bulk leukocytes. Other MHC class II-positive cells, including B cells and macrophages, were weak or inactive (14,115,116). Potency in MLR stimulation is still the most convenient assay for demonstrating the function of mature dendritic cells, in which both $CD4^+$ helper and $CD8^+$ killer responses are induced (23,117).
These MLR findings provided the first clues to the physiologic role of dendritic cells.

Antigen presentation on MHC molecules (signal 1) alone is necessary, but not sufficient to initiate a response by resting T cells *in vitro*. Also B cells and macrophages can function as APCs (i.e. show signal 1), but these cell types lack the accessory or costimulatory molecules (signal 2) that are strongly expressed by dendritic cells. Once activated by dendritic cells, T cells can efficiently interact with other APCs in either the antibody response or the MLR (20,22). As to an antibody response it is worth to be reminded, that activation of T cells develops in two phases: a first step, in which priming is induced by dendritic cells, and a second step, in which the T helper cell interacts with B cells as the APCs to induce antibody formation. Also in the MLR priming of naive T cells requires antigen presented on dendritic cells, but once activated, T-cell blasts respond vigorously to antigen presenting B cells and reciprocally cause B cells to proliferate and make antibody *in vitro* (20).
Dendritic cells strongly express a broad spectrum of second signals that mediate T-cell binding and costimulation: CD40, that interacts with CD40L on T cells and enhances maturation, CD54 [intracellular adhesion molecule (ICAM)-1], CD58 [lymphocyte function associated antigen (LFA)-3], and B7s (CD80/B7-1, CD86/B7-2) (100,103,118,119). Especially CD54/ICAM-1 and CD86/B7-2 are expressed at higher levels on dendritic cells than on others (120).
The number of interactions between MHC-peptide and T cell receptor (TCR) that are required to stimulate a T cell is decreased in the presence of costimulators (121). As to the *in vivo* situation costimulatory molecules probably play a crucial role in promoting an immune response at the beginning of an infection, because only low concentrations of antigen are present and the number of MHC-peptide molecules on dendritic cell surface is lower than in artificial *in vitro* systems. When an organism can eliminate the antigen, for example bacteria, quickly after infection the manifestation of a disease can be avoided, what in turn can be crucial for its survival.
Dendritic cells can bind T cells loosely in an antigen-independent fashion. This may provide the opportunity for small amounts of MHC-peptide to be recognised (122,123). In current opinion, the couples of molecules function in a cascade-like manner to optimise the APC-T cell interaction, even if the mechanisms involved are not yet fully understood. After a first loose contact the TCR can recognise the antigen on the MHC molecule. Then ICAMs solidify the cell-cell binding and successively the other costimulators (B7-2, CD40) come into play (124-126). This interaction has the characteristics of a dialogue, as each of the partners sends and receives information and signals.
One of the signals that is received by dendritic cells is to produce cytokines and to stay alive for further stimulation of T cells. Maturing dendritic cells secrete high levels of IL-12, which enhances the formation of Th1 type helper and killer cells (127,128), and are resistant to the effects of IL-10 (129). IL-10 can induce apoptosis in developing dendritic cells, what can be prevented by the addition of TNF-α or CD40L (130). The life span of dendritic cells can be prolonged by interaction of CD40 (on mature dendritic cells) and CD40L (on activated T cells) (126), and by TRANCE receptor (on dendritic cells) and TRANCE (on activated helper and cytotoxic T cells (131).

In a vaccination it is necessary to add artificial substances, like oils in complete Freund's adjuvans or colloidal aluminium salts, to induce immunity to a certain antigen. A postulated mechanism of action of adjuvants is to enhance the expression of costimulators on macrophages and other APCs. Dendritic cells can activate naive and quiescent T cells, both $CD4^+$ T helpers (132-135) and $CD8^+$ T killers (136-141) *in vivo,* and thereby initiate a primary immune response, in the absence of any other adjuvant.
These findings led to the conclusion, that dendritic cells were acting as "nature's adjuvant" (113).
To denominate all cells that have both, antigen-presenting and costimulatory functions the term "professional APC" is now used.

1.3 Ontogeny of Langerhans cells

Since Katz *et al.* and Frelinger *et al.* in 1979 demonstrated that Langerhans cells derive from a mobile pool of cells that originates in the bone marrow (25,26), many attempts have been made in humans and rodents, especially mice, to establish intermediate stages and the ontogenetic relationship between Langerhans cells and other hematopoietic cell lineages. Even if a broad variety of approaches have been undertaken a definitive ontogenetic pathway or even culture conditions that give rise to pure Langerhans cell populations have not yet been identified. In the following I want to give a short overview of our current thinking.

1.3.1 Human system

There are some clear advantages about the identification of Langerhans cells in the human as compared to the widely used murine system. First of all, antibodies exist, that selectively stain human Langerhans cells, like "Lag", an antibody that recognises a Langerhans-associated granule antigen of unknown function, and Langerin, an endocytic receptor involved in the induction of Birbeck granules (142,143). No such specific antibody exists for the murine system. The murine homologue for Langerin has just been cloned and the first mAb´s are becoming available (S. Saeland, Schering-Plough, Dardilly, France, personal communication) Furthermore Birbeck granules of human Langerhans cells in culture remain stable for long periods, whereas Birbeck granules of murine Langerhans cells disappear within 72 hours when cultured with keratinocytes or keratinocyte-supernatant (52). Therefore, in the mouse the only unequivocal markers for the identification of Langerhans cells are missing, and the approaches to find answers to the above mentioned questions are far behind those in the human system.

Generation of Langerhans cells from $CD34^+$ progenitors

The first ones to establish culture conditions for the generation of Langerhans cells were Caux *et al.* in 1992 (144). $CD34^+$ hematopoietic progenitor cells (HPC) isolated form **human umbilical cord blood**, cultured in serum-containing medium supplemented with GM-CSF and TNF-α for 12 days, gave rise to a $CD1a^+$ population (20 – 50% of all $CD34^+$ HPC) of dendritic/Langerhans cells, of whom 1 out of 5 cells contained Birbeck granules as assessed by electron microscopy (~ 4 – 10% of $CD34^+$ HPC).

Four years later, in 1996, Strobl *et al.* presented a serum-free culture system of $CD34^+$ HPC (145). The medium was supplemented with GM-CSF, SCF, TNF-α and TGF-β1, the latter being essential for the appearance of Lag^+ Langerhans cells (yield 21% of all CD34+ cells), that regularly showed Birbeck granules.
In the same year Caux *et al.* extended our understanding of Langerhans cell development by their discovery of two apparently independent pathways, only one of which led to Langerhans cells, namely the $CD1a^+/CD14^-$ subpopulation of $CD34^+$ HPC (146). From this subpopulation, after 12 – 14 days of culture in serum-containing medium, supplemented with GM-CSF and TNF-α, a subset expressing E-cadherin, Lag (19 – 34% Lag^+) and Birbeck granules (31 – 56% BG^+) arose (12 – 14% Lag^+ of the total $CD34^+$ population). When these results are compared with those of Strobl (145) TGF-β1 seems to increase the total percentage of Langerhans cells and to push development of progenitors towards this special cell type *in vitro*. The other pathway, $CD14^+/CD1a^-$ cells, gave rise to $CD1a^+$ dendritic cells lacking E-cadherin, Lag and Birbeck granules, that were supposed to be dermal dendritic cells.
In 1997 Strobl *et al.* published another paper about serum-free culture of Langerhans cells (147). In this system the culture medium was supplemented with flt3-ligand in addition to TGF-β1 and the above mentioned cytokines (GM-CSF, SCF, TNF-α). The addition of flt3-ligand only slightly increased the percentage of Lag^+ cells (from 19% to 21%) and thereby seemed not to be crucial for the development of Langerhans cells.
Of much more interest are two recently published papers, one from Jaksits *et al.* (148) and one from Caux *et al.* (149), that further describe the two different developmental pathways of Langerhans cells Caux *et al.* have already described in 1996 (146).
Jaksits *et al.* found, that the addition of TGF-β1 to a serum-free culture medium further supplemented with GM-CSF and TNF-α, was only necessary for the development of Langerhans cells (Lag^+, BG^+) from $CD14^+/CD1a^-$ precursors (the first six days cells were grown in a serum-containing medium, then sorted for CD14 or CD1a and transferred to a serum-free culture medium conditioned with defined cytokines). The $CD1a^+/CD14^-$ population could develop into Langerhans cells independently of TGF-β1 (serum-free medium supplemented with GM-CSF and TNF-α). As to the yields of Langerhans cells in both pathways, the majority of cells were Lag^+ and 10 – 20% showed Birbeck granules, but the $CD14^+/CD1a^-$ precursor population underwent one more cell cycle as compared to the $CD1a^+/CD14^-$ precursor population, and thereby seems to be the major "route" for the development of Langerhans cells. Basically the findings of Caux et al. suggested the same (149).
These newer results in the human system strongly add to the importance of TGF-β1 for the development of Langerhans cells. The finding, that TGF-β1 knock-out mice are devoid of Langerhans cells, but not of other dendritic cell types, reflects the importance of this cytokine for Langerhans cell-development also in the murine system (150,151).
Parallel to the culture systems that used $CD34^+$ HPC from human cord blood another source of $CD34^+$ HPC was examined, the **human peripheral blood**. The first ones to describe such a culture system were Mackensen *et al.* (152). The blood was taken from cancer patients, $CD34^+$ HPC were enriched and then cultured in serum-containing medium supplemented with

a whole armada of cytokines (IL-4, GM-CSF, SCF, erythropoietin, IL-1β, IL-3, IL-6) for 15 days. The yields of Langerhans cells at first glance were impressing: over 75% of the sorted $CD1a^+$-population were Lag^+; but the $CD1a^+$ cell population made up approximately 10% of all nucleated cells on day 15 of culture. This system was not further examined, maybe because of the scarce availability of this specific source of $CD34^+$ HPC (cancer patients with solid tumours that are undergoing an autologous peripheral blood precursor cell-transplantation) and of the low total yield of Langerhans cells.
The year after another culture system for Langerhans cells was published: Strunk *et al.* developed a procedure for the development of human dendritic and Langerhans cells from $CD34^+$ HPC isolated from buffy coats and leukapheresis products of healthy volunteers (153). $CD34^+$ HPC were cultured for 14 – 21 days in serum-containing medium supplemented with GM-CSF, TNF-α and IL-4. The cell population that arose after that time was approximately 6% Lag^+ and 24% $CD1a^+$. IL-4 decreased total cell numbers, but increased the percentage of $CD1a^+$ cells. In the paper following this work Strunk *et al.* characterised two different subpopulations of $CD34^+$ HPC derived from human peripheral blood of healthy volunteers by the marker cutaneous lymphocyte-associated antigen (CLA) (154). This marker is a skin homing receptor and thereby strongly suggests that the destiny of dendritic cells expressing this marker might be dermis or epidermis. After 10 – 18 days of culture in serum-containing medium supplemented with GM-CSF and TNF-α only the CLA^+ subset expressed Birbeck granules and Lag (12% - 22,5% $CD1a^+$, 5 – 20% Lag^+; 4 – 7.5% BG^+ of $CD34^+/CLA^+$ population). The CLA^- subset never showed Lag reactivity nor Birbeck granules. These results indicate another good and useful marker for Langerhans cell development in the human system: CLA.
All culture systems suggested, that commitment to the Langerhans cell lineage happens at an early stage during the precursors life.

Generation of Langerhans cells from other progenitors from human peripheral blood

Two more publications in this field seemed to be of particular interest to me. One is a paper published by Geissmann *et al.* in 1998, in which it could be demonstrated that Langerhans cells also arise from $CD14^+$ peripheral blood monocytes (155). These cells differ from the above mentioned $CD14^+$ cells as they are not derived from $CD34^+$ hematopoietic progenitor cells. The $CD14^+$ peripheral blood monocytes were isolated from peripheral blood mononuclear cells (PBMC) by negative magnetic depletion and cultured for 5 – 7 days in serum-containing medium supplemented with GM-CSF, IL-4 and TGF-β1, the latter being again essential for the generation of Langerhans cells. Of the thereby generated cells 26 – 33% were BG^+ and 20 – 40% Lag^+ (82% E-$cadherin^+$, 93% $CD1a^+$, 80 – 90% CLA^+). Monocyte derived-dendritic cells obtained in the presence of GM-CSF and IL-4 could be further induced to differentiate into E-$cadherin^+$, CLA^+ and BG^+ Langerhans cells in the presence of GM-CSF, IL-4 and TGF-β1, thus confirming that Langerhans cells and dendritic cells can originate from the same population of $CD14^+$ monocytes and share a common precursor.

The other publication that deserves a close look is the paper published in 1999 by Ito *et al.* (156). $CD4^+/CD3^-/CD14^-$ cells were sorted from peripheral blood mononuclear cells (PBMC) and cultured in serum-containing medium supplemented with GM-CSF, IL-4 and TGF-β1 for 1(!) – 6 days. Already after 24 hours of culture a defined subset of dendritic cells ($CD1a^+/CD11c^+$) started to upregulate E-cadherin and CD1a and to express Langerin. This upregulation and expression increased during the whole time of culture (6 days), leading to a 20 – 25% BG^+ population. Only the $CD1a^+/CD11c^+$ fraction gave rise to Langerhans cells and because of the very short time needed to acquire the specific phenotype was supposed to be a direct precursor of Langerhans cells in peripheral blood.

Langerhans cell ontogeny

Only few publications describe results about Langerhans cell ontogeny in the human system. First hints concerning the arising of Langerhans cells from bone marrow progenitors came from a case report by Volc-Platzer et al. (157) and the paper published by Perreault *et al.* in 1984 (158). As to the latter, the number of Langerhans cells in the epidermis was determined by fluorescent immunolabelling before and after bone marrow transplantation. Directly after transplantation numbers of Langerhans cells decreased and reached a nadir around day 11. Normal values were gained 120 – 365 days post transplant. Considering the experimental evidence in the murine system (25,26) it could be concluded that Langerhans cells in humans originate from the bone marrow. As to the development of Langerhans cells in the epidermis of the human fetus Foster *et al.* described the appearance of $HLA\text{-}DR^+/ATPase^+$ Langerhans cells in human fetal skin already ˆ ı 6 – 7 weeks estimated gestational age, the earliest time point examined (159).
Because of evident difficulties as to experimental material and ethical aspects *in vivo* studies in humans were not extended much further.

1.3.2 Rodent systems

As mentioned above there are clear disadvantages in the rodent systems, taking the murine system as example, in clearly identifying Langerhans cells *in vitro*. As usually in life, though, there are also clear advantages: *in vivo* experiments can be performed easily and the availability of progenitor cells usually is not a limiting factor. Therefore, there are many publications about examination of the ontogeny of Langerhans cells during gestation and a few about immigration of Langerhans cells into the epidermis.

Langerhans cell ontogeny

First of all, as already mentioned, Katz *et al.* and Frelinger *et al.* showed that Langerhans cells are bone marrow derived (25,26).
It could be shown, that in fetal epidermis only very few Ia^+ Langerhans cells could be detected, but Ia^- cells expressing Langerhans cell and macrophage markers (Fc- and C3bi-receptor, F4/80, ATPase) were found before birth (160). These Langerhans precursor cells quickly became Ia^+ after birth and reached levels superior to LC-densities in adult mice on day 7 post partum (160,161). Densities afterwards decreased, but normal values were not detected within 6 weeks. In fetal rats the spreading of Langerhans precursor cells from cranial

to caudal was described (162). Mature Ia^+, BG^+ Langerhans cells appeared from day 21 of gestation on.
Data obtained in these experiments strongly suggested for a major contribution of the epidermis to the maturation of Langerhans cell precursors (163).

Langerhans cell immigration into the epidermis

A field that has only recently became a focus of interest is the immigration of Langerhans cells or Langerhans cell precursors into the epidermis.
As to the migration of fully differentiated Langerhans cells it was shown that when injected intravenously they preferentially migrate to the skin (164). The migration rate is not changed by UVB-irradiation or by working in an allogeneic system. The migrated Langerhans cells are functional and can induce contact hypersensitivity.
When the skin is depleted of Langerhans cells by daily topical application of clobetazole propionate and is then put, epidermal side down, onto a culture medium that contains syngeneic epidermal cells, and thereby fully differentiated Langerhans cells, the latter actively and strongly immigrate into the epidermis (165). In contrast to the above-mentioned intravenous injection experiments semi-syngeneic or allogeneic Langerhans cells hardly immigrated at all. Anti-IL-6– and anti-TNF-α– antibody markedly decreased the numbers of immigrated Langerhans cells by 45 – 60%, what appears to be in contrast with the pro-inflammatory and emigration-enhancing function attributed to these two cytokines.
When the pattern of repopulation with Langerhans cells after mild heat injury is assessed *in vivo*, and thereby the immigration of Langerhans cell precursors, precursors coming from the blood or the dermis reconstitute the epidermal population (166). Langerhans cells residing in the epidermis are neither able to divide nor to migrate to depleted areas.
Another interesting situation is the inflamed skin. Immature bone marrow-derived dendritic cells tether and roll on P- and E-selectin *in vitro* and on murine dermal endothelium *in vivo* (intravital microscopy) (167). They are preferentially recruited to inflamed skin (upregulation of P- and E-selectin in neighbouring endothelia) and were shown to extravasate *in vivo*.
Preincubation of dendritic cells with TGF-β1 further enhanced migration of immature bone marrow-derived dendritic cells to inflamed skin (168). In this work epidermis and Langerhans cells and dermis and dermal dendritic cells were not evaluated separately.

Generation of Langerhans cells from bone marrow-derived progenitors

The only data available on such attempts were the unpublished results of Dr. Franz Koch, presented in a talk at the ÖGAI-meeting 1999. By evaluation with electron microscopy bone marrow-derived precursor cells, cultured with GM-CSF alone for 13 days, were found to differentiate into Langerhans cells. The impressively high rate of 60% of total cells were Birbeck granule-positive.

When the research mentioned in this chapter is considered it becomes evident that the conditions for Langerhans cell development and immigration *in vivo* in the mouse are a promising field of investigation. To find models in which different cytokines, chemokines and culture conditions can be tested is essential for elucidation of the processes that happen in a

healthy organism. This would contribute to a better understanding of this cell type and thereby facilitate its manipulation and specific use.

2. Goals and aims of this study

2.1. Immigration of Langerhans cell precursors into the epidermis

The most important question was to find a model where Langerhans cell precursors immigrate into the epidermis. This chapter of the life of Langerhans cells has been hardly studied at all and so we expected to obtain very basic informations as to the regulation of this process. Several different parameters were analysed in more detail.

2.1.1 Detection and staining

PKH26, a fluorescent dye repeatedly mentioned in literature as being suitable for long term in vivo migration studies, seemed to be ideal for identifying the injected cells in the mouse. In preliminary experiments we wanted to test the properties of the substance as indicated by the manufacturer:

- Would the marker stay stably linked to the cell surface and keep the intensity for the period needed in our experiments?
- Would stained cells keep the marker inside them or lose it to unstained cells? This could give false positives in our experiments.
- How would dying with PKH26 alter the viability of bone marrow-derived dendritic cells? This might alter their migratory behaviour *in vivo* and therefore give false informations. Moreover, because of their unfit constitution they could be eliminated by the immune system itself and end in the liver without ever reaching their destination.

As an alternative to the fluorescent dye we evaluated an experimental setting in which the target cells could be identified by **selective staining of their MHC class II-isotype**.

2.1.2 "Creation of a demand"

It is a general believe, that Langerhans cells in the epidermis have a certain "closeness", that does not allow new, additional Langerhans cells to enter the epidermis. There never was any real proof of that, however. So, another point was the idea of "creating a demand" for Langerhans cells in the epidermis. With the technique of tape stripping we studied the emigration of Langerhans cells from the epidermis after irritation and their re-immigration afterwards. So the questions we wanted to answer are:

- Does tape stripping create a demand for Langerhans cells in the epidermis?
- How can we time this demand with the injection of progenitor cells in the mouse?

2.1.3 Culture and treatment prior to injection

There is evidence, that the decision of becoming a Langerhans cell takes place early during the development from haematopoietic stem cells. So it was of interest to vary culture conditions so that an increase of Langerhans cell precursors in the cultured population might

be detectable. Here my work could profit notably from the research of Dr. Franz Koch and Hella Stössel, who analysed the effects of different culture conditions on dendritic cells under the aspect of their development into Langerhans cells by electron microscopy.

These informations would also allow conclusions about the kind of signal(s) given by the epidermis, when Langerhans cells are needed.

We cultured pure keratinocytes from mouse epidermis in a monolayer, supposing that the signal for the recruitment of Langerhans cells would be secreted into the culture medium. This keratinocyte (KC) supernatant then was added to cultures of bone marrow derived dendritic cells.

- Would Langerhans cells be detectable when the bone marrow derived dendritic cells are cultured in our routine way?
- Would the addition of keratinocyte supernatant influence the development of the cultured cells towards Langerhans cells?

2.1.4 Injection

As application form we choose the intravenous injection, actually because it would be the most elegant solution. If we could "grow" Langerhans cells they would migrate through the endothelium of the venules like precursor cells were there would be a need for them, for example our stripped ear. There we would then be able to detect them. This setting would imitate the theory we have about the events *in vivo*.

As an alternative we thought about intradermal injection. It would be more direct, more "in place" and without the circulation and the complicated system of the body, specially lung, spleen and liver, between point of application and destination. So with this application form we would not lose injected cells in the body, but as a precondition the identification of them must be unequivocal.

2.2 Migration of intravenously injected bone marrow-derived dendritic cells into lymphoid organs

Another point we wanted to study is the general distribution of dendritic cells, injected intravenously into mice, in lymphoid organs (spleen, bone marrow, lymph nodes) and the skin. Dermis and epidermis were of particular interest.

Dendritic cells were cultured from the bone marrow into immature and mature populations.

The injected cells were stained with PKH-26 and their distribution analysed in a time-course study. Cell suspensions were prepared in a routine way, Cytospin preparations done and evaluated with the fluorescence microscope.

Epidermis and dermis were made into "sheets". These then were screened under the fluorescence microscope for positive cells without any further dying.

We wanted to know:

- Would we find positive cells in the analysed organs? The skin was of special interest.
- Would there be differences in the distribution between the single organs?

- Would there be differences in the migration behaviour of immature and mature dendritic cells?
- Would the distribution vary in time?

3. Materials and methods

3.1 Mice

Inbred strains of BALB/c (H-2^d) and C57BL/6 (H-2^b) were purchased from Charles River Germany, Sulzfeld, Germany, and were used for all experiments at an age of 8 - 12 weeks. F1 (BALB/c x C57BL/6) hybrid mice (H-$2^{d/b}$) were bred in our breeding facility at the Department of Dermatology and Venereology, University of Innsbruck, Austria.

3.2 Equipment

CO_2-Auto-Zero (incubator 37°C)	Heraeus Instruments, Martinsried, D
Glass petri dishes	Gatt-Koller, Absam, Austria
Laminar flow (Danlaf HF 1206)	Uniequip, Martinsried, D
Falcon ® Pipettes (5 ml, 10 ml, 25 ml)	Becton Dickinson Labware, Franklin Lakes, NJ, USA
Plastic petri dishes (6 cm, 10 cm)	Becton Dickinson Labware, Franklin Lakes, NJ, USA
Polypropylene Falcon ® tubes 15 ml	Corning Incorporated, Corning, NY, USA
Sterile filter (50 ml)	Becton Dickinson Labware, Franklin Lakes, NJ, USA
Stripette (anti drop pipette)	Costar, Cambridge, MA, USA
Tooth picks	Billa, Innsbruck, Austria
Tubes 15 ml, 50 ml	Becton Dickinson Labware, Franklin Lakes, NJ, USA

Centrifugation:

Cytospin 2 (Cytocentrifuge)	Shandon, Pittsburgh, PA, USA
Filter cards (Cytospin)	Shandon, Pittsburgh, PA, USA
Centrifugation tubes (superspeed centrifuge)	Beckman Instruments, Palo Alto, CA, USA
Omnifuge 2.0 RS	Heraeus Sepatech, Vienna, Austria
Sorvall RC-5B Refrigerated Superspeed Centrifuge	Du Pont Instruments, INULA, Vienna, Austria

Dissection tools:

Blades for scalpel nr. 3	Feather, pfm (Exclusive distributor for Europe), Köln, D
Saatilene Hitech (nets suitable for sterilisation)	Saati, Como, Italy
Scalpel (size nr. 3)	Aesculap, Tuttlingen, Germany
Scissors in different sizes	Aesculap, Tuttlingen, Germany
Tweezers in different sizes	Aesculap, Tuttlingen, Germany

Microscopy:

BX60 Fluorescence microscope	Olympus, Optical Co. Ltd., Japan

Hemacytometer	Reichert Biovar, Leitz, Austria
Light microscope	Reichert Biovar, Leitz, Austria
Nikon TMS microscope	Nikon, D

Injection:

Restraining device for intravenous injection of mice	
Brunswick ® 501-TB 1 ml syringe	Sherwood, Crawley, Sussex, GB
1 ml tuberculin syringe with 25G needle	Braun, Barcelona, Spain
Microlance ® needle, 30G	Becton Dickinson, Drogheda, Ireland
Microlance ® needle ,27G	Becton Dickinson, Dublin, Ireland
Norm Ject ® 10 ml syringe	Henke-Sass, Wolf GMBH, Tuttlingen, D
Omnifix ® 30 ml syringe	B. Braun Melsungen AG, D

Tissue culture:

Tissue culture dishes (6 cm, 10 cm)	Becton Dickinson Labware, Franklin Lakes, NJ, USA
24-well-tissue-culture-plates	Costar, Cambridge, MA, USA
96-well- tissue-culture-plates	Becton Dickinson Labware, Franklin Lakes, NJ, USA
6-well-tissue-culture-plates with filter insets catalog number 3414 (3,0 mm poresize, Polycarbonate Membrane)	Costar, Cambridge, MA, USA

3.3 Chemicals

Ammoniumthiocyanate (NH_4SCN)	Merck, Darmstadt, D
Bovine serum albumine (BSA)	Biomex, Mannheim, D
Collagenase Type 3	Worthington Biochemical Corp., Lakewood, NJ, USA
DNase from bovine pancreas (grade II, stock solution of 5 mg/ml in PBS)	Boehringer-Mannheim, Mannheim, D
4´,6-Diamidino-2-Phenylindole (DAPI)	Sigma Immuno Chemicals Co., St. Louis, MO, USA
Disodium phosphate Dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$)	Merck, Darmstadt, D
Ethanol	Fluka, Buchs, CH
Fetal calf serum (FCS)	Biological Industries, Beth Haemek, Israel
Gentamycine (20 mg/ml)	Amimed AG, Allschwill, CH
L-glutamine (200 mM)	Life Tech. Ltd., Paisley, Scotland
Hank´s salt solution w/o Phenol Red	Seromed, Biochrom KG, Berlin, D
1 M HEPES buffer	Seromed, Biochrom KG, Berlin, D
Low Tox-M Rabbit Complement	Sera-lab, Crawley Down, Sussex, England
2-Mercaptoethanol	Sigma Immuno Chemicals Co , St. Louis, MO, USA
Phosphate buffered saline (PBS)	Biological Industries, Beth Hamerk, Israel

PKH26 red fluorescent cell linker kit	Sigma Immuno Chemicals, St. Louis, MO, USA
Potassium Phosphate Monobasic (KH_2PO_4)	Merck, Darmstadt, D
RPMI 1640 (w/o L-glutamine)	Biological Industries, Beth Haemek, Israel
Trypanblue	Seromed, Berlin, D
Vectashield Mounting Medium for Fluorescence	Vector Laboratories, Burlingame, CA, USA

3.4 Antibodies

Table 2: Primary antibodies used for staining epidermal and dermal sheets.

Antigen specificity	Clone	ATCC	Ig-class	Origin
MHC class II I-$A^{b,d}$	B21.2	TIB 229	rat IgG2b	The Rockefeller University, New York, USA
mouse B 220	RA3-3A1/6.1	TIB 146	rat IgM	The Rockefeller University, New York, USA
mouse CD8	HO-2.2	TIB 150	mouse IgM	The Rockefeller University, New York, USA
murine granulocytes	RB 6	-	rat IgG2b	The Rockefeller University, New York, USA
mouse CD4, L3T4	GK 1.5	TIB 207	rat IgG2b	The Rockefeller University, New York, USA
DNP group Used as isotype-matched control antibody	LO-DNP11	-	rat IgG2b	Dr. H. Bazin University of Brussels
Intracellular organelles in mature DC and B-cells	2A1	-	rat IgG2a	The Rockefeller University, New York, USA
murine CD86 (B7-2)	GL 1	-	rat IgG2a	PharMingen, San Diego, USA
MHC class II I-A^d, I-E^d FITC labelled	2G9	-	rat IgG2a	PharMingen, San Diego, USA
MHC class II I-A^d	AMS-32.1	-	mouse IgG2b	PharMingen, San Diego, USA
isotype-matched control antibody for AMS-32.1	A95-1	-	rat IgG2b	PharMingen, San Diego, USA
MHC class II I-$E^{d,k}$	14-4-4S	HB 32	mouse IgG2a	The Rockefeller University, New York, USA
MHC class II I-A^b	25-9-3S	HB 38	mouse IgM	ATCC, Rockville, USA
human CD 8	Leu 2	-	mouse IgG2a	Dr. Yum Yanng, New York, USA
Protein in the cell wall of Chlamydia used as isotype-matched control antibody	MAC-1	-	rat IgG2a	European Collection of animal Cell Cultures, Salisbury, UK

Secondary antibodies:

Sheep anti-rat Ig, biotinylated, species specific	Amersham Life Science, Amersham, UK
Sheep anti-mouse Ig, biotinylated, species specific	Amersham Life Science, Amersham, UK
FITC polyclonal anti-rat Ig	PharMingen, San Diego, USA

Fluorescence conjugates:

Streptavidin-FITC	Amersham Life Science, Amersham, UK
Streptavidin-Texas Red	Amersham Life Science, Amersham, UK

Blocking agents:

Rat γ globulin	Jackson Immuno Research Laboratories, Avondale, PA, USA

3.5 Media and solutions

Collagenase stock solution

1 g	collagenase
25 ml	Hank´s salt solution w/o Phenol Red

Aliquot 1,5 ml and store at -20°C.

Culture medium "R10"

500 ml	RPMI 1640
55 ml (=10%)	fetal calf serum, heatinactivated (56°C, 30 minutes)
0,5 ml (=0,05 μM)	2-mercaptoethanol solution
0,5 ml (= 20 mg)	Gentamycine
5 ml	L-glutamine

Fetal calf serum, 2-mercaptoethanol, gentamycine and L-glutamine must be sterile filtered before added to the RPMI 1640!

"Cytotox medium" (used for complement-mediated lysis of cells)

500 ml	RPMI 1640 (take out 18,1 ml!!!)
12,5 ml	HEPES buffer
5,6 ml	dense BSA stock solution (35%)

2-Mercaptoethanol solution (0,05 mM)

14,2 ml	RPMI 1640
50 μl	2-mercaptoethanol (14,3 M)

PBS / 10% BSA

5 g	BSA
50 ml	PBS

Sterile filter! Dilute 1:10 with PBS before use to obtain the required concentration of 1% BSA!

Sheets: Ammoniumthiocyanate-solution

1,9 g (= 0,5 M)	Ammoniumthiocyanate (NH_4SCN)
50 ml (= 0,1 M)	Phosphate buffer

Sheets: Phosphate buffer

8,9 g (= 0,1 M)	Disodiumhydrogenphosphate ($Na_2HPO_4 \cdot 2\ H_2O$)
6,8 g (= 0,1 M)	Potassiumdihydrogenphosphate (KH_2PO_4)
500 ml	distilled water
pH = 6,8	

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

The culture supernatant of a cell line transfected with the gene for murine GM-CSF (plasmacytoma line X63-Ag8; kind gift of Drs. Lanzavecchia and Ruedl, Basel Institute for Immunology, Basel) was used as a source of GM-CSF. Typically, supernatant was added at a ratio of 1:200 (i.e. 5 µl per ml). The biological activity of these supernatants had previously been determined in comparison with purified recombinant murine GM-CSF (Immunex Corp., Seattle, WA, USA).

3.6 Preparations

All the preparations and manipulations are made under sterile conditions. Murine bone marrow cells were obtained according to standard protocols published previously (12,169,170). Typically, one needs 1 – 3 mice for one experiment.

3.6.1 Preparation of bone marrow cells for the generation of dendritic cells

1. Mice were killed by suffocation with carbodioxide.
2. The skin of the dead mouse is incised with small scissors in the peritoneal fold and is slipped off the posterior legs. To avoid hairs from flying around, wet this part of the skin with some 70% ethanol before cutting it.
3 A second small cut into the muscles nearby the hip joint makes it easier to detach the posterior legs from the rest of the body. Muscles, cartilage and tendons are removed carefully from femur and tibia. The bones are collected in a small petri dish.
4. Fill up the dish with 70% ethanol and immerse for 1 minute. The bones must be completely submersed in the alcohol to become sterile.
5. Rinse the bones: prepare three small petri dishes, two with PBS and one with RPMI 1640. Just pass the bones briefly through each dish using a pair of sterile tweezers.

6. Prepare two petri dishes with RPMI 1640. Take the first bone with tweezers and cut off the ends (epiphyses) with a pair of strong scissors ("cartilage-scissors"). Then continue in the same way with the other bones. Collect the ends in the first dish and the tube-shaped shafts of the bones in the second

7. Mince the epiphyses with two tweezers. Take up the cell suspension with a pipette, rinse the crushed bones a few times and then pipet it through a sterile nylon net into a Falcon 50 ml tube. Rinse again with some fresh RPMI 1640

8. Flush the bone marrow cells out of the tube-shaped bone shaft, that is secured in place with the tweezers with a syringe. Collect the cells.

9. Centrifugation: 1200 rpm (= 295 g), 8 minutes, 4°C.

10. While the cells are in the centrifuge: prepare the rabbit serum containing the complement system: dissolve the content of one flask (1 ml rabbit serum lyophilized) in 1 ml of sterile H_2O. Mix 3,5 ml Cytotox-medium, 0,5 ml DNAse (final concentration 160 µg/ml) and 1 ml complement solution (if only 1 mouse is used these volumes are halved).

11. When the centrifugation is finished aspirate the RPMI 1640 almost completely and resuspend the cells in 2 ml (for 3 mice) or 1 ml (for 1 mouse) each of the following antibody supernatants:

    TIB 146: marks B lymphocytes
    TIB 150: marks CD8+ T cells
    GK 1 5: marks CD4+ T cells
    B 21/2: marks MHC class II-expressing cells
    RB 6: marks granulocytes

    Then add the complement mix to the cell suspension by filtering it through a sterile filter (pore size 0,45 µm).

11. Incubate the Falcon 50 ml tube with the cell suspension under gentle agitation in a shaking water bath at 37°C for one hour. Now the antibody-marked cells, i.e. the undesired cells that already belong to established cell lineages, are being lysed by the complement system.

12. After the incubation wash out the complement by filling up the tubes with R10 and centrifuge:

    1200 rpm (= 295 g), 8 minutes, 4°C.

13. Aspirate the supernatant with a vacuum pump.

14. Count the cells: resuspend with R10. The volume added will vary depending on how many dendritic cells can be isolated from the bone marrow. Normally, if the procedure is done with 1 mouse add 10 - 15 ml R10, with 3 mice 20 - 25 ml R10.

15 Take out 40 µl from the well resuspended (!) cell suspension and pipette it into an Eppendorf tube.

16. Add 10 µl Trypan blue and mix well

17. Then pipette 10 µl into the hemacytometer and count the cells in the inner 25 squares under the light microscope.
18. Calculate the cell density per ml and the absolute cell amount.

Viable cells (white-yellow) in the cell suspension can be distinguished from dead cells (blue, the cell membrane is no longer intact and the dye can penetrate the cell).

3.6.2 Culture, feeding and harvesting of bone marrow cells

Day 0 until day 6

The cells are cultured in 24-well plates in an incubator (37°C, 5% CO_2). Into each well: $0{,}5 \times 10^6$ cells in 1,5 ml R10 supplemented with 200 units per ml GM-CSF.

1. 48 and 96 hours after the culture was started the cells are fed by changing the medium in the following way:

   Tilt the plate and aspirate the medium gently, leaving a small residue inside the well. Proceed this way with the first two rows of wells. Then refill the wells with 1,5 ml fresh R10 medium supplemented with 200 units GM-CSF. Continue with the other two rows of the plate.

   This step is crucial on day 2: the dendritic cells are slightly adherent, other cells are not. It is important therefore to aspirate nearly all the medium, but it should be done gently and carefully, so as not to pull off the dendritic cells as well. Try to reduce the time the plates are outside the incubator to a minimum.

2. On day 6 harvest the cells:

   With a mechanical pipette fixed on 750 µl resolutely, but not roughly, suspend the cells in the medium by sucking up and down, especially in the edges. Carefully avoid foam bubbling. Then collect the cell suspension in a Falcon 50 ml tube. Continue with the next well. This way harvest the cells of the first two rows.

   Add 0,5 ml of fresh R10 to the harvested wells

   Harvest the cells of the next two rows.

   Transfer the rinsing medium from the upper two rows to the lower 2 rows, well by well (e.g. from A1 to C1, A2 to C2, and so forth) and collect the cell suspension in a Falcon 50 ml tube.

   Centrifugation: 1200 rpm (= 295 g), 8 minutes, 4°C.

   Resuspend the cells in a suitable volume R10 for counting them.

   Then, depending on the experimental setting, the cells can be split: for example take the amount needed for the injection and the rest can be cultured until day 8 or 10. The majority of dendritic cells on day 6 are immature.

Culture from day 6 until day 10:

1. Cells are plated on tissue culture petri dishes (diameter 10 cm). In each dish:

   $8x10^6$ dendritic cells in 12 ml R10 supplemented with 200 units per ml GM-CSF.

2. On day 8 cells are harvested and then centrifuged: 1200 rpm (= 295 g), 8 minutes, 4°C.

3. The consumed culture medium is aspirated with a vacuum pump.

4. The cells are resuspended in fresh R10 supplemented with 200 units GM-CSF and put again onto the same culture dishes.

5. On day 10 cells are again harvested, centrifuged (1200 rpm (= 295 g), 8 minutes, 4°C), resuspended in R10 and counted.

Culture can also be ended on day 8, which is the common procedure.

3.6.3 Identification of migrated precursor and Langerhans cells

- "Pre-injection approach". Labelling of cells with a cell tracing dye (PKH26)

All media, centrifugations and incubations are used and made at room temperature! The given volumes are suitable for staining $20x10^6$ cells. The following protocol is based on what is recommended by the manufacturer. It was modified and improved in several points in our laboratory (Dr. Franz Koch).

1. In a Polypropylene Falcon 15 ml tube mix 4 µl PKH-26 stock solution and 1 ml solution C (supplied with the kit).
2. Centrifuge the cell suspension, that is typically in a 50 ml tube at this point, at 1500 rpm (= 460 g), 8 minutes, 20°C.
3. Aspirate as much of the culture medium as possible and resuspend in 10 ml PBS. Transfer the cell suspension to a Polypropylene Falcon 15 ml tube.
4. Centrifugation: 1200 rpm (= 295 g), 8 minutes, 20°C.
5. Aspirate the PBS leaving approximately 25 µl of it and resuspend the cells with 1 ml of solution C.
6. Then transfer this 1 ml cell of suspension to the tube containing the PKH diluted in solution C, mix. Incubate exactly (!) for 4 minutes, gently shake from time to time. Do not do the transfer vice-versa.
7. Add an equal volume (here 2 ml) of sterile filtered FCS and incubate for exactly 1 minute.
8. Add the same volume (here 4 ml) of R10, mix and then centrifuge: 1200 rpm (= 295 g), 8 minutes, 20°C.
9. First washing: Aspirate almost all the liquid and resuspend the pellet in 5 ml R10. Transfer to a new Polypropylene Falcon 15 ml.
10. Centrifuge: 1200 rpm (= 295 g), 8 minutes, 20°C.

11. Wash twice more: Aspirate almost all the liquid and resuspend the pellet in 5 ml R10. Centrifuge: 1200 rpm (= 295 g), 8 minutes, 20°C.
12. Resuspend the cells in 5 ml R10 and count them. Now the cells are ready to be injected.
13. Check fluorescence of the cells on cytospins under the fluorescence microscope.

- "Post injection approach": Immunohistochemistry of epidermal and dermal sheets

The staining is done in 96-well plates: there are 12 columns of wells on one plate and in each two sheets of the same sample should be stained. The freshly prepared or thawed sheets can be placed directly into the wells or in special "sheet shifters", a device originally developed in our laboratory by Dr. P. Stöger. To be sure that the sheets are entirely covered with antibody solution pipette 100 µl into each well.

Procedure for single-colour labelling of MHC II molecules (tape stripping without injection)

1. Primary antibody: B21/2 supernatant, isotype control LO-DNP11 and PBS control. Incubate overnight at 4°C.
2. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
3. Second antibody: anti-rat biotinylated, diluted 1:100 in PBS / 1% BSA. Incubate for 90 minutes at 37°C.
4. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
5. Third step: Streptavidin-FITC, diluted 1:100 in PBS / 1% BSA. Incubate for 90 minutes at 37°C.
6. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
7. Use tooth picks to embed the sheets on a slide in Vectashield fluorescence mounting medium. Be careful about folds, overlappings and air bubbles. Store at 4°C in the dark (!).

Procedure for double-labelling of MHC II molecules (injection and tape stripping) (see also 3.6.5)

1. Primary antibody: B21/2 supernatant, isotype control LO-DNP11 and PBS control. Incubate for 90 minutes at 37°C.
2. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
3. Second antibody: mouse anti-rat Ig-FITC, diluted 1:30 in PBS / 1% BSA. Incubate for 90 minutes at 37°C.
4. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
5. Block residual anti-rat binding sites with rat γ gobulin for 30 minutes at 37°C.
6. Put on directly the third antibody: HB32 supernatant, isotype control Leu2 and PBS control. Incubate overnight at 4°C.
7. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.

8. Fourth antibody: Ig biotinylated anti-mouse, diluted 1:100 in PBS / 1% BSA. Incubate for 90 minutes at 37°C.
9. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
10. Fifth step: Streptavidin-Texas Red, diluted 1:100 in PBS / 1% BSA. Incubate for 90 minutes at 37°C.
11. Wash 3 times in PBS / 1% BSA for 20 minutes at room temperature.
12. Use tooth picks to embed the sheets on a slide in Vectashield fluorescence mounting medium. Be careful about folds, overlappings and air bubbles. Store at 4°C in the dark (!).

3.6.4 Intravenous injection of dendritic cells

1. Dendritic cells are harvested on the day of the injection and dyed with PKH-26, following the protocol (3.6.4 Labelling of bone marrow derived dendritic cells with PKH-26).
2. Centrifugation: 1200 rpm (= 295 g), 8 minutes, 4°C.
3. Aspirate the R10 and resuspend the cells in PBS (room temperature). Usually we injected $5x10^6$ dendritic cells in 200 µl PBS into each mouse.
   200 µl is the maximum volume that can be injected in a mouse intravenously at any one time. We varied the cell number (up to $25x10^6$ dendritic cells in 200 µl PBS into each mouse).
4. Before injection mice are irradiated with an heat lamp until they sweat behind the ears. With the increased blood circulation the vessels are enlarged, so that it becomes easier to see the veins. Turn the cut of the needle up, so that the scale can be seen at the same time. The mouse is put into a restraining device. With the left hand immobilize the mouse by pulling the tail towards you. With the right hand take the syringe, cut upwards, and introduce the needle in a proximal way, nearly parallel to the tail. The injection was successful if it is observed that the vein turns white as the cell suspension is inserted and there is no swelling of the surrounding tissue.

Detection of the injected cells is a simple process: cell suspensions of the organs of interest, or dermal and epidermal sheets are made. With the fluorescence microscope migrated cells can be distinguished from resident cells by their red fluorescence. (See also 3.3.10 Preparation of skin and lymphoid organs for migration studies).

3.6.5 Intradermal injection of dendritic cells

In this experimental setting the target cells are detected by selective staining of their MHC II-haplotypes. For this purpose F1 (BALB/c x C57BL/6) hybrid mice ($H\text{-}2^{d,b}$, fig. 1) were bred in our breeding facility at the University of Innsbruck, Austria. A pseudo-syngeneic "parent into F1" system was used: the parental bone marrow-derived dendritic cells of C57black mice were injected into the F1 generation (fig. 2 and 3).

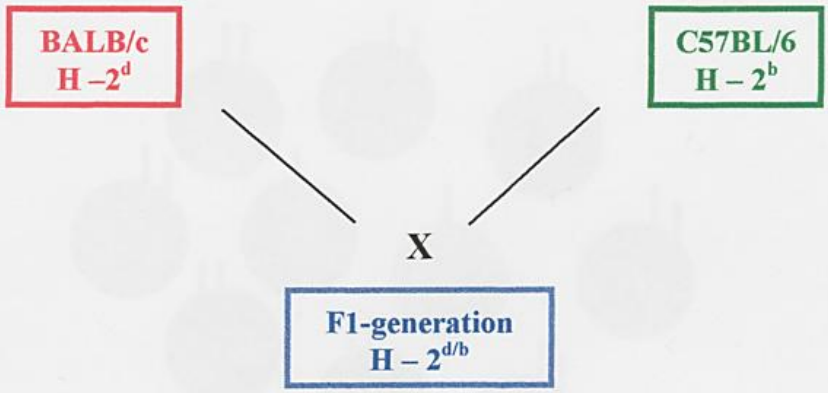


Figure 1: Concept and experimental setting for the intradermal injection of bone marrow derived dendritic cells. Following the Mendel rules for genetics the F1 generation possesses both alleles for the MHC class II haplotype.

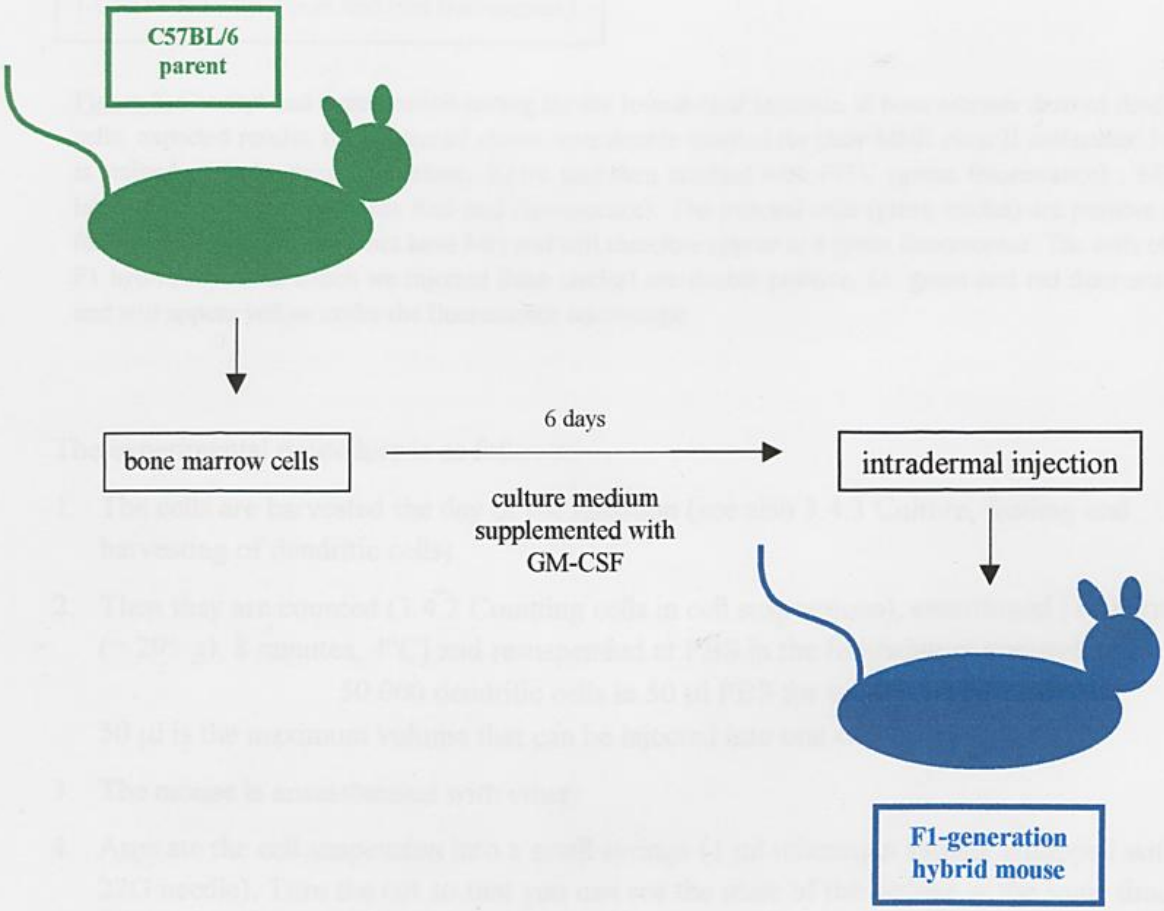
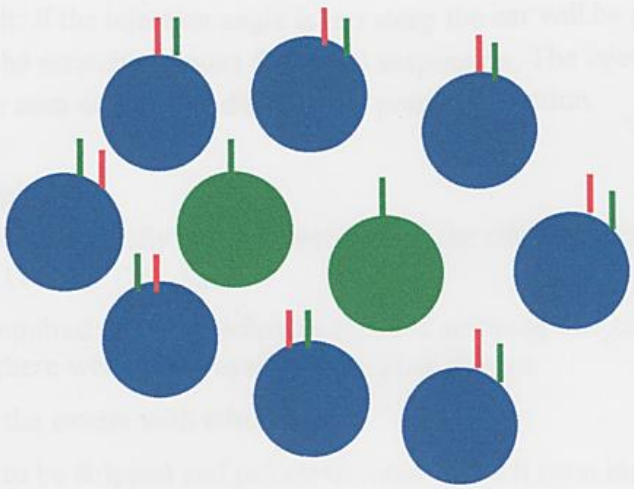


Figure 2: Experimental setting for the intradermal injection of bone marrow derived dendritic cells. The bone marrow of a C57BL/6 parent mouse is cultured for 6 days in our routine fashion and is then injected in the dermis of a F1-generation hybrid mouse. The ears of the F1-generation hybrid mouse were treated with tape stripping (see also). At defined time points after the injection F1 hybrid mice were sacrificed and epidermal sheets analysed.



I-A^b labelled with FITC (green fluorescence)
I-E^d labelled with Texas Red (red fluorescence)

Figure 3: Concept and experimental setting for the intradermal injection of bone marrow derived dendritic cells; expected results. the epidermal sheets were double labelled for their MHC class II molecules: I-$A^{b,d}$ is stained with the primary antibody B21/2 and then labelled with FITC (green fluorescence) , I-E^d is labelled with HB32 and Texas Red (red fluorescence). The injected cells (green circles) are positive only for I-A^b (C57BL/6 mice do not have I-E) and will therefore appear in a green fluorescence. The cells of the F1 hybrid mouse in which we injected (blue circles) are double positive, i.e. green and red fluorescence, and will appear yellow under the fluorescence microscope.

The experimental procedure is as follows:

1. The cells are harvested the day of the injection (see also 3.4.3 Culture, feeding and harvesting of dendritic cells).
2. Then they are counted (3.4.2 Counting cells in cell suspensions), centrifuged [1200 rpm (= 295 g), 8 minutes, 4°C] and resuspended at PBS in the following concentration:
   50.000 dendritic cells in 50 µl PBS for each ear.
   50 µl is the maximum volume that can be injected into one ear.
3. The mouse is anaesthetised with ether.
4. Aspirate the cell suspension into a small syringe (1 ml tuberculin syringe equipped with a 27G needle). Turn the cut so that you can see the scale of the syringe at the same time. Slightly bend the needle with the cut upwards. Place the empty corpus of a 10 ml syringe on the worktop, the anaesthetised mouse parallel to it. With the middle finger of the left hand stretch the ear of the mouse over the syringe corpus. Immobilise its head with the forefinger and thumb.
5. Now take the syringe containing the cell suspension with the right hand and gently slide the needle into the dermis. The cut is upwards and the scale of the syringe is visible. This

step is difficult: if the injection angle is too steep the ear will be punctured, if it is too flat it will simply be scratched. Inject 50 µl cell suspension. The injection is successful if a bubble can be seen developing distal to the point of injection.

3.6.6 Tape stripping

This procedure was essentially done as described in the classical work of Streilein, Lonsberry and Bergstresser (171).

1. Prepare the required pieces of adhesive tape for stripping one ear (in most of our experiments there were 12). I used regular TIXO® tape.
2. Anaesthetise the mouse with ether.
3. Take the ear to be stripped and put on the tape. Press it onto the ear and then pull it off. Repeat until the required number of strippings have been made. Usually the mouse comes out of the anaesthesia during this procedure and must be anaesthetised again.

The efficiency of tape stripping in our hands was controlled by measuring the Transepidermal water loss (TEWL) as described in the results (4.2.1 Epidermal water loss after tape stripping)

3.6.7 Preparation of epidermal and dermal sheets

1. Prepare a small petri dish with ammoniumthiocyanate-solution.
2. Cut off the ears of the mouse with curved scissors, pull off the dorsal half (i.e. the side of the ear that has received the injection or the tape stripping) from the cartilage with two tweezers. Put the dorsal half with the inner, moist part of the ears gently on the ammoniumthiocyanate-solution, so that it floats on the surface (the epidermis is not in contact with the ammoniumthiocyanate!). Incubate at 37°C for 20 minutes.
3. Prepare for each sample: 2 small petri dishes with PBS, 2 glass petri dishes with acetone (one for the epidermis and one for the dermis, respectively).
4. Separate the epidermis from the dermis with fine, curved tweezers and put each in one of the dishes with PBS. Continue until you have taken all the samples out of the ammoniumthiocyanate. It is harmful for them and therefore the incubation time should not be exceeded.
5. Take the pieces of epidermis and dermis and cut them into small squares of approximately 2 x 3 mm size ("sheets") with a scalpel. The sheets must not be too large to allow the antibodies to penetrate the skin entirely. One common shape, for example squares, is preferable since it will be easier to avoid overlappings or folds when they are embedded in the mounting medium after staining.
6. Collect the sheets and fix them for 20 minutes in acetone at room temperature.
7. Washing steps: in a 24-well culture plate (or small petri dishes if there are just 1 or 2 samples) prepare twice PBS and twice PBS / 1% BSA in each vertical row, one for each sample. After the fixation in acetone collect the sheets immediately and wash them at room temperature in PBS (2 x 20 minutes) followed by PBS/1% BSA (2 x 20 minutes).

8. After the washing the sheets are ready for immunohistochemistry. Alternatively, they may be conserved by freezing them at -20°C in a drop of PBS / 1% BSA.

3.6.8 Preparation of skin (epidermis and dermis) and lymphoid organs (bone marrow, spleen, mesenterial lymph nodes) for migration studies

Once the cell suspensions are prepared they should be stored as a pellet in fresh RPMI 1640 on ice, to prevent cells from adhering to the tubes or dying. Everything should proceed rapidly during this preparation.

1. Prepare 3 small petri dishes with RPMI 1640 for the extracted organs (mesenterial lymph nodes, femur and spleen) and 1 small petri dish with gauze, moist with PBS, for the ears. Thaw the aliquoted collagenase and dilute it 1:10 (final volume 5 ml) and 1:40 (final volume 30 ml) in Hank´s balanced salt solution.
2. Dissect the mouse and prepare the spleen, the mesenterial lymph nodes and 1 femur (as described in the standard preparation of the bone marrow, point 1 – 2). Put each in one small petri dish filled with RPMI 1640. Cut off the ears and put them on the moist gauze; store them at 4°C.
3. **Bone marrow**: continue as described in the standard preparation of the bone marrow, point 5 - 8. Store the cells as a pellet on ice for eventual counting.
4. **Lymph nodes**: hold the extracted tissue with fine tweezers and tease it into small pieces with cogged tweezers. The cells will be seen to burst out from the lymph nodes. Aspirate the RPMI 1640 plus cells and tissue with a pipette, rinse the dish a few times and then pipette it through a sterile net into a Falcon 50 ml. Try to squeeze the tissue with the sterile tip of the pipette. Rinse dish and sterile net again with some fresh RPMI 1640.
5. Lymph nodes: centrifugation: 1200 rpm (= 295 g), 8 minutes, 4°C. Store the cells in a pellet on ice until counted.
6. **Spleen**: Prepare one big petri dish (diameter 10 cm) with a few ml of collagenase diluted 1:40 with Hank´s ("collagenase-1:40"). Suck up approximately 10 ml of this collagenase with a syringe.
7. Put the spleen in the dish and hold it with tweezers. Slowly inject collagenase-1:40 into it. First from the ends towards the centre and then from the middle towards each side. The organ inflates a bit and becomes somewhat transparent, cells are washed out. With the cogged tweezers tease it into small pieces.
8. With a gentle circular movement of the dish group the tissue pieces in the middle. Suck up the liquid with the cells, as far as possible without tissue, and collect it in a Falcon 50 ml. Rinse the tissue pieces again with fresh collagenase-1:40 and collect liquid and cells.
9. Add collagenase-1:10 to the tissue and incubate for approximately 10 minutes at 37°C. In the meantime centrifuge the already extracted cells: 1200 rpm, 8 minutes, 4°C.
10. Prepare another petri dish with a sterile sieve. When the cells are centrifuged aspirate the collagenase-1:40 down to 3 ml. Resuspend the cells and filter the cell suspension through the sieve. Take out the tissue pieces from the incubator, suck them up and filter them as well. With the pestle of a 30 ml syringe push and squash the tissue through it. Put away the

sieve with the leftovers. Suck up the cell suspension and filter it through a sterile net into a Falcon 50 ml. Rinse the petri dish and the net again with collagenase-1:40.

11. Centrifugation: 1500 rpm (=460 g), 8 minutes, 4°C.

12. Aspirate the collagenase-1:40 entirely and resuspend the isolated cells in 2,5 ml dense BSA. Try to avoid bubbles.
Dense BSA (bovine serum albumin)" is a separation medium, that was specifically developed for the isolation of dendritic cells from spleen (172,173). After centrifugation the low density fraction is enriched in immature dendritic cells. The preparation of dense BSA is tricky. Detailed protocols can be found in the following publications: (12,170,174).
Transfer the cell suspension to Beckmann centrifugation tubes.

13. With a special anti-drop pipette gently layer 1 ml R10 on top of the cell suspension, carefully avoiding mixing the two phases! Tilt the tube, with the pipette enter the tube until contact is made with the surface of the dense BSA. Then start to pipette R10 very gently and slowly and at the same time take the pipette higher, so that the distance to the surface of liquid stays the same. When 1 ml R10 have been pipetted take out the pipette, still tilting the tube. Then slowly put it into an upright position. The better the two phases remain separated the better the enrichment step will work.

14. Pipette 2,5 ml dense BSA and 1 ml R10 in a counter-balance tube.

15. Centrifugation in the Sorvall "super speed"-centrifuge: 7.000 rpm (= 10.800 g), 14 minutes, 4°C, no brakes.

16. Aspirate the interphase, on which the dendritic cells float, with a Pasteur pipette and collect it in a Falcon 15 ml. Be careful about cells sticking on the side of the tube. Aspirate nearly all the liquid, but the pellet should not be interfered with. Conserve the pellet until the cells have been counted.

17 Fill up the Falcon 15 ml with RPMI 1640, shake a few times upside down to avoid formation of a gradient! Then centrifuge: 1200 rpm (= 295 g), 8 minutes, 4°C.

18. Check the pellet after the centrifugation. Suck off the supernatant completely with a vacuum pump and resuspend the cells in a suitable volume of R10 for counting.

19. **Ears**: the preparation of the ears is the same as described for epidermal and dermal sheets (point 1 - 7). The sheets are directly embedded in Vectashield fluorescence mounting medium.

3.6.9 Cytospin preparation

1. The slides are clamped together with filter cards and funnel. Cell concentration is set to 150.000 cells per ml R10.
200 µl cell suspension (= 30.000 cells) are applied per slide.

2. Centrifugation in the Cytospin: low acceleration, 6 minutes, 450 rpm.

3. First two test slides are run and cell density on the slide is checked in the Nikon TMS phase contrast microscope. The single cells should be clearly distinguishable from each other. For migration studies cell density was varied: In this experimental setting it was more useful to cover the slide with a densely packed cell layer, in order to be able to sample as

many cells as possible. In spite of the high density, detection of PKH26-positive cells under the fluorescence microscope was easy due to their bright fluorescence. Moreover, the above mentioned concentration is optimal for Cytospin preparations of mature dendritic cells. These cells are relatively big. The cells studied here are of different smaller sizes. Therefore cell density in the cell suspension could even be doubled in migration studies.

5. The slides are dried for 1 hour at room temperature and can then be frozen at -20°C.

6. For microscopical analyses the slides are taken out from the freezer (-20°C) and dried at room temperature for at least an hour.

7. Then they must be fixed 5 minutes in acetone and dried for another 10 minutes at room temperature.

8. The slides can be directly embedded with half a drop of Vectashield fluorescence mounting medium and half a drop of DAPI if the target cells are already stained with PKH26. Store at 4°C in the dark.

4. Results

4.1. PKH26

The first step was to test the dying properties of PKH26 in our own experiments and to compare the results with the information given by the manufacturer and in the literature (68,175-177). This would give us a better knowledge about the suitability of PKH26 for the planned experiments.

4.1.1 At what levels can PKH26-stained cells be detected by FACS analysis in a mixed population?

Our purpose is to inject cells stained with PKH26 into a mouse and at defined time points thereafter analyse cell suspensions of lymphoid organs. It can be assumed that the injected cells will constitute a very small percentage of all the cells of these organs. The easiest and quickest way to detect the target cells in these cell suspensions would be by FACS analysis, but as a prerequisite it would need to be very sensitive.
Dendritic cells were cultured form bone marrow-derived precursor cells for 12 days. On day 12 cells were harvested, counted and half the population stained with PKH26. To get an idea about the sensitivity of flow cytometric analysis we mixed different percentages of stained and unstained cells, as listed in table 3. FACS analysis was done the same day.

Table 3: Detection of dendritic cells stained with PKH26 by FACS analysis, list of experiments and results. The sensitivity of FACS analysis of stained and unstained dendritic cell populations mixed in different proportions was tested.

Exp. no.	µl unstained cell suspension	µl stained cell suspension	% unstained cells	% stained cells	% stained cells detected by FACS analysis (20.000 counts)
1	200	0	100%	0%	set 0%
2	0	200	0%	100%	99,58%
3	100	100	50%	50%	67,48%
4	150	50	75%	25%	39,01%
5	180	20	90%	10%	14,54%
6	190	10	95%	5%	6,06%
7	196	4	98%	2%	4,19%
8	198	2	99%	1%	2,18%

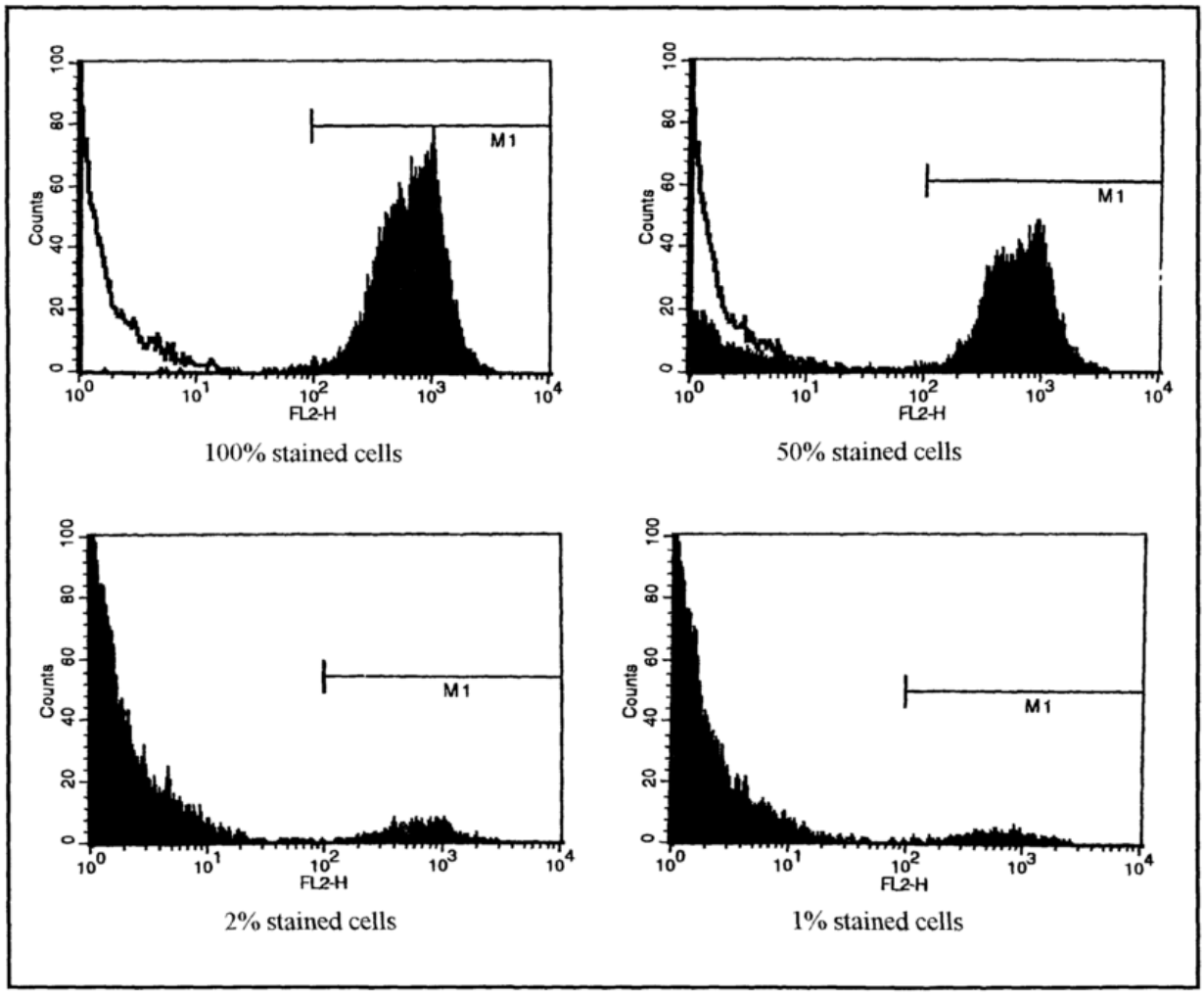


Figure 4: Sensitivity of FACS analysis of stained and unstained dendritic cell populations mixed in different proportions was tested on the day of PKH26 staining. Cells stained with PKH26 can be detected even at low levels, i.e. if only 1% of all cells are positive. 20.000 events were analysed. Open histogram represents unstained control cells.

Cells stained with PKH26 can be detected even at low levels, i.e. if only 1% of all cells are positive (fig. 4). Positive cells are clearly distinguishable. So, FACS analysis seems to be a valid means for quantifying PKH26 positive cells in cell suspensions of lymphoid organs.

4.1.2 Does PKH26 stay stably linked to the cell surface in co-culture? Do the percentages of positive cells change in time?

According to the information given by the manufacturer, PKH26 stays stably linked to the cell surface and the wash out in time can be ignored. To test this statement, dendritic cells from bone marrow-derived precursor cells of BALB/c mice were cultured for 12 days (no enriching steps) and then half the population was stained (= day 0). Populations of stained and unstained cells were mixed and co-cultured for 6 days.

The following cultures were set up:

- 100% PKH26-stained cells, as positive control and to check for a possible loss of intensity in the fluorescence of PKH26.
- A mixed population of 50% stained and 50% unstained cells, the "real" co-culture. These cells differ only in the staining.
- 100% unstained cells as negative control.

On days 0, 3 and 6 FACS-analyses and Cytospin preparations of all samples were done.

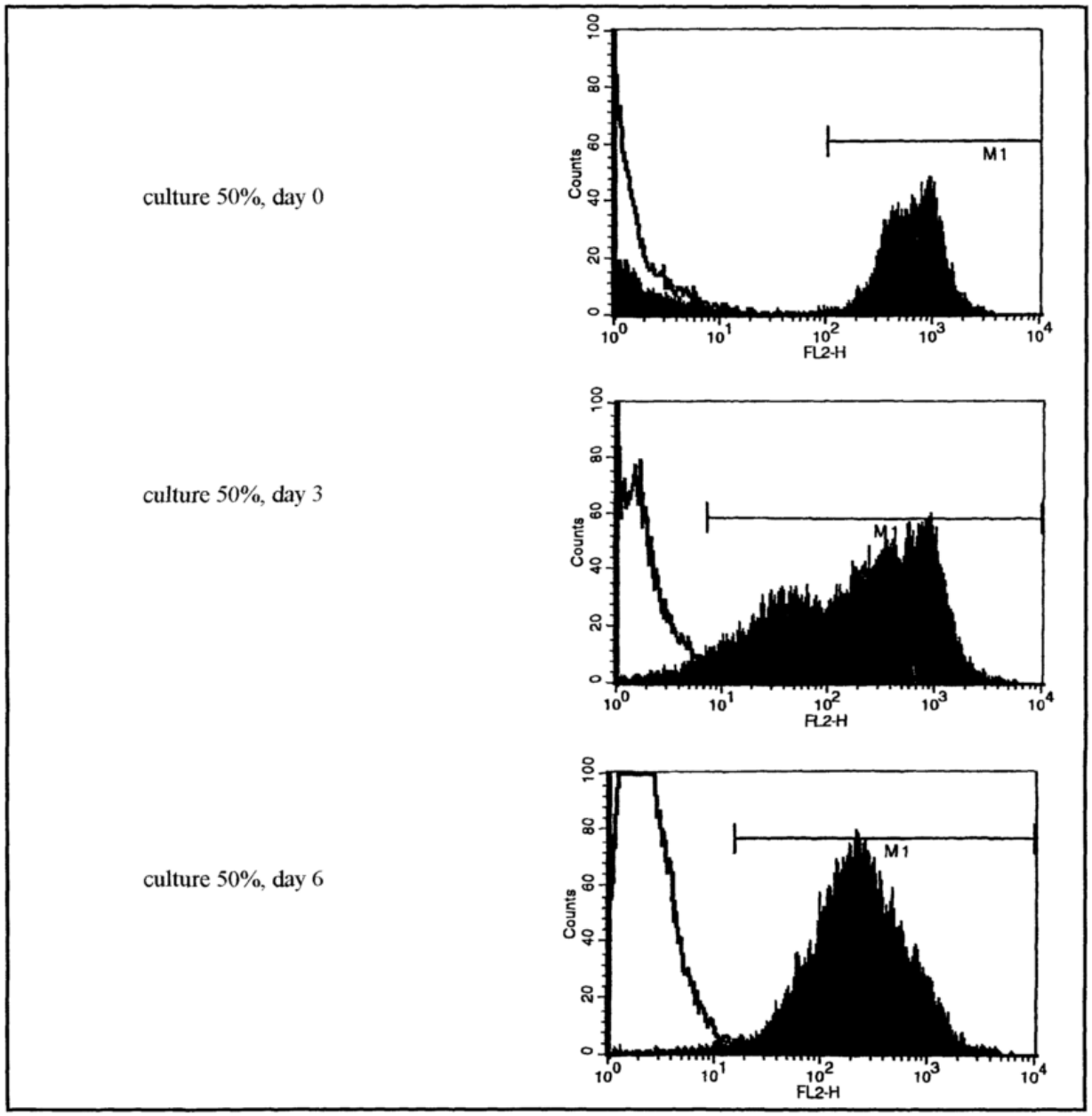


Figure 5: Analysis of co-cultures consisting of 50% PKH26-stained cells and 50% unstained cells with time (same data as in table 4). Note that the percentage of PKH26-positive cells increases to >90% on days 3 (middle panel) and 6 (bottom panel) of co-culture. 10 – 20.000 cells were analysed. Open histogram represents unstained control cells.

The percentage of positive cells in the co-culture (= culture 50%) increases. On day 3, two populations can be distinguished (fig. 5) that seem to "melt" on day 6, on which 98,8% of all cells are PKH26-positive (see also table 4 and fig. 6). Direct control with the fluorescence microscope could confirm the data given by FACS analysis: virtually all cells were PKH26-positive on day 6 (fig. 7).

Table 4: Flow cytometric analysis of a co-culture experiment of bone marrow-derived dendritic cells. PKH26 positive cells detected after 0, 3 and 6 days of co-culture in per cent of the total counts.

Culture	% positive cells detected on day 0	% positive cells detected on day 3	% positive cells detected on day 6
culture 0% (unstained cells, negative control)	0%	0%	0%
culture 50% (50% PKH26-stained cells and 50% unstained cells)	67,48%	96,80%	98,80%
culture 100% (PKH26-stained cells, positive control)	99,58%	97,92%	96,02%

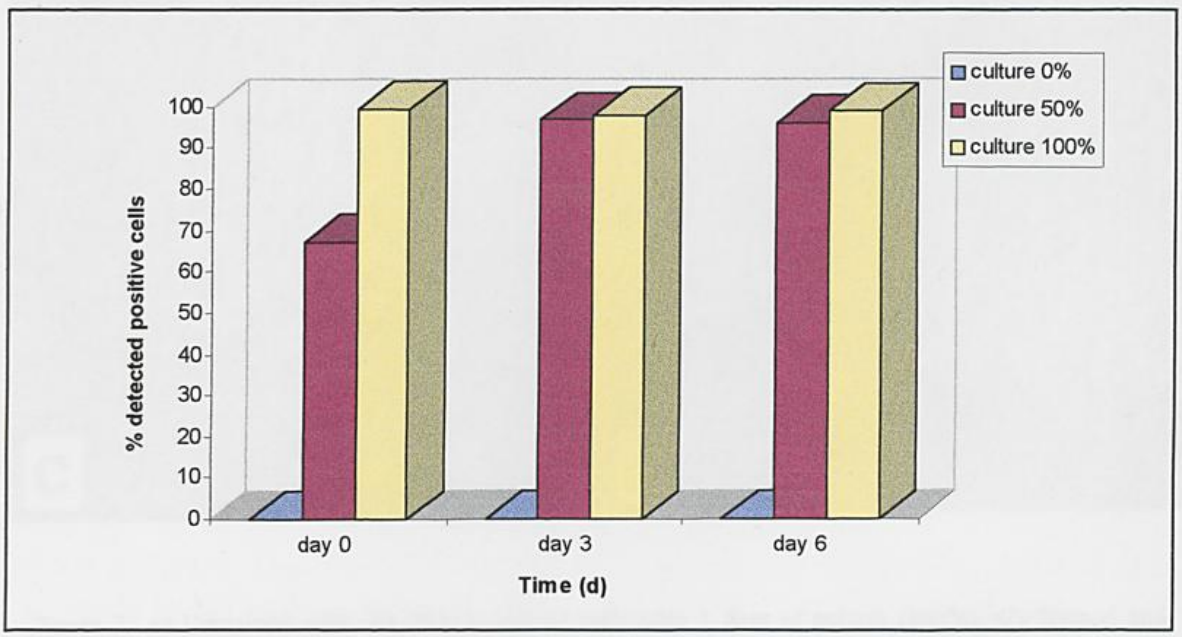


Figure 6: Change of the percentages of PKH26-positive cells during co-culture with unstained cells (same data as in table 4). Note that in the co-culture experiment on days 3 and 6 nearly all dendritic cells are PKH26-positive.

Already after 3 days, all the cells of the co-cultured cell populations are stained with PKH26. In figure 7 it can be seen that the intensity of the fluorescence is not equally bright on all the cells, but nevertheless they are all positive.

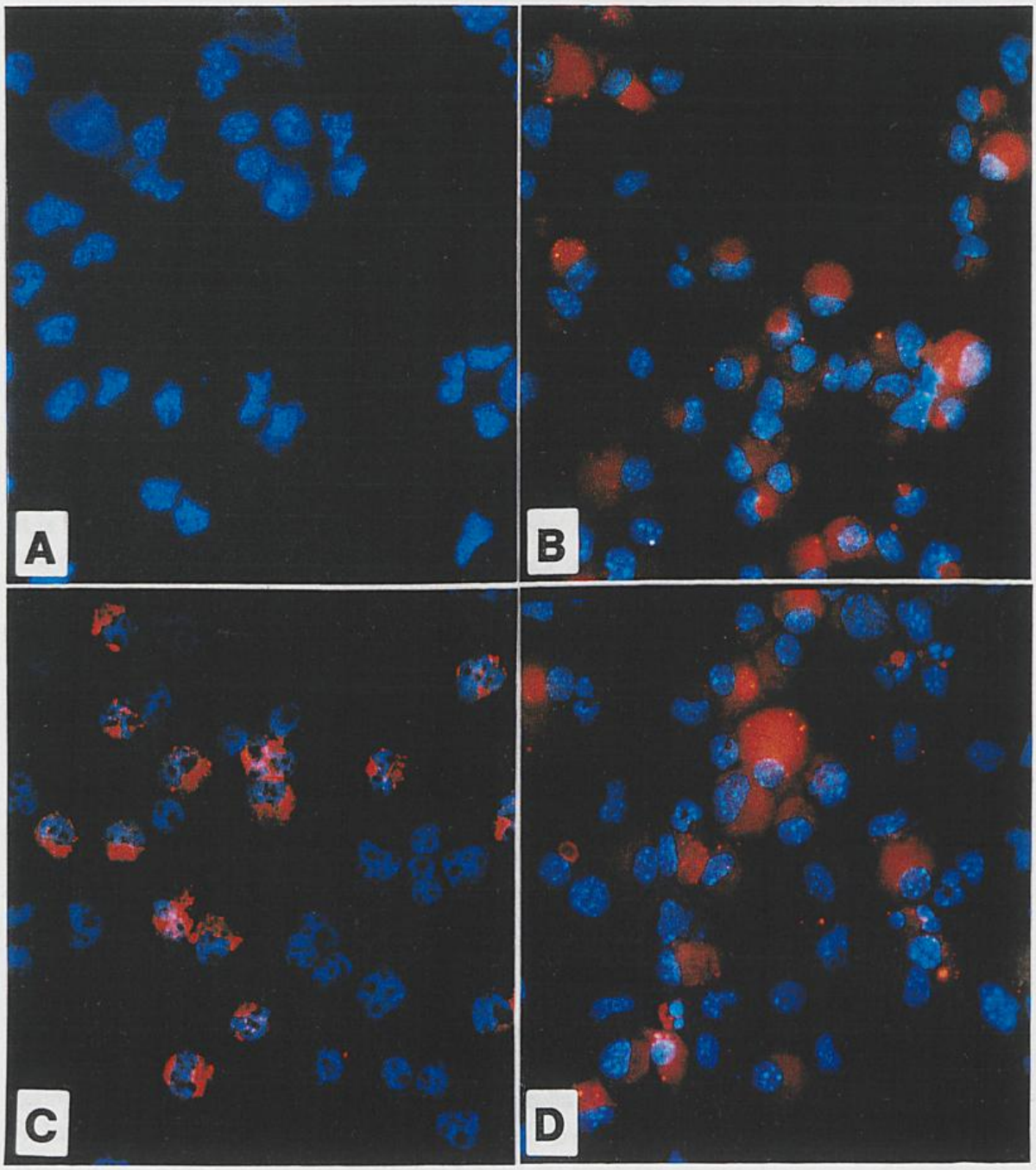


Figure 7: **A)** Unstained cells, **B)** PKH26-stained cells after 3 days of culture (100%), **C)** Stained and unstained dendritic cells the day of staining (=day 0), **D)** Stained and unstained dendritic cells after 3 days of co-culture (400x). It can be seen, that the vast majority of cells is PKH26-positive. The intensity of PKH26-fluorescence becomes more diffuse during the culture period (=6 days).

4.1.3 Is there a transfer of PKH26 between the populations?

To understand the mechanism responsible for this *in vitro*-effect and perhaps to prove a transfer of PKH26 to the unstained cells, the following experiment was done:
Dendritic cells from two different mouse strains, BALB/c and C57BL/6, were used. These two mouse strains differ in the haplotype of their MHC II molecules and they can therefore be selectively stained with appropriate monoclonal antibodies. One dendritic cell population is stained with PKH26 (red fluorescence) and the other one is immunohistochemically stained with the antibody against its MHC II molecule (FITC, green fluorescence). Double-positive cells after 3 days of co-culture would confirm a transfer of PKH26 from stained to unstained cells.
Bone marrow-derived precursor cells are cultured for 6 days to obtain standard immature dendritic cells. Dendritic cells of the C57BL/6 mouse strain were stained with PKH26. Dendritic cells from BALB/c mice were stained with mAb HB 32 followed by biotinylated sheep anti mouse and streptavidin-FITC. This combination was chosen because of the availability of a better primary antibody against BALB/c MHC class II molecule ($I\text{-}A^d$), i.e. HB 32 supernatant. Seven samples were cultured until day 9, again comprising 3 days of co-culture (day 6 –9). Equal numbers of BALB/c and C57BL/6 cells were mixed. This set-up is schematised in table 5.
It is known that PKH26 stresses the dendritic cells. Approximately 30-50% of the cells are lost or die during the staining procedure. To check for possible differences in the ability of PKH26 -stained cells to adhere to the bottom of the culture dish and to proliferate we counted the harvest from each dish and compared it to unstained cells (table 6). Such differences would affect the percentages of stained and unstained cells in the population and be reflected in the FACS analysis.
PKH26-stained dendritic cells could be recovered in equal, if not better yields than unstained cells. This confirms and extends the manufacturer's indications with regard to BMDC. Also, the density of the cell layer that still adhered to the bottom of the dish after harvesting was equal for all cultures. PKH26-stained dendritic cells apparently adhere and proliferate in a normal way. This again suggests that the observed augmentation of positive cells after co-culture is caused by a transfer of PKH26.
FACS analysis and Cytospin preparations were again the methods of choice to analyse the percentage of PKH26-positive cells on day 6, the day of staining, and day 9. Flow cytometric analysis on day 6 before the onset of co-cultures (fig. 8) confirmed the correct mixing of cell populations. After 3 days of co-culture the changes described earlier (4.1.2) became apparent again: The proportions of stained and unstained cells shifted; more cells became stained (fig. 9). In the cultures 2, 4, 5, and 6 nearly all the cells are now PKH26-positive. Cultures 2 (BALB/c + C57BL/6) and 4 (C57BL/6 + C57BL/6) show the identical increase of positive cells in co-culture. Therefore, this effect is not dependent of the mouse strain. Moreover, the increase in PKH26-positive cells also occurred in cultures 4,5 and 6 (fig. 9), indicating that it is also not dependent on cell density.

Table 5: List of experiments performed to prove a transfer of PKH26 from stained to unstained dendritic cells during co-culture. C57BL/6-dendritic cells stained with PKH26 and unstained BALB/c-dendritic cells were mixed in equal numbers and co-cultured for 3 days. After co-culture cells were harvested and only BALB/c dendritic cells were immunohistochemically stained for MHC class II (I-A^d). Co-cultures were analysed for red PKH26-fluorescence and green I-A^d-fluorescence by flow cytometry. Double positive cells, for example in culture 2, would show a transfer of PKH26 (see also figure 10, lower panel).

Exp. no.	Dendritic cells from BALB/c mice	Dendritic cells from C57BL/6 mice	cells per culture dish	comments
1	unstained	-	$5x10^6$	negative control (for BALB/c)
2	unstained	stained	$5x10^6$	co-culture to check for a transfer of fluorescent cell linker from the C57BL/6 population to the unstained BALB/c population
3	-	stained	$5x10^6$	positive control
4	-	50% unstained 50% stained	$5x10^6$	control for interactions between the two mouse strains, comparison with nr.2, and wash out of PKH26 due to high cell density *in vitro*, comparison with nr.5 and 6
5	-	50% unstained 50% stained	$2x10^6$	control for wash out of PKH26 due to high cell density *in vitro*, different cell numbers in the culture dishes
6	-	50% unstained 50% stained	$1x10^6$	control for wash out of PKH26 due to high cell density *in vitro*, different cell numbers in the culture dishes
7	-	unstained	$5x10^6$	negative control (for C57BL/6)

Table 6: Numbers of dendritic cells harvested from the single cultures after 3 days of co-culture. PKH26-stained dendritic cells could be recovered in equal numbers as compared to PKH26-unstained cells. They adhere and proliferate in a normal way, confirming the manufacturer's indications .

Exp. no.	Culture	dendritic cells plated in 1 dish on day 6	dendritic cells harvested on day 9	recovery
1	BALB/c unstained	$5x10^6$	$2,3x10^6$	46%
2	50% BALB/c unstained, 50% C57BL/6 stained	$5x10^6$	$2,8x10^6$	56%
3	C57BL/6 stained	$5x10^6$	$2,4x10^6$	48%
4	50% C57BL/6 unstained, 50% C57BL/6 stained	$5x10^6$	$2,9x10^6$	58%
5	50% C57BL/6 unstained, 50% C57BL/6 stained	$2x10^6$	$1,3x10^6$	65%
6	50% C57BL/6 unstained, 50% C57BL/6 stained	$1x10^6$	$0,38x10^6$	38%
7	C57BL/6 unstained	$5x10^6$	$1,8x10^6$	36%

When analysing co-cultures consisting of immunolabelled BALB/c dendritic cells (green fluorescence) and C57BL/6 dendritic cells stained with PKH26 (red fluorescence), it becomes apparent, that some cells are double-positive (fig. 10). For a better understanding it must be explained that BALB/c dendritic cells are immunolabelled at the end of the co-culture with an anti-MHC II mAb (HB 32), followed by a secondary antibody (biotinylated sheep anti mouse) and streptavidin-FITC (green fluorescence). C57BL/6 dendritic cells are stained with PKH26 (red fluorescence) before the onset of co-culture.
Double positive cells express both red PKH26 fluorescence and green MHC II fluorescence at the end of co-culture. This indicates that BALB/c dendritic cells must have taken up PKH26-positive C57BL/6 dendritic cells, or at least free dye. In further experiments it was tried to study the way of PKH26-uptake (4.1.4).

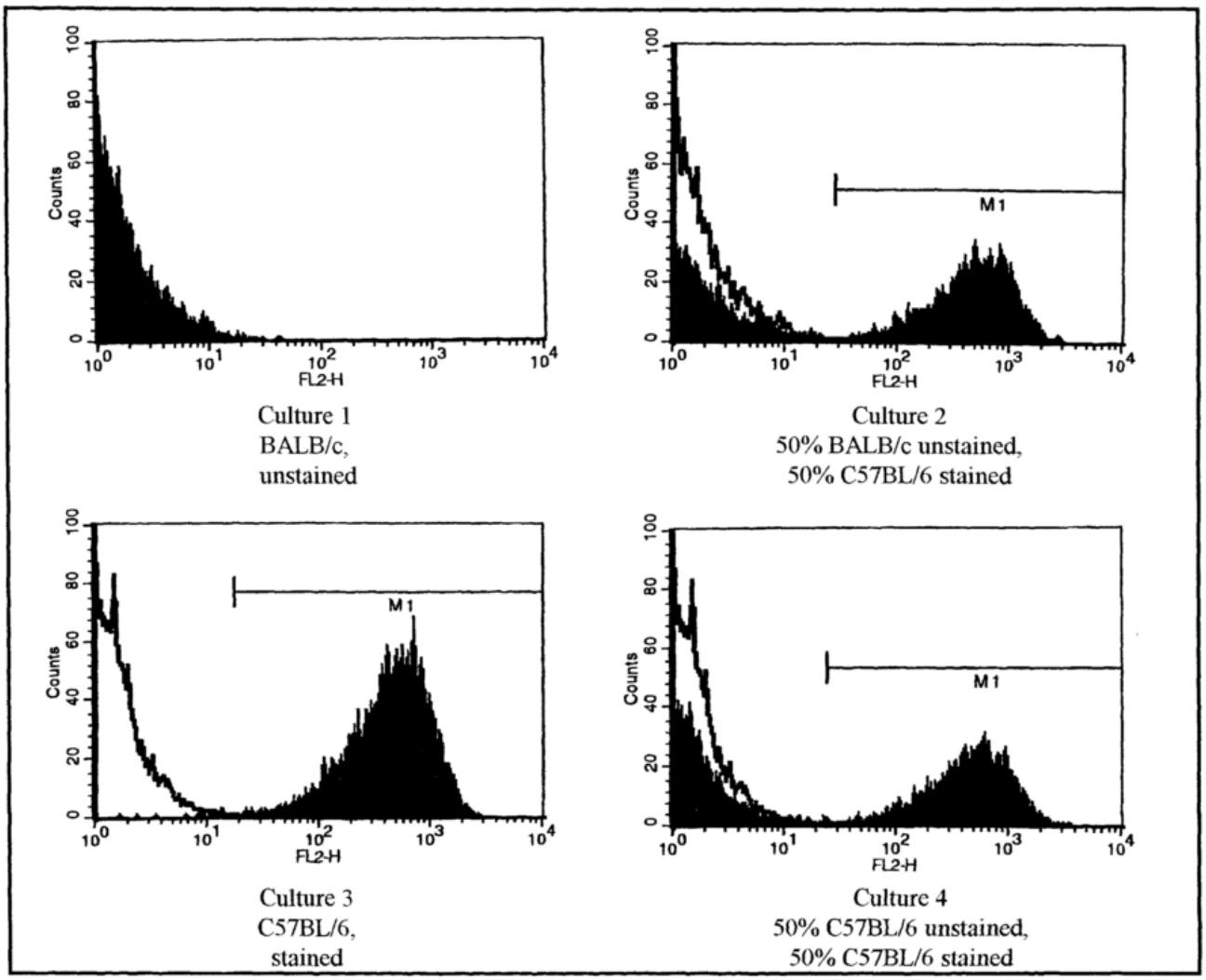
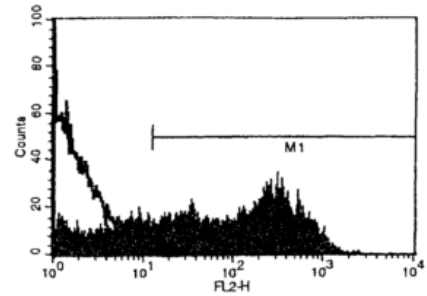
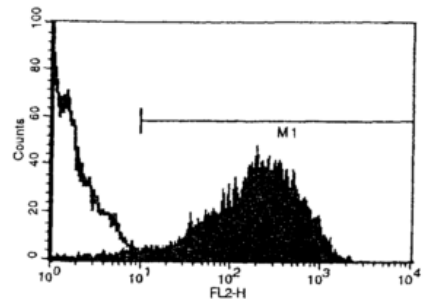


Figure 8: Flow cytometric analysis to confirm the correct mixing of cell populations. Analysis is performed before the onset of co-culture on day 6, the day of staining with PKH26. In each dish a total number of $5x10^6$ dendritic cells was plated. Open histogram represents unstained cells, 10.000 events were analysed respectively.

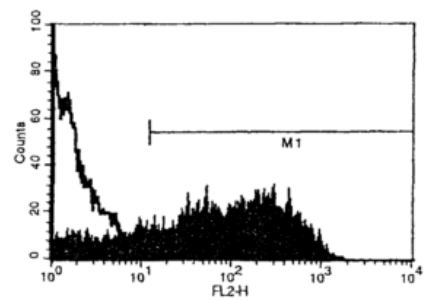
culture 2
50% BALB/c unstained,
50% C57BL/6 stained
$5x10^6$ cells per culture dish



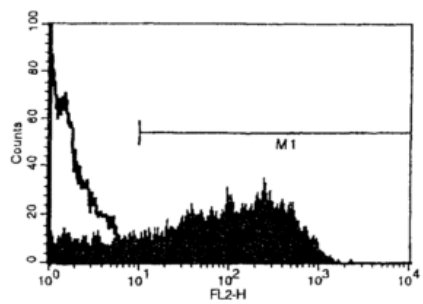
culture 3
C57BL/6, stained
$5x10^6$ cells per culture dish



culture 4
50% C57BL/6 unstained,
50% C57BL/6 stained,
$5x10^6$ cells per culture dish



culture 5
50% C57BL/6 unstained,
50% C57BL/6 stained,
$2x10^6$ cells per culture dish



culture 6
50% C57BL/6 unstained,
50% C57BL/6 stained,
$5x10^6$ cells per culture dish

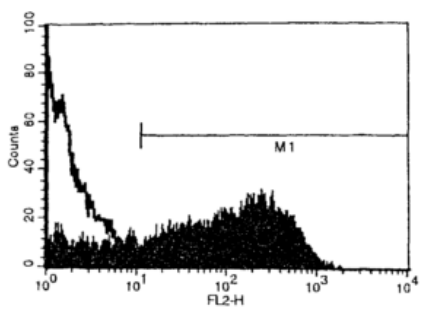


Figure 9: Flow cytometric analysis of the cultures listed in table 5 on day 9, after 3 days of co-culture. A prominent increase of PKH26-positive cells in cultures 2, 4, 5, and 6 can be seen. This increase is neither dependent on the mouse strain (compare 2 and 4), nor on cell density in the culture dishes (compare 4, 5 and 6). Open histogram represents unstained cells, 10.000 events were analysed respectively.

In the dot plots in figure 10 it is shown that a transfer of PKH26 from the stained population to the unstained one is in fact happening. The MHC II molecules of BALB/c dendritic cells are immunohistochemically labelled with a specific primary antibody (i.e. HB32), a secondary, biotinylated antibody (i.e. sheep anti mouse Ig, biotinylated) and streptavidin-FITC (green fluorescence, FL1-H). The C57BL/6 dendritic cells are stained with PKH26 (red fluorescence, FL-2H).
The hypothesis of a transfer of PKH26 from stained dendritic cells to unstained cells has been confirmed by this experiment.

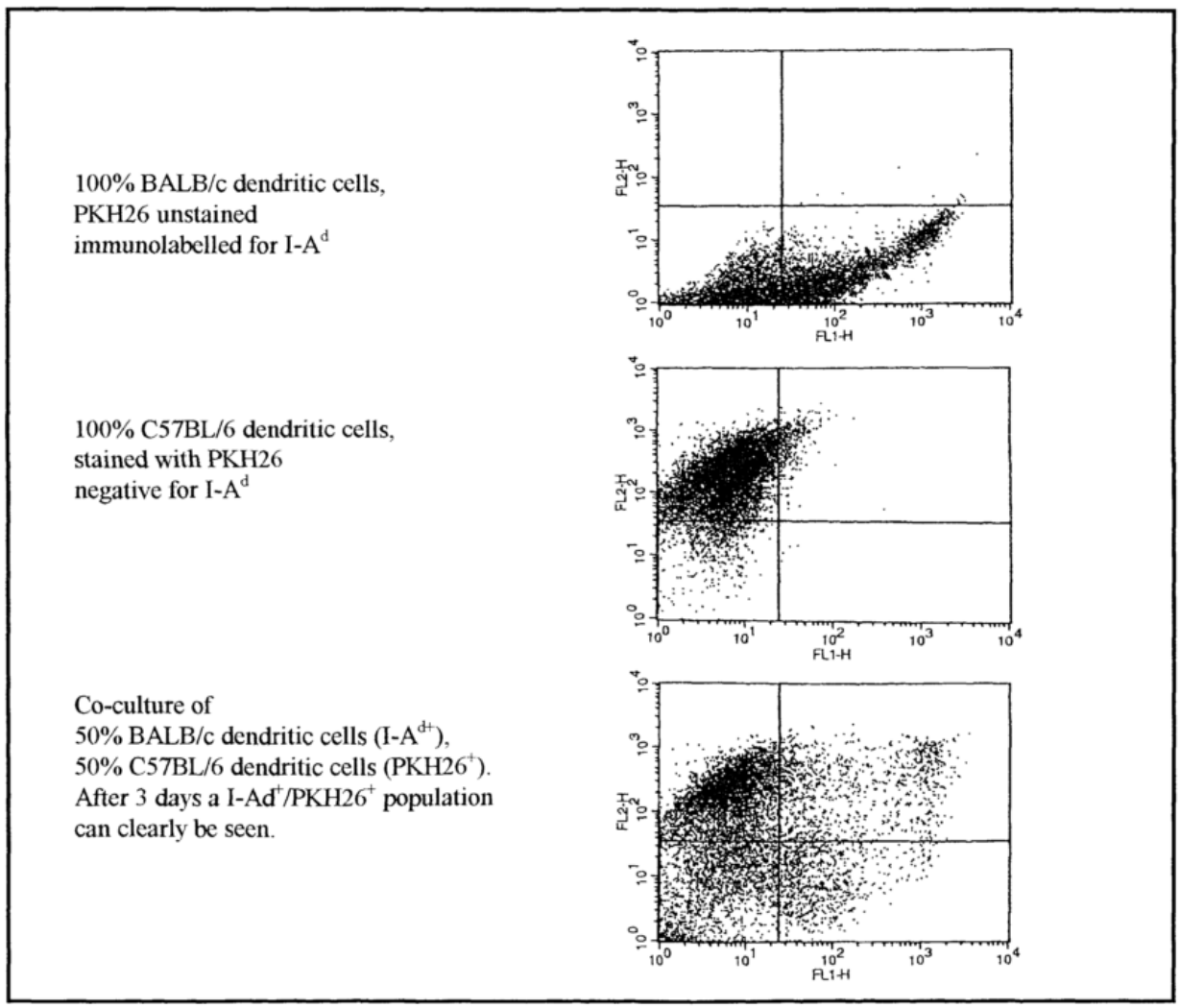
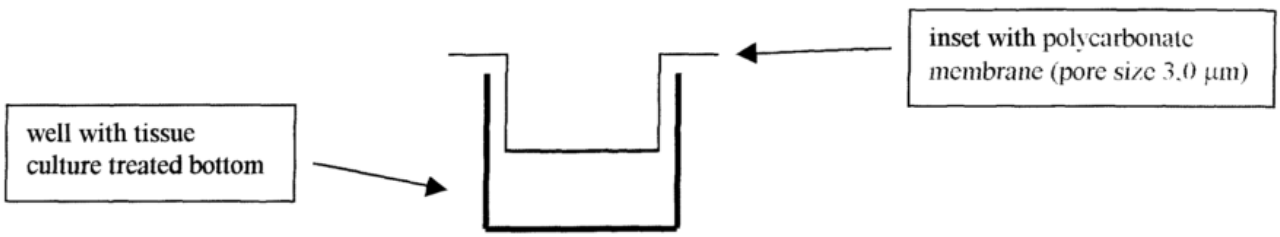


Figure 10: Transfer of PKH26 to the unstained cell population after 3 days of co-culture. **Upper panel**: distribution of BALB/c dendritic cells selectively immunolabelled for I-A^d after culture (FL1-H). These cells are PKH26$^-$ (FL2-H). **Middle panel:** C57BL/6 dendritic cells stained with PKH26 before the onset of culture (FL2-H). Because of the haplotype of the mouse strain these cells are I-A^{d-} (FL-1H). **Lower panel:** FACS analysis after 3 days of co-culture of BALB/c dendritic cells (PKH26$^-$) and C57BL/6 dendritic cells (PKH26$^+$). At the end of co-culture BALB/c dendritic cells were selectively immunolabelled for I-A^d. The appearance of a double-positive cell population (I-A^{d+}/PKH26$^+$, upper right quadrant) shows that an uptake of PKH26 by BALB/c dendritic cells has happened.

4.1.4 Is PKH26 transferred by phagocytosis of stained cells?

As mentioned above, question of interest was whether whole cells were phagocytosed in this transfer, or if any cell-cell-contact was necessary. Bone marrow-derived precursor cells were cultured for 6 days to obtain immature dendritic cells. On day 6 they are harvested, counted and half the population was stained with PKH26.

We used a 6-well-plate with tissue culture treated bottom, PVP free, and an inset with a polycarbonate membrane (pore size 3,0 µm) for the experiment. The pore size guarantees that dendritic cells can not squeeze through the membrane into the other compartment, whereas dissolved dye and stained cell membrane fragments can pass through.



The wells and insets were filled in the following way:

- first row: inset filled with 300.000 stained dendritic cells, well filled with 300.000 unstained dendritic cells.
- second row: inset filled with 300.000 unstained dendritic cells, well filled with 300.000 stained dendritic cells.

The cells were suspended in 4 ml R10 culture medium supplemented with GM-CSF, so that the polycarbonate membrane was covered and the two compartments were joined together by the liquid phase. After 3 days of co-culture inset and well were harvested separately and FACS analysis was done (fig. 11).

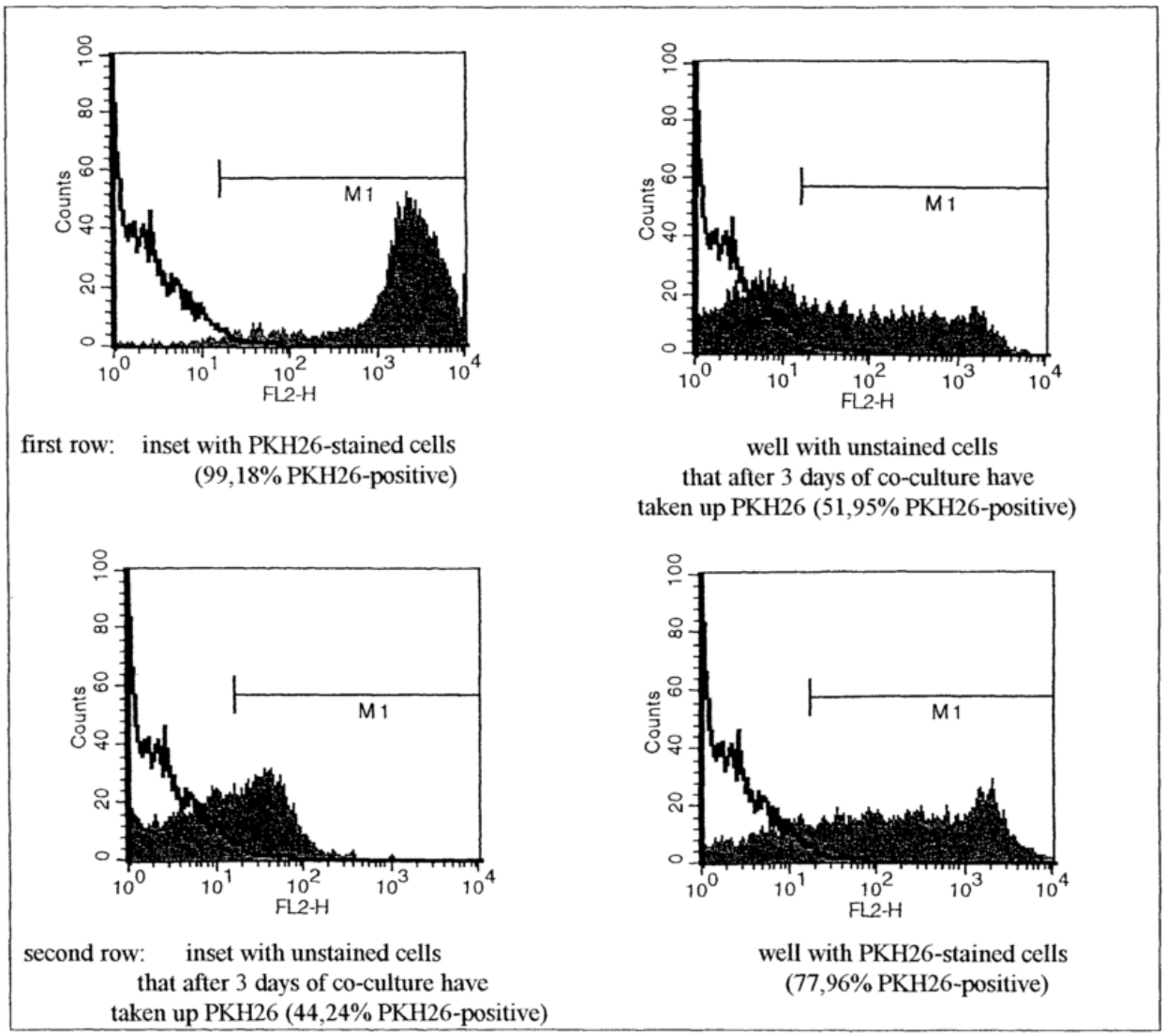


Figure 11: Transfer of PKH26 from stained to unstained dendritic cells separated by an inset with a polycarbonate membrane (pore size 3,0 µm). After 3 days of co-culture unstained dendritic cells, independently of the compartment in which they were plated, have become PKH26-positive (first row, right panel; second row, left panel).

The transfer of PKH26 fluorescent dye is not due to phagocytosis of whole cells and does not need any cell-cell-contact.

To find out if the sterile filtered supernatant of stained dendritic cells was sufficient to transfer PKH26, a further experiment was performed.

BALB/c bone marrow derived dendritic cells were stained with PKH26 on day 6, and plated on culture dishes. Culture went on until day 9, cells were harvested, spun and the supernatant was collected. This supernatant was sterile filtered and added to unstained, immature (=cultured until day 6) BALB/c bone marrow derived dendritic cells. After 3 days of incubation with the supernatant, flow cytometric analysis of the unstained cells was performed. Hardly any PKH26-positive cells could be detected (fig. 12).

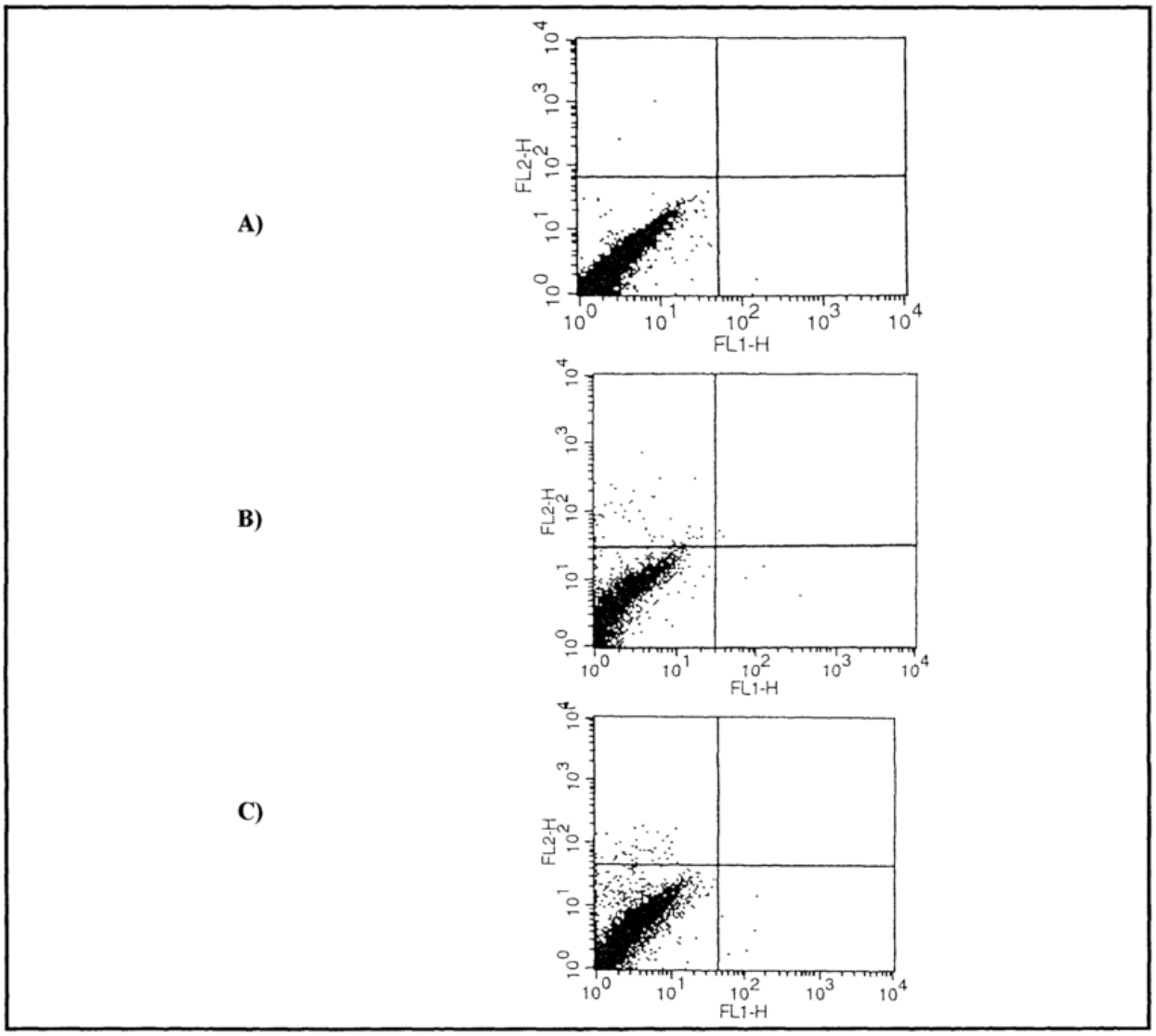


Figure 12: FACS analysis of bone marrow-derived dendritic cells incubated with sterile filtered supernatnat of PKH26-stained dendritic cells after 9 (**A**), 13 (**B**) and 15 days (**C**). Hardly any PKH26-positive cells (FL2-H) can be detected.

4.2 Tape stripping

Langerhans cells in the epidermis have a certain density during a steady state. If this closeness is reached it is an unproved assumption that the epidermis is "full". Immigration could only thake place when resident Langerhans cells leave the epidermis to fulfill their duty as guardian of the immune system, and a "hole" in the net of Langerhans cells appears. The target in these experiments therefore was to create a demand for new Langerhans cells in the epidermis by "emptying" it in as gentle a way as possible and without destroying the histological structures into which (injected) progenitors could immigrate. The method of choice was tape stripping.

4.2.1 Epidermal water loss after tape stripping

At first it was necessary to get an idea about how many tape strippings would be useful in this specific experimental setting, so the epidermal loss of water after different numbers of strippings was checked. This parameter is an indicator for the barrier disruption caused by this mechanical treatment and thereby for the strength of the irritation of the skin.

Table 7: Epidermal water loss measured after tape stripping with adhesive tape (TIXO®).

Number of tape strippings	water loss
Control (0 strippings)	+5,5
4 strippings	+9 - +10
6 strippings	+15
10 strippings	+26 - +30

The values obtained in this experiment (table 7) have to be viewed with some caution due to the fact that the instrument used to determine the epidermal water loss is constructed for humans and not mice. The area for measuring is standardised, but the ear of a mouse is smaller than this area. Nevertheless, for our purpose these results are sufficient. After determining the number of strippings that would cause a certain effect we tested them in a pilot experiment.

Mice were stripped 6 or 10 times on the left ear. The right ear of the same mouse served as untreated control. At defined time points thereafter mice were sacrificed and epidermal sheets were prepared. Langerhans cells were immunohistochemically stained. The sheets were evaluated with the fluorescence microscope using counting lenses with squares: Langerhans cells in 5 - 20 small squares were counted. Emigration is described in % Langerhans cells still found in the epidermis, setting the control ear of each mouse as 100%. Because the cell numbers differ from mouse to mouse, the counts of the control ears were not pooled and each mouse was calculated separately.

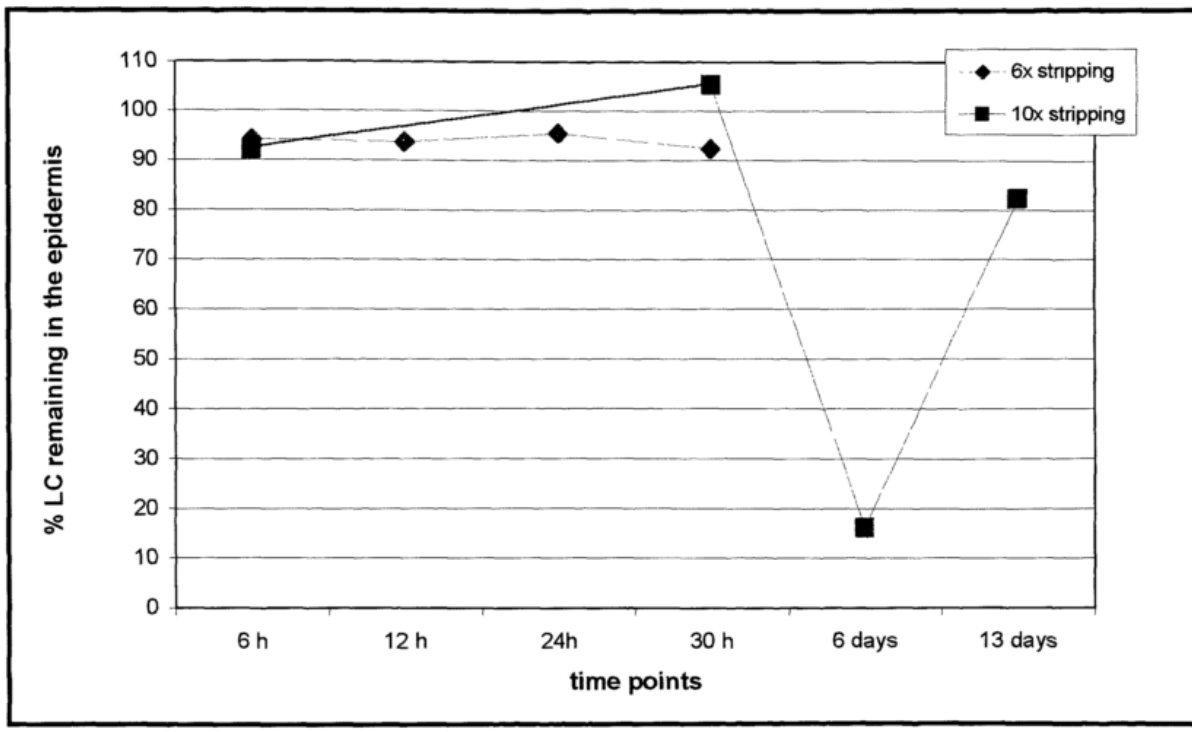


Figure 13: Emigration of Langerhans cells from the epidermis detected in a preliminary experiment. Two different intensities of tape stripping (6 strippings vs. 10 strippings) were tested for their effect on the emigration of Langerhans cells from the epidermis. Apparently only a higher number of strippings can induce the desired effect (10 strippings, 6 days).

On day 6, patches without Langerhans cells are visible for the first time in samples of ears stripped 10 times (fig. 13). Only 16% Langerhans cells of total numbers (=control ear) can still be found in the epidermis. There was no evidence of emigration in samples stripped 6 times. It can be concluded, that tape stripping can induce a reduction of Langerhans cells in the epidermis, but it is dependent on the number of strippings.

4.2.2 Mechanism of Langerhans cell reduction in the epidermis

- Are Langerhans cells pulled out from the epidermis in this specific experimental setting?

The number of strippings were raised to test whether the Langerhans cells can be pulled out in this experimental setting, as described in the literature (171,178). Half an hour after the stripping epidermal sheets were prepared. The Langerhans cells are immunohistochemically stained and counted under the fluorescence microscope using a counting ocular with squares.

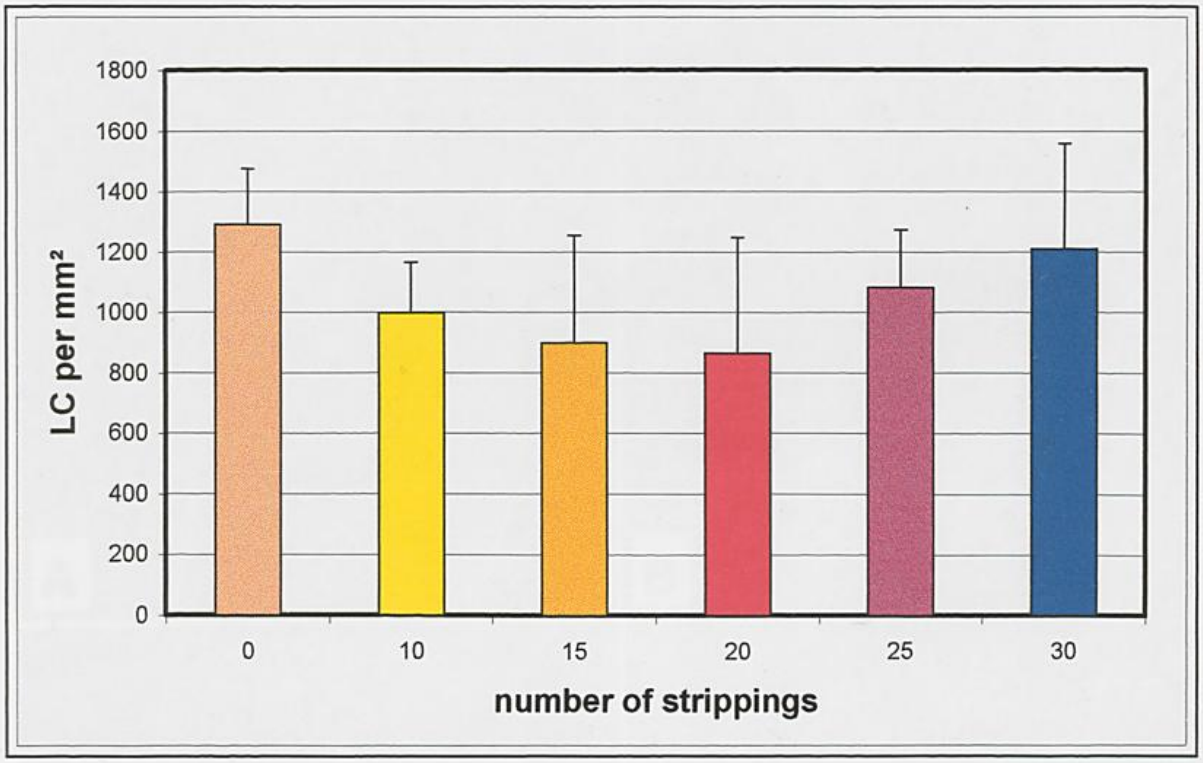


Figure 14: Density of Langerhans cells in the epidermis detected immediately after tape stripping. Even if the number of strippings is raised up to 30 Langerhans cell numbers are not substantially changed. Thereby it can be concluded that Langerhans cells are not pulled out of the epidermis.

Even if we raised the number of strippings up to 30, the Langerhans cell density did not change significantly (fig. 14). This indicates that the basal and suprabasal layers of the epidermis in which Langerhans cells reside could not be reached. A physical extraction of Langerhans cells can therefore be excluded in this experimental setting.

- Can "cords" with emigrating Langerhans and dendritic cells be detected in the dermis and do they express maturation markers?

A BALB/c mouse was stripped 12 times (as in the standard experiments, 4.2.3) on the left ear, the right ear served as untreated control. Four days afterwards the mouse was sacrificed and epidermal and dermal sheets prepared. The sheets were immunohistochemically stained for MHC class II molecules (fig. 15).

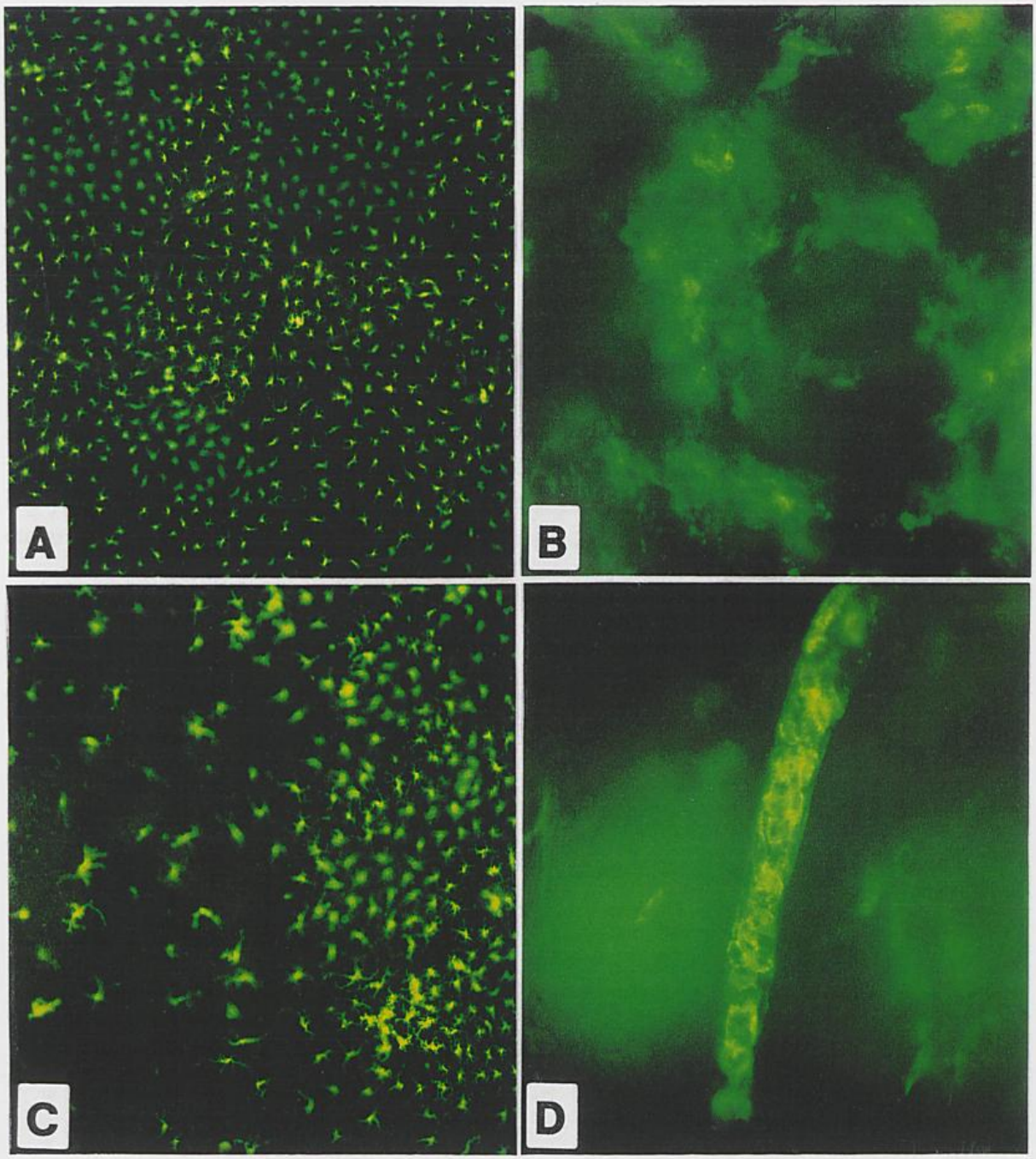


Figure 15: Overview of untreated epidermis (**A**, 100x) and untreated dermis (**B**, 400x). Epidermis 4 days after tape stripping, with visible patchy emigration (**C**, 100x). Dermis, 4 days after tape stripping, with a lymphatic vessel filled with emigrating Langerhans and dendritic cells, a so called "cord" (**D**, 400x). MHC class II molecules are immunohistochemically stained.

The results were astonishingly clear:

- in the stripped ear Langerhans cells were reduced in the epidermis and in the dermis lymphatic vessels filled with emigrating Langerhans cells and dermal dendritic cells, so called "cords", were visible.

- in the control ear the net of Langerhans cells in the epidermis was untouched and in the dermis no cords could be found.

In a further experiment we wanted to determine the degree of maturation of the Langerhans and dendritic cells in the cords.
BALB/c mice were stripped 12 times. Dermal sheets were prepared on the day of stripping (=day 0) and 4 and 6 days afterwards. The sheets were double-labelled:

- for MHC class II and CD86, a co-stimulatory molecule necessary for the activation of naive T-cells.
- for MHC class II and organelles of unknown function that appear only in mature dendritic and B-cells (primary antibody "2A1").

Emigrating cells in "cords" express the costimulatory molecule CD86 on their surface (figure 16) and have organelles, that are characteristic for mature dendritic cells (figure 17). This shows that after tape stripping, epidermal Langerhans cells and dermal dendritic cells emigrate in lymphatic vessels to the draining lymph node.

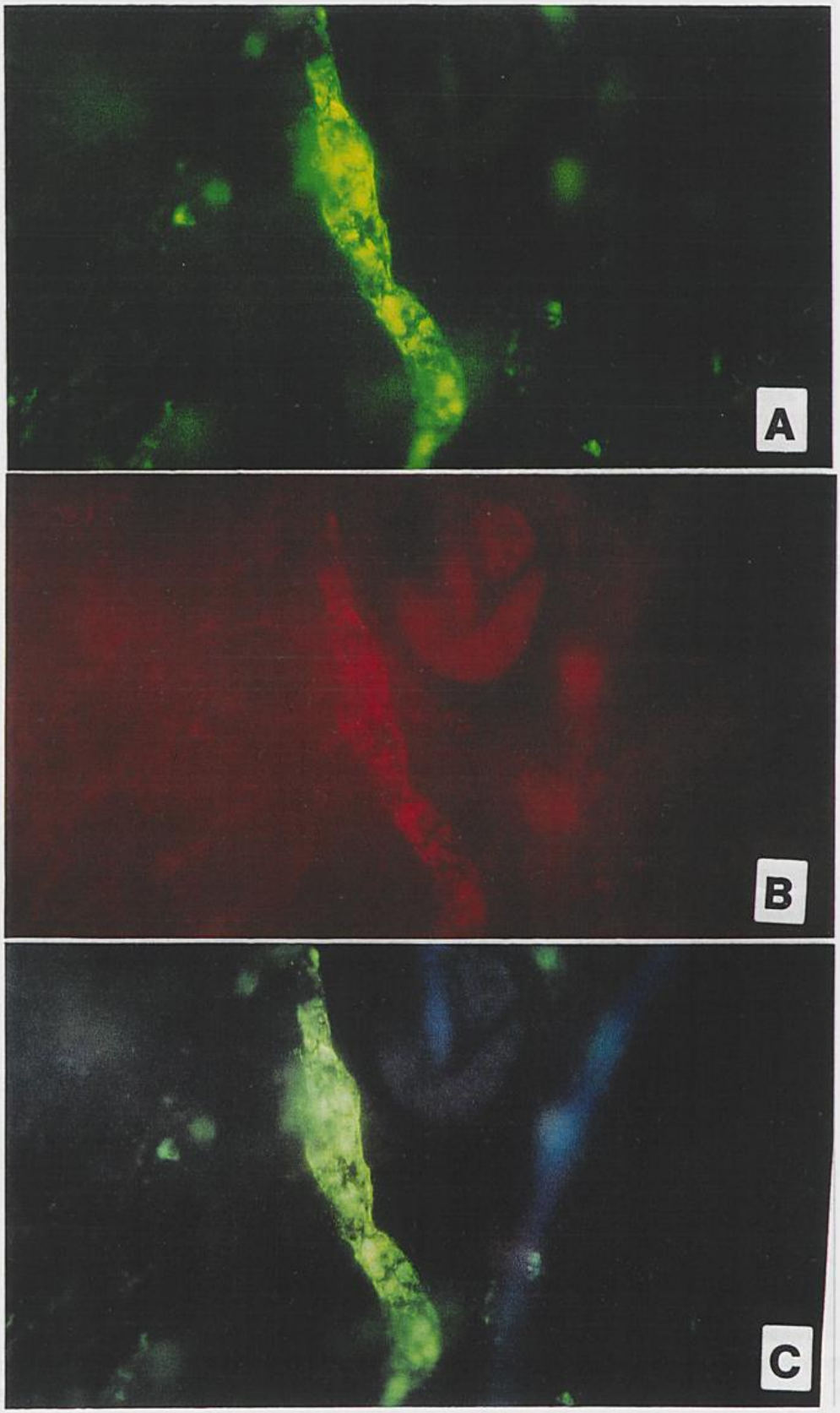


Figure 16: Maturing Langerhans and dendritic cells can be found emigrating the skin of tape stripped ears (4 days after stripping, 400x). Cells were stained for MHC class II (green fluorescence, **A**) and the costimulatory molecule CD86 (red fluorescence, **B**). **C**: double exposure.

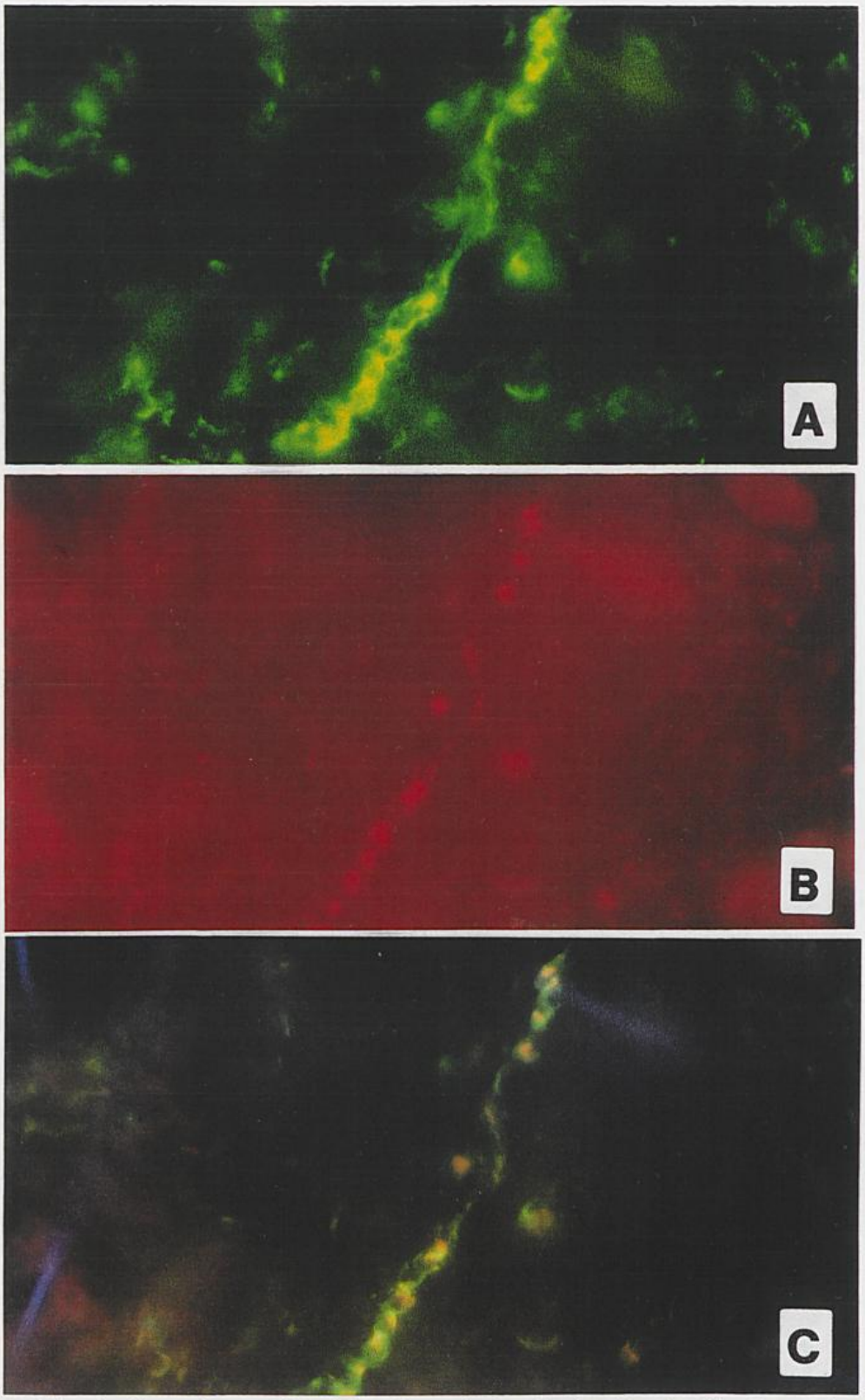


Figure 17: Maturing Langerhans and dendritic cells can be found emigrating the skin of tape stripped ears 6 days after stripping (400x). Cells were stained for MHC class II (green fluorescence, **A**) and an intracellular organelle typical for mature dendritic cells with the primary antibody 2A1 (red fluorescence, **B**). Observing the double exposure (**C**) it can be imagined that the stained organelles are located inside the emigrating dendritic cells.

- Does tape stripping provoke an inflammation in the skin?

Ears of BALB/c mice were stripped 12 times (= day 0). Routine H&E sections were done of an untreated control ear and of stripped ears after 4 days and after 6 days (fig. 18).

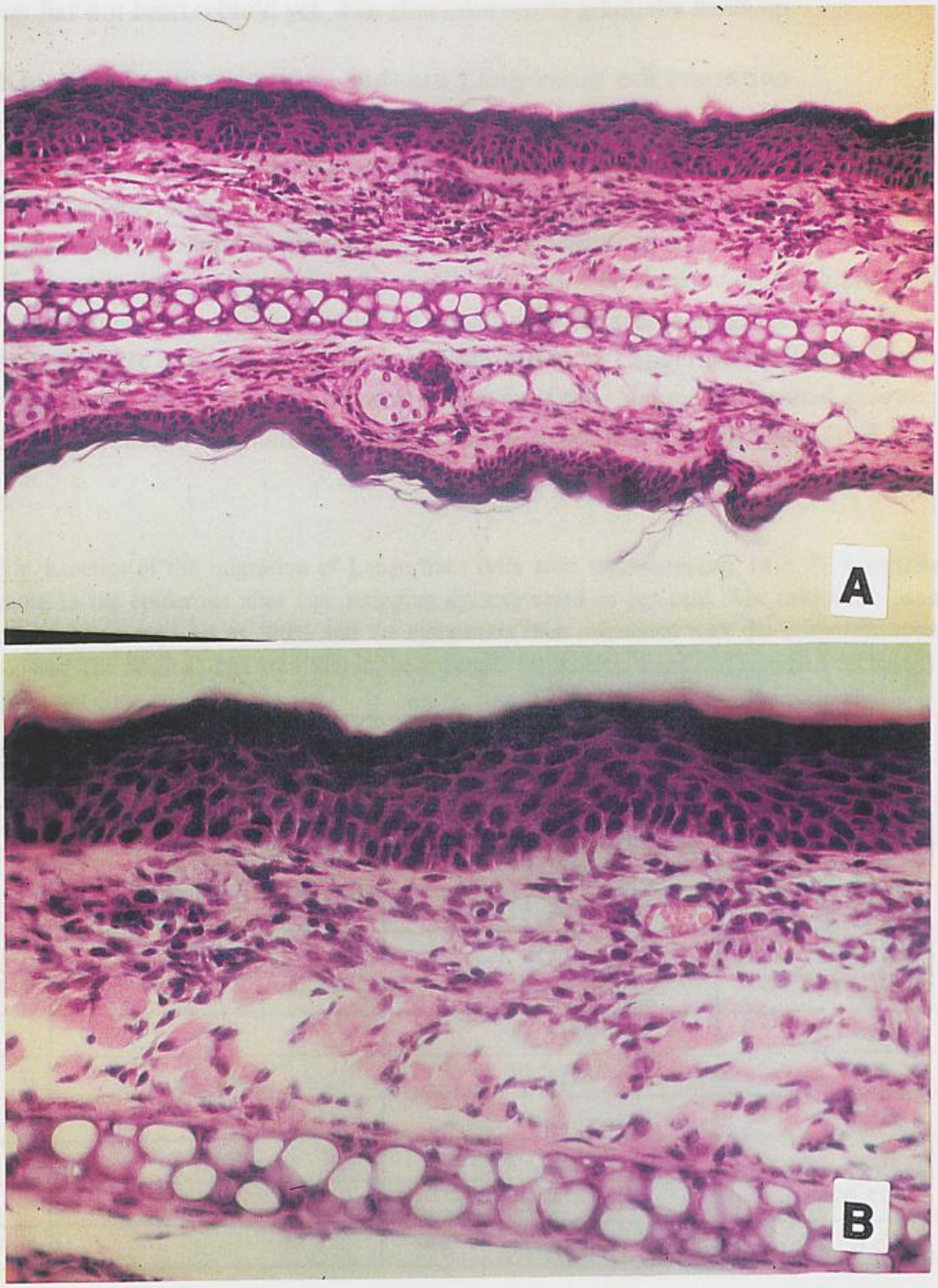


Figure 18: Routine H&E-stained sections of a mouse ear 4 days after tape stripping. **A)** The epidermis of the tape stripped dorsal half is swollen compared to the untreated ventral half (100x). **B)** Lymphocytes and macrophages infiltrate the moderately inflamed skin (400x).

In the sections it can be distinguished which side of the ear was stripped. A moderate inflammation can be seen histologically on days 4 and 6, being stronger on day 4. Macroscopically the following features are characteristic: during the stripping the hairs are pulled out and the skin starts to glisten. The lymph then dries and the surface becomes papery. When sheets are prepared it can be noticed, that the epidermis is thin, the *stratum corneum* has not been rebuild yet. The skin afterwards gradually heals up.

4.2.3 Kinetics of tape stripping – induced Langerhans cell migration

Even though in the pilot experiment an emigration could be shown, it went on very slowly. Therefore the number of strippings was raised to set a more intensive stimulus: BALB/c mice were stripped 12 times on the left ear. The right ear of the same mouse was the untreated control. At defined time points mice were sacrificed and epidermal sheets prepared. Langerhans cells were immunohistochemically stained. The sheets were evaluated under the fluorescence microscope using a counting ocular with squares. Langerhans cells in 5 – 20 small squares were counted. Emigration is described in % Langerhans cells still found in the epidermis, setting the control ear of each mouse as 100%. Values for control ears are not pooled: each mouse is calculated separately. Three experiments were done (table 8).

Table 8: Kinetics of the migration of Langerhans cells after tape stripping. (n = 3). Langerhans cells remaining in the epidermis after tape stripping are expressed in per cent. The arithmetic mean of the control ear was always set as 100% and the emigration then calculated with the arithmetic mean of the stripped ear. The original data are listed in the appendix (arithmetic mean and standard deviation). n.d. .... time point not done.

Day	experiment 1	experiment 2	experiment 3
0	n.d.	115,45%	88,52%
1	n.d.	n.d.	75,88%
2	61,38%	52,08%	n.d.
4	44,1%	35,91%	42,58%
6	17,71%	n.d.	48,10% 35,42%
7	n.d.	37,61%	n.d.
8	43,18%	33,31%	n.d.
10	44,35%	n.d.	n.d.
16	74,17%	89,52%	n.d.
17	n.d.	n.d.	50,59%
33	n.d.	126,88%	n.d.

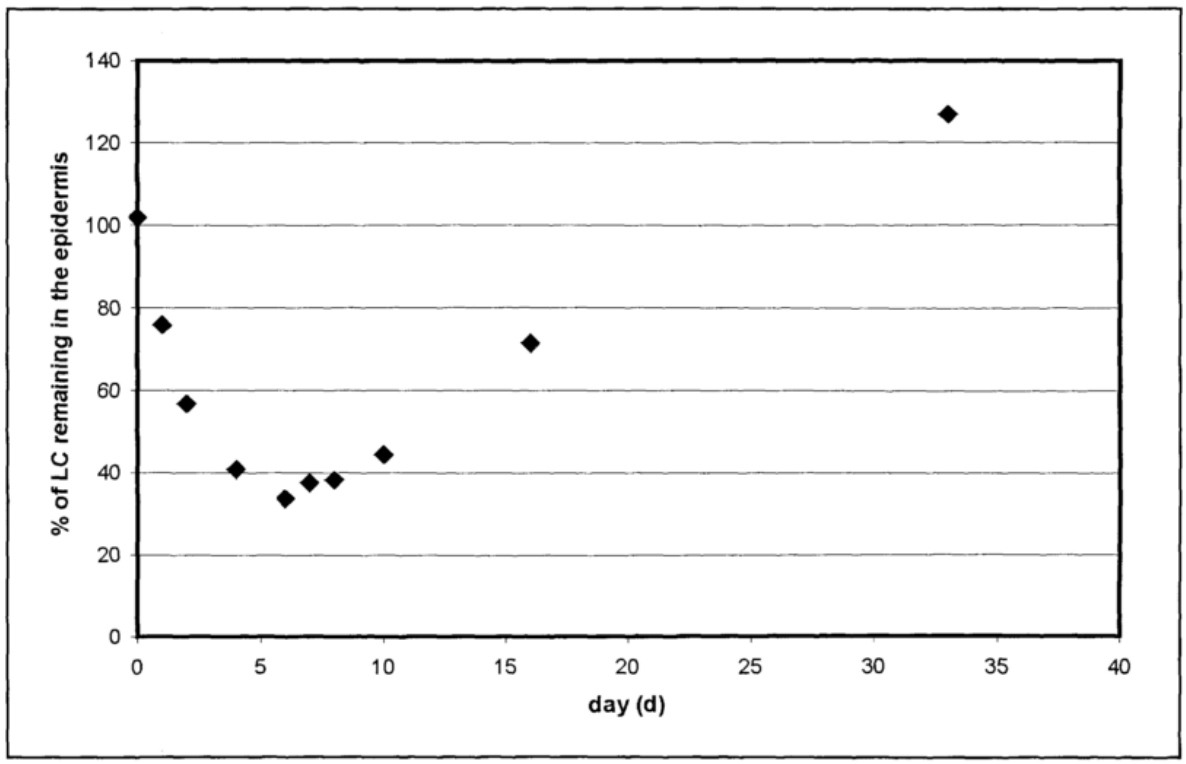


Figure 19: Time course of the migration of Langerhans cells after mechanical irritation of the skin by tape stripping (n = 3). The number of Langerhans cells remaining in the epidermis decreases continually after tape stripping and reaches a nadir on day 6 (33,74% LC still found in the epidermis). Afterwards the number slowly increases and reaches control values about 1 month after stripping.

Emigration is visible from day 2 on and reaches its maximum on day 6 with only 33,74% Langerhans cells remaining in the epidermis (fig. 19). After 30 days normal densities are reached again. The repopulation of the epidermis with Langerhans cells is slower than the emigration.

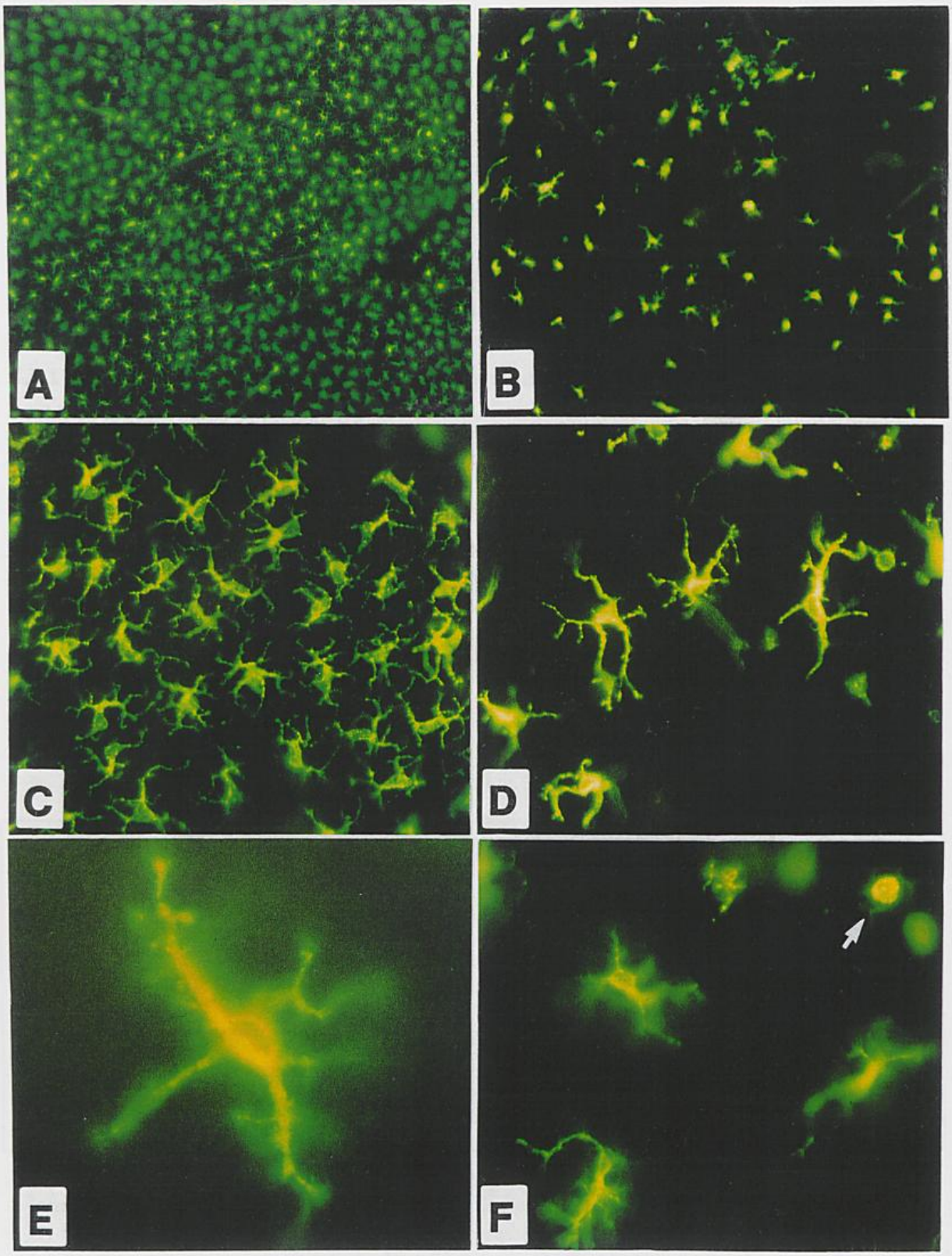


Figure 20: Comparison between untreated epidermis (**A**, **C**) and tape stripped epidermis (6 days after stripping, **B**, **D**, **E**, **F**). Langerhans cells in untreated epidermis seem to form a regular net of defence against foreign antigens (**A**, 100x; **C**, 400x). In tape stripped epidermis this net is in a turmoil (**B**, 100x; **D**,**F**, 400x): Langerhans cells strongly express MHC class II and some of them round off before they emigrate (**F**, arrow). Other Langerhans cells stay in the epidermis, appearing enlarged (**D**, 400x; **E**, 1000x).

Emigration is patchy (see figure 20). It appears to spread from strongly activated cells, that express more MHC class II and start to round off before they then emigrate to still resting Langerhans cells. LC remaining in the epidermis appear to be enlarged and express more MHC II compared to cells in a steady state, reminiscent of explant cultures or contact hypersensitivity.

4.3 Migration of intravenously injected bone marrow derived dendritic cells in lymphoid organs and the epidermis

4.3.1 Comparison between dendritic cells day 6 and day 8, first experiment

In this first experiment bone marrow-derived dendritic cells were injected into mice without any further treatment. The cells were cultured for 6 or 8 days in R10 supplemented with 200 units per ml GM-CSF. The day of their harvest dendritic cells were stained with PKH26 and $4x10^6$ injected i.v. in each mouse. At defined time points thereafter, mice were sacrificed. Cell suspensions of bone marrow, mesenterial lymph nodes and spleen were prepared and flow cytometric analysis and Cytospin preparations (60.000 cells per slide) done. Epidermal and dermal sheets were prepared and embedded into Vectashield fluorescence mounting medium without further staining.

No PKH26-positive cells could be identified in epidermal and dermal sheets. Positive cells in cell suspensions of lymphoid organs could not clearly be detected by flow cytometric analysis (fig. 21).

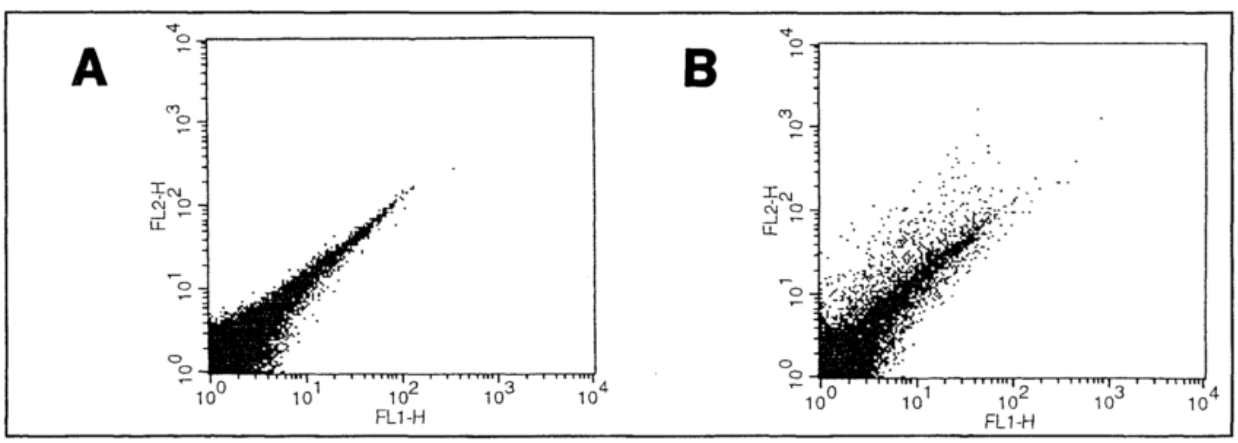


Figure 21: Flow cytometric analysis of single cell suspensions of lymphoid organ taken from mice injected intravenously with PKH26-stained dendritic cells. **A**) Suspension of spleen cells, negative control. **B**) Suspension of spleen cells 7 days after intravenous injection of PKH26-stained dendritic cells of intermediate maturation stage (standard culture for 8 days). 50.000 cells were analysed.

Some cells can be seen to split off from the negative cell population. This fraction is very heterogeneous regarding staining and size of the cells and there is no distinct, spot-shaped cell population. Therefore these few cells could not be quantified.

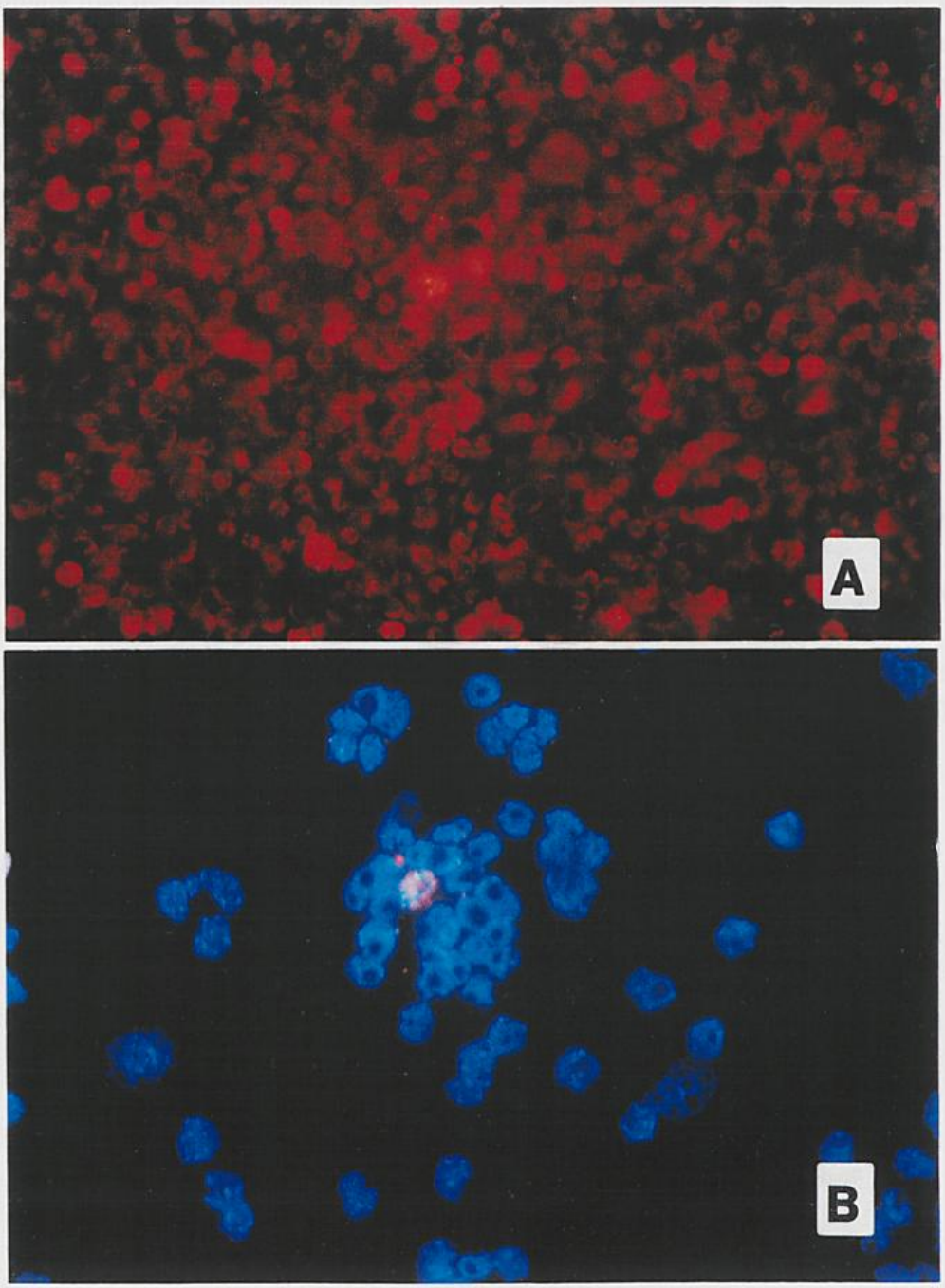


Figure 22: **A**) Cell suspension of bone marrow containing PKH26-stained immature dendritic cells injected 8 days before. Cells are as densely packed as possible (100x). **B**) Cytospin preparation of spleen cell suspension, showing one PKH26-positive cell (red fluorescence) amongst spleen cells stained with DAPI (400x).

For the evaluation of the samples a semi-quantitative system was adopted. Cytospin preparations (5 slides per sample) were fixed in acetone and embedded with Vectashield fluorescence mounting medium containing DAPI (fig. 22). DAPI stains the DNA in the cell nucleus and therefore it is possible to identify all cells under the fluorescence microscope. Vectashield prevents the red fluorescence of the injected cells form early fading.
Assuming that the distribution of cells on the slide is homogenous, we counted the PKH26-positive cells of at least 100 small squares (counting ocular). The unstained cells were calculated as follows: the number of cells in 5 squares (one on each slide) was counted. The arithmetic mean was calculated. We therefore had an average of how many negative cells could be found in one small square. This value was multiplied with the number of squares evaluated for PKH26-positive cells. Finally the number of PKH26-positive cells per 10.000 negative cells was calculated.

Example: In 101 squares 26 PKH26-positive cells could be identified. On average in each square there are 74,14 cells, as determined by their DAPI staining.
The number of negative cells in all counted squares therefore is:

101 squares x 74,14 PKH26-negative cells = 7.488,14
~ 7.500 negative cells

The number of PKH26-positive cells per 10.000 unstained, negative cells is calculated as follows:

26 positive cells : 7.500 negative cells = x positive cells : 10.000 negative cells
x = 34,666 ~ 35 PKH26-positive cells/10.000 negative cells

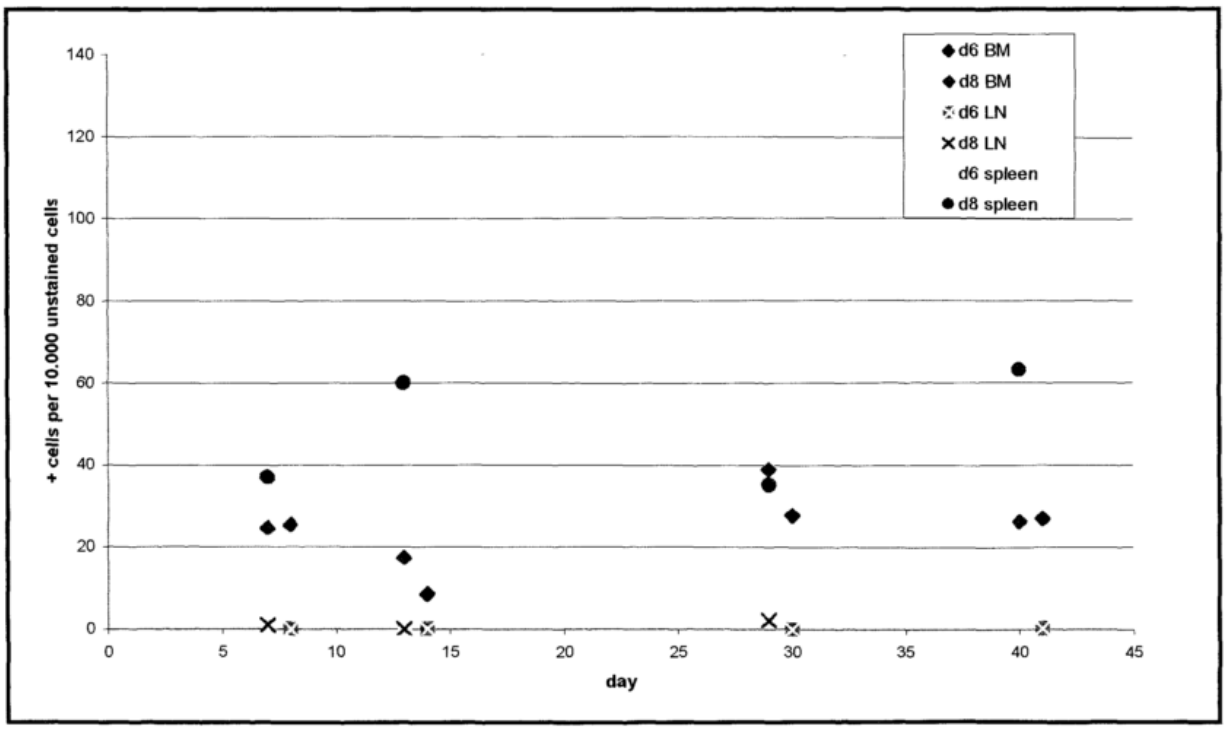


Figure 23: Time course for PKH26-positive cells found in single cell suspensions of lymph node (LN), bone marrow (BM) and spleen after intravenous injection of PKH26-stained immature (d6...6 days of standard culture) and intermediate (d8...8 days of standard culture) bone marrow-derived dendritic cells. The highest level of $PKH26^+$ cells can be found in the spleen, followed by the bone marrow. Only rarely $PKH26^+$ cells were detected in the mesenterial lymph nodes.

There is no striking difference between dendritic cells cultured for 6 or for 8 days, nor at the different time points. But there are clearly visible differences between the type of lymphoid organs (fig. 23).

Only very few dendritic cells could be found in the mesenterial lymph nodes (0 – 2,2 $PKH26^+$ cells per 10.000 lymph node cells). There are some more in the bone marrow (9 – 39 $PKH26^+$ cells per 10.000 bone marrow cells) and, as expected, the highest amount was found in the spleen (35 – 94 $PKH26^+$ cells per 10.000 spleen cells).

The cells seem to home in on the different organs and stay there. Only in the spleen 13 and 14 days after injection there was an increase of $PKH26^+$ cells, especially if the injected cells are immature day 6 dendritic cells. No significant turn-over could be identified with our evaluation system, not even after 40 days.

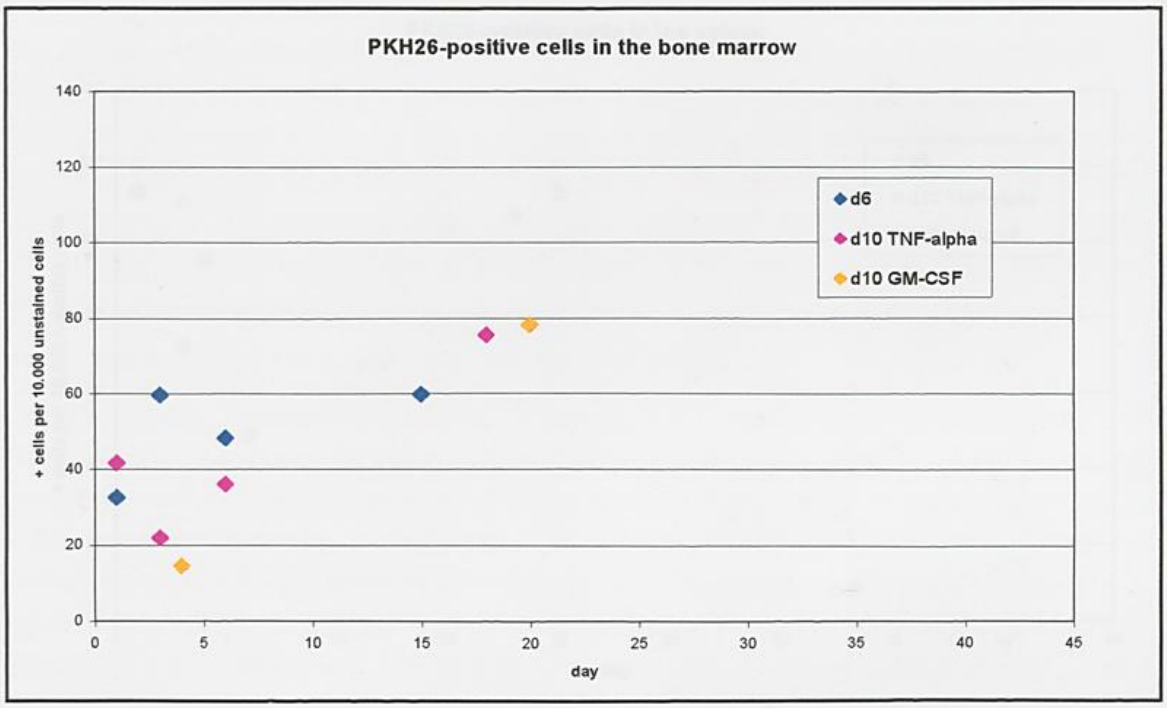
4.3.2 Comparison between dendritic cells enriched for mature and immature cells

In this experiment a few parameters were changed. The dendritic cells were cultured until day 6 in R10 supplemented with 200 units per ml GM-CSF. More mature, single dendritic cells were separated from "balls" of rather immature dendritic cells by floatation of 50% FCS columns (one gradient per 3 wells) on day 6.

> 3 ml of sterile FCS and 3 ml of RPMI 1640 were pipetted into a Falcon 15 ml tube to get this dense medium and foamed with a Pasteur pipette until the foam was approximately 1 cm high. Then the very gently harvested cells of 3 wells were pipetted homogeneously on top of the foam and allowed to sediment for 30 minutes. Because of their physical properties the "balls" sank quickly to the ground of the tube and the single cells found in the upper part of the dense medium. The two fractions were separated at 4 ml (volume of the dense medium still left in the tube): in the upper part there were the more mature, single dendritic cells, in the lower part the rather immature dendritic cells still adhering to each other, forming the so called "balls".

The immature cells of the lower fraction were stained with PKH26 and then injected intravenously the same day ($4{,}25 \times 10^6$ dendritic cells per mouse). The single, rather mature dendritic cells of the upper fraction were cultured until day 10 in R10 supplemented with 200 units per ml GM-CSF and with or without 500 units per ml TNF-α. On day 10 mature and fully matured (i.e. with TNF-α) dendritic cells were harvested, stained with PKH26 and injected intravenously ($4{,}5 \times 10^6$ fully mature dendritic cells per mouse, $4{,}7 \times 10^6$ mature dendritic cells per mouse). In each mouse only one cell type was injected. Earlier time points than in the first experiment were examined. Mice were sacrificed and cell suspensions of bone marrow, mesenterial lymph nodes and spleen were prepared and Cytospin preparations (60.000 cells per slide) done, fixed in acetone and embedded with half a drop Vectashield fluorescence mounting medium and half a drop DAPI. Again the semi-quantitative evaluation system described in the previous experiment was adopted. The immigration into the epidermis was not assessed this time.

A)



B)

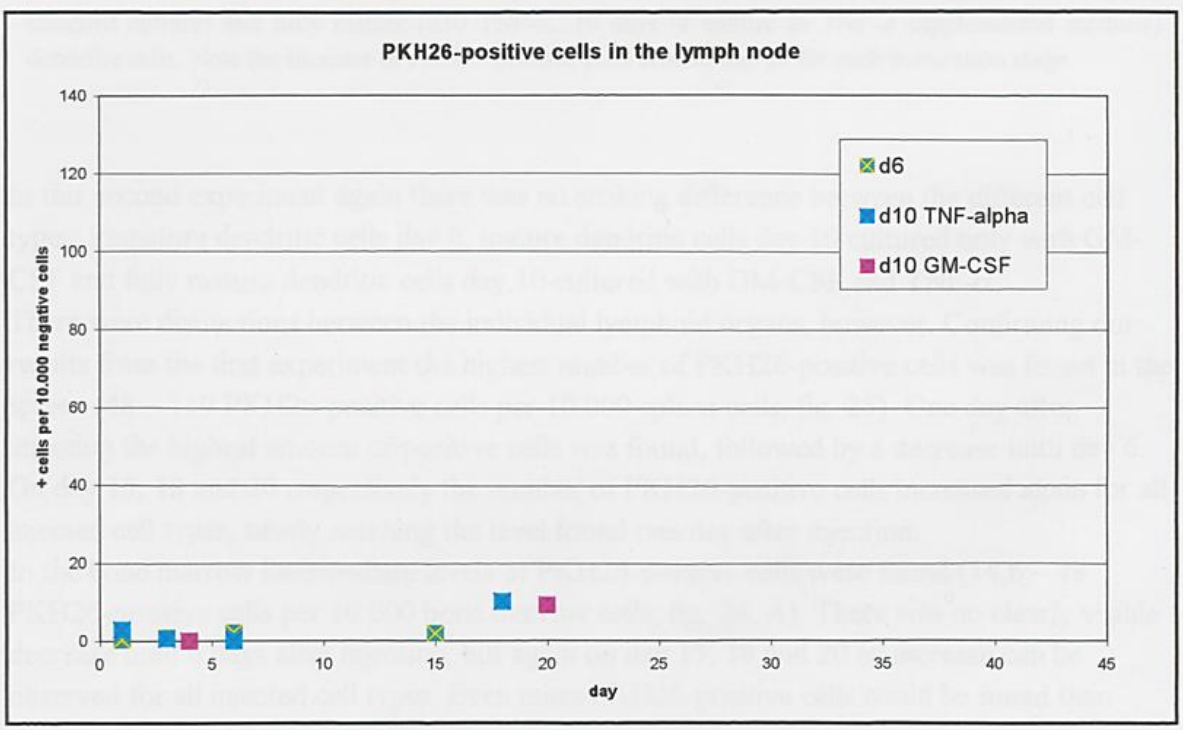


Figure 24: Time course for PKH26-positive cells found in single cell suspensions of bone marrow (**A**) and mesenterial lymph nodes (**B**) after intravenous injection of immature (d6...6 days of standard culture), mature (d10 GM-CSF...10 days of standard culture) and fully mature (d10 TNF-α...10 days of culture in TNF-α supplemented medium) dendritic cells. Note the increase of PKH26-positive cells around day 18 for both organs and each maturation stage.

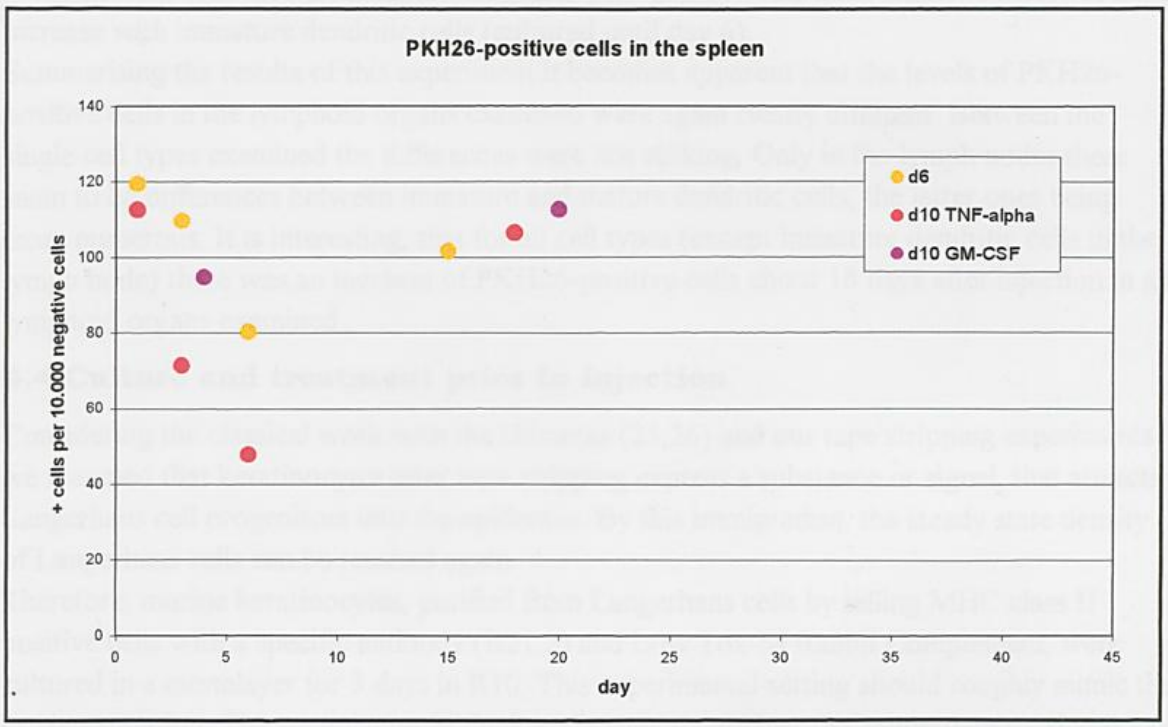


Figure 25: Time course for PKH26-positive cells found in single cell suspensions of the spleen after intravenous injection of immature (d6...6 days of standard culture), mature (d10 GM-CSF...10 days of standard culture) and fully mature (d10 TNF-α...10 days of culture in TNF-α supplemented medium) dendritic cells. Note the increase of PKH26-positive cells around day 18 for each maturation stage.

In this second experiment again there was no striking difference between the different cell types: immature dendritic cells day 6, mature dendritic cells day 10 cultured only with GM-CSF and fully mature dendritic cells day 10 cultured with GM-CSF and TNF-α.
There were distinctions between the individual lymphoid organs, however. Confirming our results from the first experiment the highest number of PKH26-positive cells was found in the spleen (48 – 119 PKH26-positive cells per 10.000 spleen cells; fig. 25). One day after injection the highest amount of positive cells was found, followed by a decrease until day 6. On day 15, 18 and 20 respectively the number of PKH26-positive cells increased again for all injected cell types, nearly reaching the level found one day after injection.
In the bone marrow intermediate levels of PKH26-positive cells were found (14,6 – 78 PKH26-positive cells per 10.000 bone marrow cells; fig. 24, A). There was no clearly visible decrease until 6 days after injection, but again on day 15, 18 and 20 an increase can be observed for all injected cell types. Even more PKH26-positive cells could be found than shortly after injection.
In the lymph nodes during the first six days the amount of PKH26-positive cells found was quite low as in the first experiment (0 – 10 PKH26-positive cells per 10.000 negative lymph node cells; fig. 24, B). Nevertheless again a 9-fold increase of positive cells on day 18 and 20 for mature dendritic cells (cultured until day 10 with GM-CSF only) and fully mature

dendritic cells (cultured until day 10 with GM-CSF and TNF-α) were visible. There was no increase with immature dendritic cells (cultured until day 6).
Summarising the results of this experiment it becomes apparent that the levels of PKH26-positive cells in the lymphoid organs examined were again clearly different. Between the single cell types examined the differences were not striking. Only in the lymph nodes there seem to be differences between immature and mature dendritic cells, the latter ones being more numerous. It is interesting, that for all cell types (except immature dendritic cells in the lymph node) there was an increase of PKH26-positive cells about 18 days after injection in all lymphoid organs examined.

4.4 Culture and treatment prior to injection

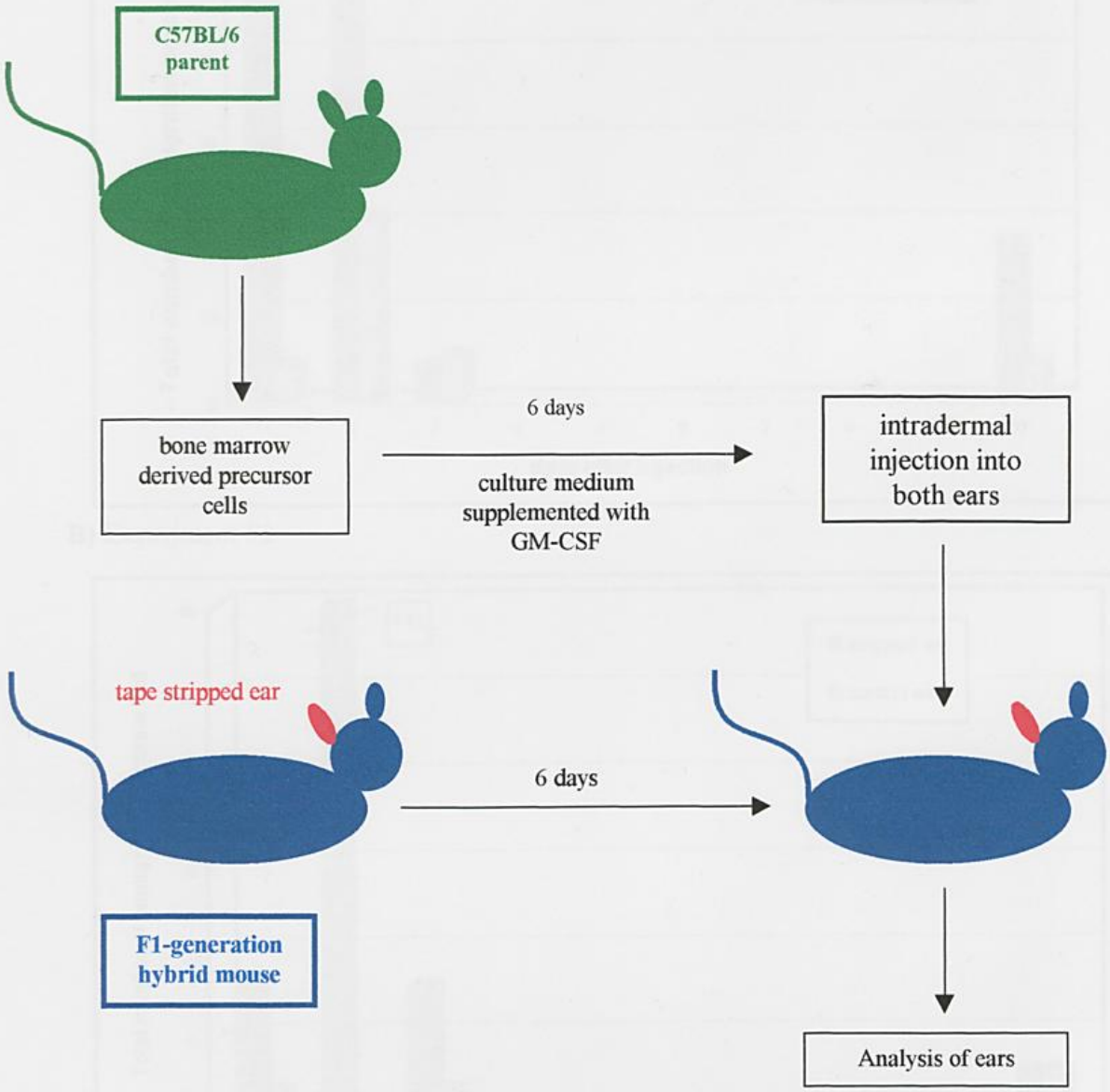
Considering the classical work with the chimeras (25,26) and our tape stripping experiments we assumed that keratinocytes after tape stripping express a substance or signal, that attracts Langerhans cell progenitors into the epidermis. By this immigration, the steady state density of Langerhans cells can be reached again.
Therefore, murine keratinocytes, purified from Langerhans cells by killing MHC class II positive cells with a specific antibody (B21.2) and Low Tox-M Rabbit Complement, were cultured in a monolayer for 3 days in R10. This experimental setting should roughly mimic the *in vivo*-situation. The supernatant of the keratinocyte monolayer (KC-supernatant), that is supposed to contain the yet unknown signal(s) for the development of Langerhans cells from progenitors, was harvested, aliquoted and frozen.
It was assumed that by culturing bone marrow-derived precursor cells with KC-supernatant the number of cells committed to become Langerhans cells could increase. By tape stripping a demand for Langerhans cells should be created in the epidermis and it is timed appropriately with the injection of PKH26-labelled dendritic cells. With these measures a detection of Langerhans cells in the epidermis might be feasible.
Bone marrow-derived precursor cells were cultured for 6 days in R10 supplemented with 200 units per ml GM-CSF and 30 Vol% thawed KC-supernatant. On day 6, dendritic cells were harvested, stained with PKH26 and injected intravenously into mice that were tape stripped 6 days before on their left ear ($6{,}6 \times 10^6$ dendritic cells per mouse). At defined time points thereafter mice were sacrificed. Epidermal and dermal sheets were prepared and embedded in Vectashield fluorescence mounting medium without further staining. The sheets were screened systematically under the fluorescence microscope for PKH26-positive cells in the epidermis or dermis. Neither in the epidermis nor in the dermis at no time point could PKH26-positive cells be detected.
In a second experiment the experimental setting was changed: for the detection of eventually immigrated cells in epidermis and dermis the MHC class II molecules were selectively stained with immunohistochemistry, the ears were pre-treated by tape stripping, and a considerably higher number of dendritic cells was injected intravenously. Bone marrow-derived precursor cells of F1 (BALB/c x C57BL/6) hybrid mice were cultured for 6 days in culture medium supplemented with 200 units per ml GM-CSF and 30 vol% KC-supernatant. On day 6 the dendritic cells were harvested and $22{,}5 \times 10^6$ dendritic cells per mouse were injected

intravenously, that were tape stripped on both ears 6 days before. On day 1, 3, 10 and 15 after the injection each time one ear was prepared as epidermal sheets. These were selectively stained for the MHC class II molecules of the injected F1-generation dendritic cells with an appropriate antibody (HB32). So the injected cells only would be stained for their MHC class II molecules. The epidermal sheets were systematically screened under the fluorescence microscope.
At no time point could injected cells be identified in the epidermis.

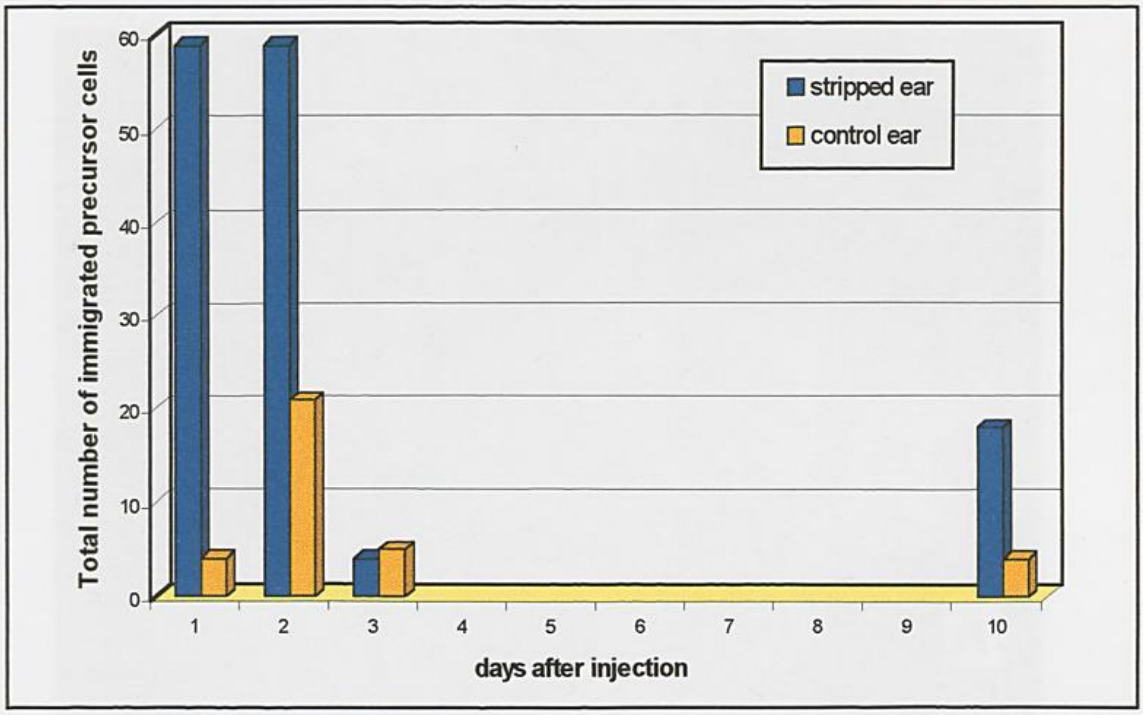
4.5 Immigration of intradermally injected bone marrow derived Langerhans cells into the epidermis

The first day (day 0) of the experiment bone marrow derived precursor cells were obtained from a C57BL/6 mouse (parent) and cultured in R10 supplemented with 200 units GM-CSF for 6 days. The same day (day 0) F1 (BALB/c x C57BL/6) hybrid mice were stripped on the left ear 12 times. The right ear remained untreated. This experimental setting was based on the kinetics of emigration of Langerhans cells from the epidermis after tape stripping determined in our previous experiments (fig. 19). By means of such timing, there was the highest emigration rate of Langerhans cells from the epidermis on the day of the injection of precursor cells, and therefore presumably the greatest "demand" for new Langerhans cells. On day 6 the bone marrow-derived dendritic cells were harvested and injected intradermally (50.000 dendritic cells in 50 µl PBS) into both ears of the F1 hybrid mice, the right ear serving as control. Thereafter mice were sacrificed and epidermal and dermal sheets prepared. Sheets were double labelled as described in 3.6.3, "Identification of migrated precursor and Langerhans cells, procedure for double-labelling of MHC class II molecules".
Detection of the injected precursor cells was possible by working in a "pseudo"-syngeneic system: bone marrow derived precursor cells of a C57BL/6 parent mouse were injected into a F1 (BALB/c x C57BL/6) hybrid mouse. Therefore the injected cells will be labelled only by FITC green fluorescence, whereas the cells of the hybrid mouse are double positive (FITC, green fluorescence and Texas Red, red fluorescence, fig. 27) (see also 3.6.3). The epidermal sheets were systematically screened under the fluorescence microscope and the total number of only FITC-positive cells in the epidermal sheets counted. A filter allowing the simultaneous detection of red and green fluorescence was used. Two experiments were carried out.

Illustration of the experimental setting:



A) Experiment #1



B) Experiment #2

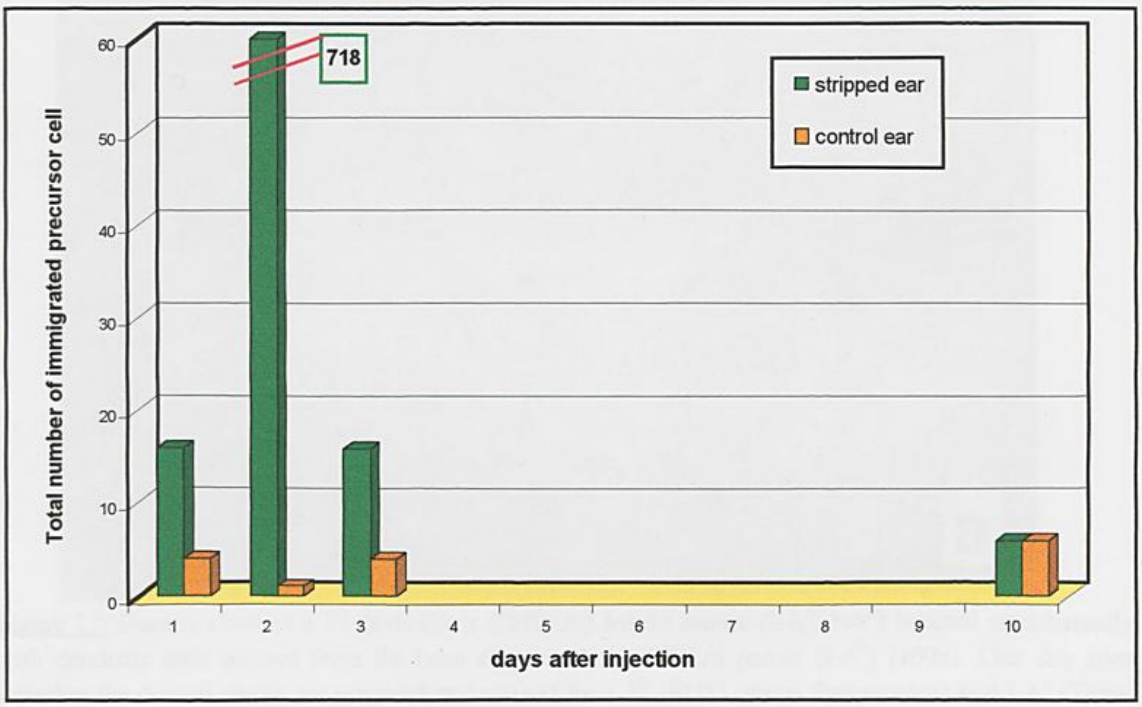


Figure 26: Time course of the immigration of Langerhans precursor cells into the epidermis. The left ear was treated with adhesive tape 6 days before intradermal injection of immature bone marrow-derived dendritic cells (stripped ear). The right ear of the same mouse remained untreated before injection (control ear). Langerhans cells were detected by selective staining of MHC class II haplotype, the whole epidermis of the dorsal half of the ear was analysed- Note the strong immigration of precursors on day 2, specially in experiment #2 (718 immigrated Langerhans cells).

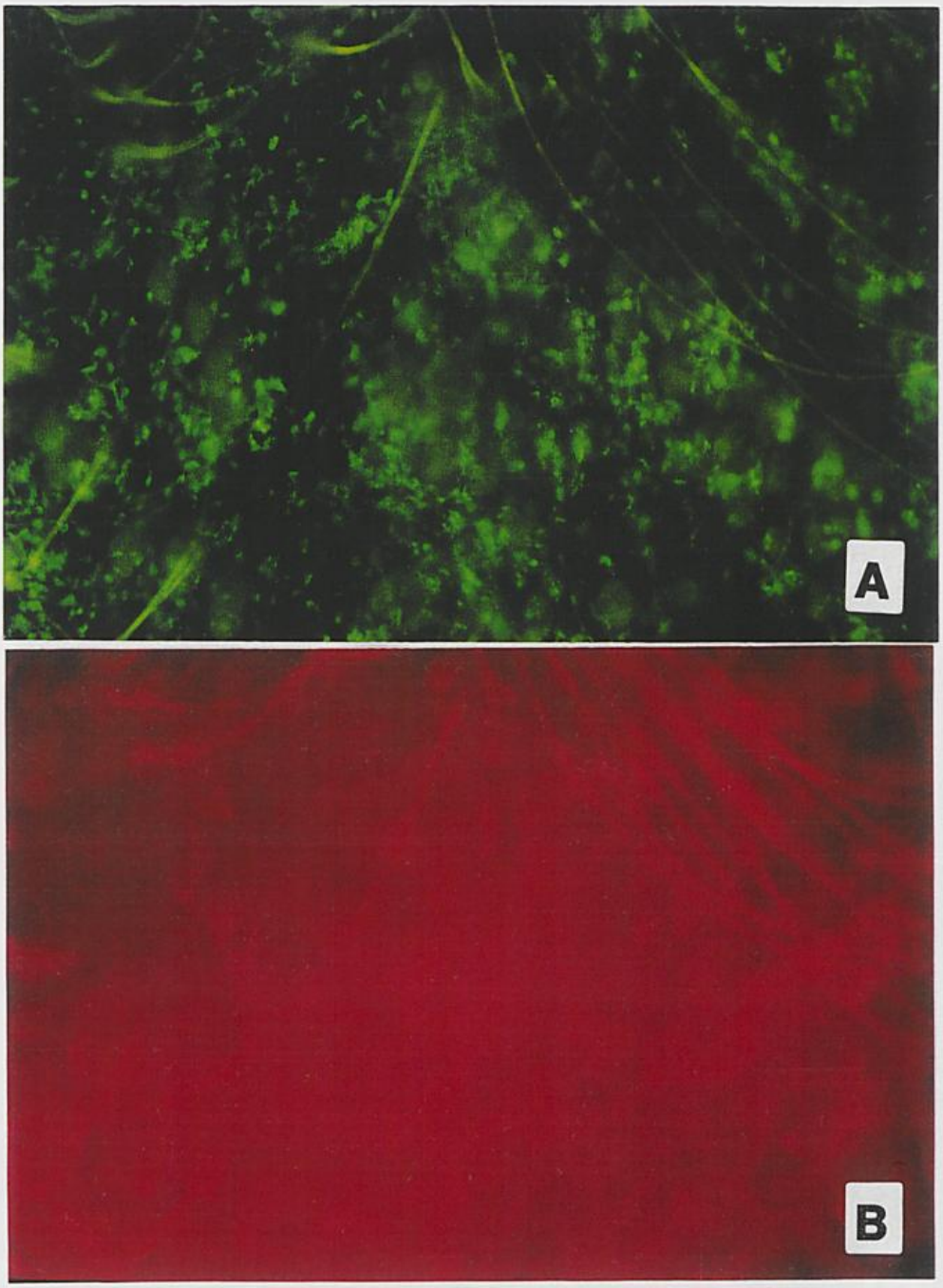


Figure 27: Dermal sheet of a F1 (BALB/c x C57BL/6) hybrid mouse (I-A^d, I-A^b) injected intradermally with dendritic cells derived from the bone marrow of a C57BL/6 parent (I-A^b) (100x). One day after injection the dermal sheets are prepared and stained for I-A^b (FITC, green fluorescence) and I-A^d (Texas Red, red fluorescence). The injected C57BL/6 dendritic cells are visible only when elicited for green fluorescence (**A**) and not for red fluorescence (**B**). The whole area shown in **A** and **B** contains only injected I-A^b positive cells, confirming that the injection was successful.

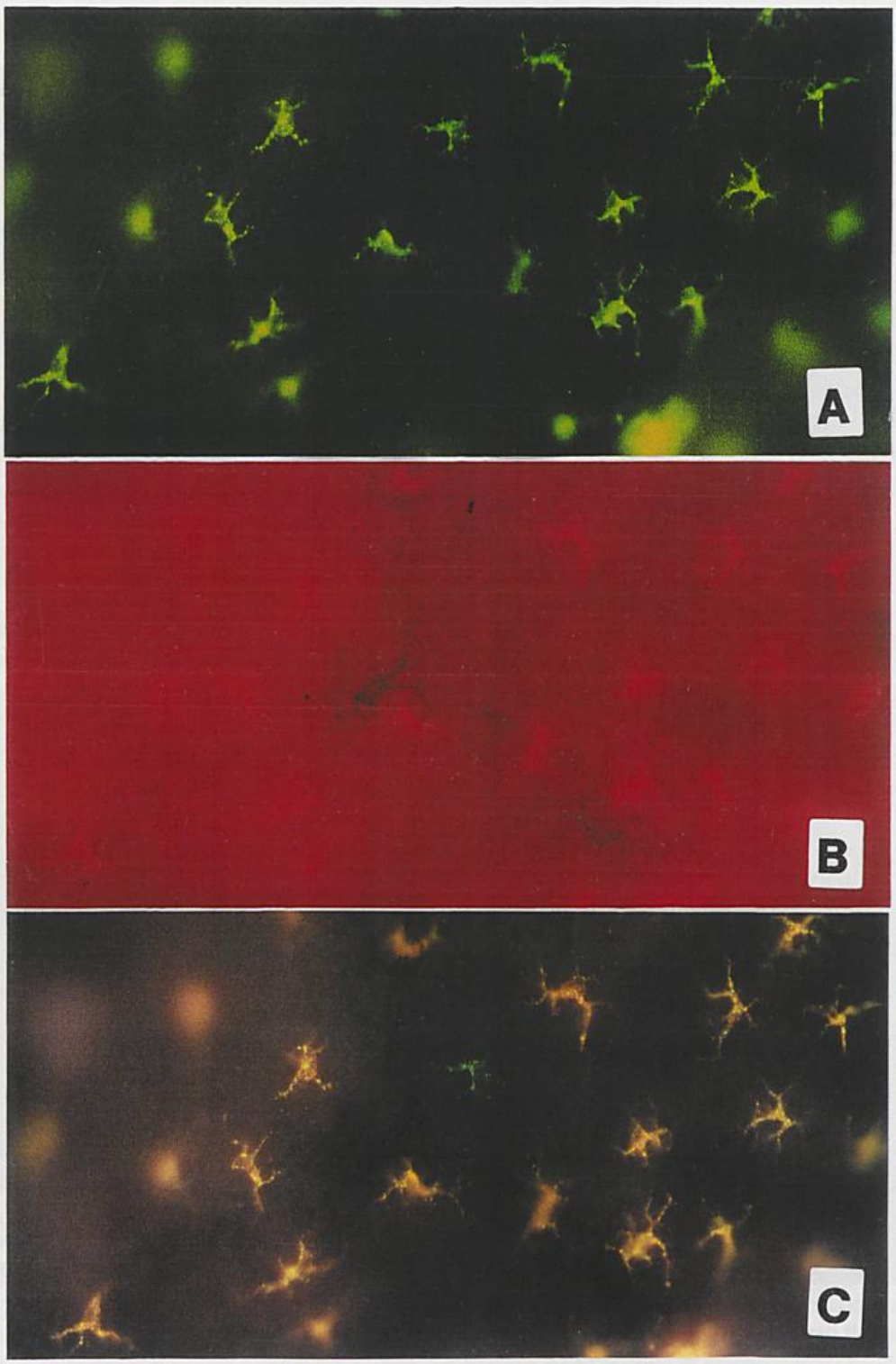


Figure 28: Single Langerhans cell immigrated into untreated epidermis 1 day after injection (400x). C57BL/6 immature dendritic cells (I-A^b) were injected intradermally into F1 (BALB/c x C57BL/6) hybrid mice (I-A^b, I-A^d). The immigrated Langerhans cell, focused in the centre, is positive for I-A^b (green fluorescence, **A**, **C**) and negative for I-A^d (not visible when elicited for red fluorescence, **B**). It can clearly be distinguished from resident F1 (BALB/c x C57BL/6) hybrid Langerhans cells that are positive for I-A^b, (green fluorescence, **A**) and I-A^d (red fluorescence, **B**). Under double exposure resident Langerhans cells appear yellow-orange (**C**).

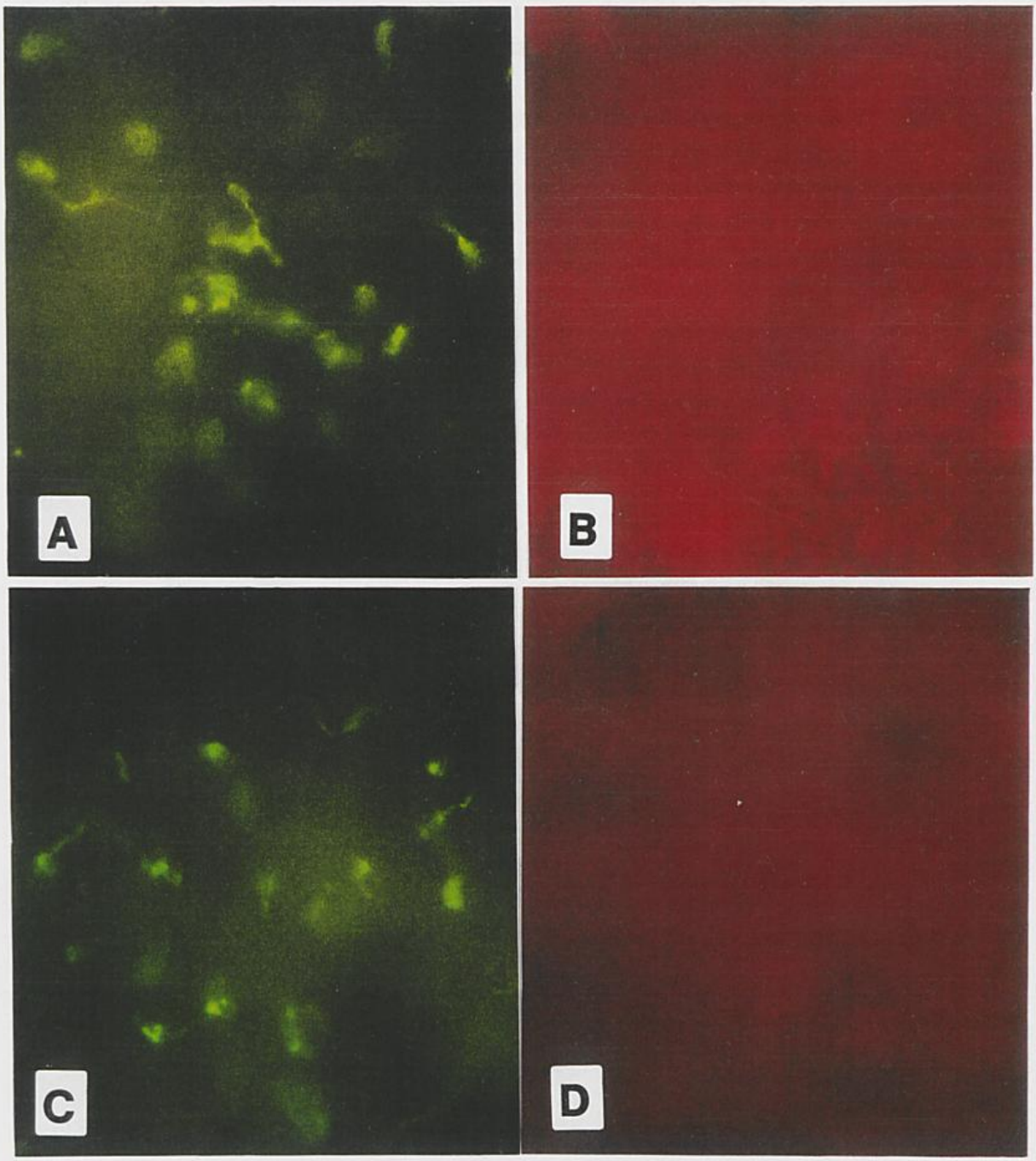


Figure 29: Groups of Langerhans cells immigrated into tape stripped epidermis 2 days after injection (400x). The staining procedure is detailed in the legend of figure (I-A^b...green fluorescence, I-A^d...red fluorescence). Groups of immigrated I-A^{b+}/I-A^{d-} Langerhans cells are positive when elicited in green fluorescence (**A**, **C**) and are negative in red fluorescence (**B**, **D**). Langerhans cells are slender with sometimes one or two dendrites. The epidermis is still reorganising and Langerhans cells are not yet neatly arranged (compare figure). In the areas shown no resident, double positive Langerhans cells are visible.

A first, very positive result of these two experiments was, that indeed, Langerhans cells immigrated into the epidermis and could be detected in this *in vivo* system. The immigration

of precursor cells into a stripped ear is 3 fold to 200 fold higher than into an untreated ear (fig. 26, 28, 29). A certain percentage of bone marrow derived dendritic cells became Langerhans cells without any further stimulus (in addition to GM-CSF) in the culture medium.

As to the changes in time (fig. 26), in both experiments the same tendency can be seen: One and two days after the injection, the highest level of cells could be found in the epidermis. In experiment 1 on both days 59 Langerhans cells could be detected. In experiment 2, on the first day 16, and on the second day 718 Langerhans cells were counted. Of the latter 681 immigrated precursor cells were found in one single epidermal sheet (in all the other sheets together there were 37).

Afterwards the number of Langerhans cells detected in the epidermis decreased. On day 3 it reached the levels of the first day (experiment 2) or even less than the control ear (experiment 1). Ten days after the injection in experiment 1, 18 positive Langerhans cells could still be detected (control ear: 4 Langerhans cells). In experiment 2 the level of the control ear was reached.

5. Discussion

5.1 PKH26

Historical aspects

In 1989 Horan and Slezak developed and patented a new technology for stable, reproducible cell labelling through the incorporation of highly aliphatic reporter molecules containing fluorochrome or radioisotope head groups into the lipid bilayer of cytoplasmic membranes. The probes are trapped once incorporated into the membrane because of their inherent insolubility in aqueous environments (179-181). This incorporation does not physically weaken the cell membrane (182).

Cell linkers like PKH26 can be used to label a wide range of different cell types, as neurones, glial cells, bacteria, yeast, marine organisms, red blood cells, platelets, endothelial cells, many tissue-cultured cells of tumour or normal cell origin (179,183), lymphocytes (184), monocytes and macrophages (185,186), spleen cells *in vivo* (187) and neutrophils *in vivo* (188).

In cultures of mixtures of labelled and non-labelled cells in complete media, Horan found little or no transfer of fluorescent dye from the labelled cells to the non-labelled cells, even after several days of culture. Also in *in vivo* cell tracking experiments with lymphocytes and *in vitro* co-cultures of endothelial cells no transfer of PKH26 to non-labelled cells could be observed (176,189).

In the literature PKH26 is mentioned to be a suitable dye for cell tracking studies (190,191).

Detection of transfer of dye

Rather than just adopting the manufacturer's protocol, I wished to test the feasibility of PHK26-labeling specifically for dendritic cells. In our *in vitro* experiments, where PKH26-stained dendritic cells and unstained dendritic cells were co-cultured, over 95% of all cells were PKH26-positive after three days, as shown by flow cytometric analysis and Cytospin preparations (fig. 5, 7). These surprising data were therefore further investigated.

The observed phenomenon was independent of the mouse strain used and the cell density in the culture dishes. Therefore we were not observing an artefact. PKH26 did not affect the ability of bone marrow-derived dendritic cells to adhere to the culture dishes and to proliferate (see table 6), in line with the data given by the manufacturer (instruction leaflet) and literature (192-194). So the general staining was not due to an impaired proliferation of the unstained cells and/or an increased proliferation of PKH26-stained cells.

In the literature there are rare reports of a transfer of fluorescent cell linkers. Teare *et al.* observed a transfer of PKH2 *in vitro* for lymphocytes (195). In long-term cell tracking experiments with lymphocytes distinct fragments of PKH26 could be found in the renal tubules of rats, indicating, that the dye is metabolised to some degree (196).

It was hypothesised, that an uptake of PKH26 by the unstained cells took place. This was proven in experiment 4.1.3: unstained BALB/c bone marrow-derived dendritic cells were co-cultured with PKH26-stained C57BL/6 bone marrow derived dendritic cells for 3 days. Flow

cytometric analysis on day 3 (figure 10) showed double labelled BALB/c cells, positive for I-A^d – immunohistochemically stained with FITC – and PKH26. So there had been a transfer of fluorescent cell linker from the stained to the unstained population.

Mechanism of dye transfer

As to the route of this transfer three possibilities were considered: uptake of

1. PKH26 in solution, i.e. not cell-bound
2. fragments of stained cell membrane,
3. whole stained cells.

Again, stained and unstained bone marrow-derived dendritic cells were co-cultured, this time separated by an inset with a polycarbonate membrane (pore size 3,0 μm[1]). Dendritic cells should not be able to squeeze through this membrane, at least in sizeable numbers. This experimental setting allowed us to harvest the cells separately after the co-culture. FACS analysis showed, that PKH26 had been transferred.

In a second experiment unstained dendritic cells were cultured with sterile-filtered supernatant (pore size 0,45μm) of stained dendritic cells. This was based on the assumption, that PKH26 dye might leak from the stained cells into the culture medium. Here, no transfer of PKH26 could be detected even after several days.

These results exclude a transfer of dissolved fluorescent dye, because then the supernatant of stained cells would have been sufficient for a transfer. Furthermore manufacturer and literature assure that the elution of PKH26 in time is minimal (176,183,184).

As to the other two possibilities the results of these two experiments are not perfectly conclusive. It may be assumed that fragments of stained cell membrane should be found in sterile-filtered supernatant. Therefore, the absence of a transfer in the second experiment would indicate, that whole cells are phagocytosed. In the first experiment though, where a transfer was detected, the polycarbonate membrane should inhibit this pathway.

So, either the dendritic cells could squeeze through the pores of the membrane, or the fragments of stained cell membrane were retained by the sterile filter. The results of the two performed experiments do not directly prove which of these two possibilities is correct. Nevertheless there are various indications:

1. In figure 10 it can be seen, that the decrease of PKH26-positive cells is less than the increase of PKH26-positive cells: the population of PKH26-stained cells becomes fewer, whereas the population of only MHC class II positive cells nearly disappears. After three days of co-culture a broad "population" that contains all possible combinations of fluorescence intensities can be seen. If whole cells were phagocytosed, there should be 3 distinct populations: one $PKH26^-$/MHC class II^+, one double positive ($PKH26^+$/MHC class II^+)and one $PKH26^+$/MHC class II^-. If stained cell fragments are taken up this variety is not surprising. The fragments would have different sizes and probably a cell could take up more than one at a time. So this scatter plot as well as figure 11 and 12 underline this impression.

[1] The pore size in migration assays für dendritic cells commonly is 5 μm.

2. In the first experiment - cells are separated by a polycarbonate membrane - there is no stimulus for the dendritic cells, to migrate through this membrane like chemotactic agents in a migration assay. This consideration and the small pore size make it unlikely that dendritic cells have squeezed through the membrane to reach the second compartment.

Therefore, in my opinion, PKH26 is transferred by fragments of stained cell membrane and these fragments were filtered out of the supernatant in the second experiment. Probably some PKH26-positive cells become necrotic, maybe due to the stress of the staining procedure. Even if this loss is not conspicuous the number of produced cell fragments is sufficient to stain the entire PKH26-negative population.

Conclusions

These experiments showed that PKH26 *in vitro* gives false positives. Nevertheless the relevance of these findings for *in vivo* cell tracking experiments can be questioned. *In vivo* the fragments of stained cell membrane would be diluted and filtered out from the circulation either in the liver, the kidney or by macrophages in the blood stream, like other cell-detritus. The chance that these fragments would give false positive dendritic cells in lymphoid organs is therefore quite small.

The labelling with cell linkers has clear advantages, as compared to radio-labelling or immunohistochemistry. Identification of injected cells in cell suspensions of lymphoid organs would be made easy and quick by flow cytometric analysis and Cytospin preparations without any further staining (196-198). In the planned experiments it would be possible to work in a syngeneic system, because no immune responses are generated against labelled isogeneic cells (182). Thereby all possible artefacts due to immune responses against allogeneic donor cells would be excluded. In our further injection experiments PKH26 was therefore used as cell label.

5.2 Tape stripping

Historical aspects and rationale

In our experiments we found that injecting bone marrow-derived dendritic cells intravenously without any further treatment of the skin did not lead to a detectable immigration of Langerhans cells in the epidermis, neither by staining the injected cells with PKH26 prior to injection, nor by selective immunohistochemical staining of the MHC class II molecules after injection. It is mentioned in literature, that the epidermis, here of guinea-pigs, contains a constant number of Langerhans cells (199), in steady state. We hypothesised, that this number is constant in mice, too, and that it can not be exceeded in untreated skin. Therefore, a depletion of Langerhans cells from the epidermis would be necessary to allow new immigration of precursors. The aim was to set a stimulus that depletes the epidermis from resident Langerhans cells without destroying the epidermal structures in which the injected precursors should immigrate.

Tape stripping of the epidermis is a simple technique first used by Wolf in 1939 to study the horny layer (200). In the beginning it was used to investigate the structure of the epidermis in human skin and its regeneration after controlled injury (201-204).

It was described as suitable for an almost complete depletion of the skin of Langerhans cells (guinea-pig, mouse), that are either shed with a parakeratotic layer four days after stripping (178,205) or are not detectable immediately afterwards and have probably been physically pulled out (171). Both techniques, if performed as described, produced pronounced damage of the epidermis and destroyed the suprabasal structures in which injected Langerhans precursor cells were supposed to immigrate.

Method of "gentle tape stripping"

It is therefore necessary to adapt the tape stripping for our specific experimental setting and a few preliminary experiments were performed. To get an impression about the intensity of the stimulus set on murine ear skin the transepidermal water loss (TEWL) was measured after a different number of strippings with TIXO®-tape. In human skin a 10- to 20-fold increase of TEWL was detected (206) after tape stripping (about 30 times). In our experiments with mice 6 strippings caused a 3-fold, and 10 strippings a 5-fold increase. Macroscopically the ear skin of mice starts to glisten, but does not bleed nor swell. The hairs of the tape-stripped areas are pulled out.

Six and ten strippings seem suitable to set a defined, yet not too invasive stimulus, and were chosen for a first time course experiment. Because only for 10 strippings an emigration was visible after 6 days, the number of strippings was slightly raised.

With 12 strippings a gradual reduction of Langerhans cells was detected, being visible first 24 h after stripping and increasing until day 6. Even if the number of strippings was raised up to 30 the density of Langerhans cells immediately after stripping did not decrease, indicating that the basal and suprabasal layers in which Langerhans cells reside could not be reached and Langerhans cells were not pulled out, in contrast to the common use (171,207-211). There was no abrupt decrease in density on day 4 and no shed pellicle could be seen. Afterwards the numbers of Langerhans cells started to increase slowly, and gained normal values only about 3 weeks after stripping, whereas the epidermis macroscopically is reconstituted much earlier. These data correspond with literature (178,207).

Migration of Langerhans cells in tape-stripped skin

It is known that Langerhans cells are the antigen-presenting epidermal cells (212). Upon antigen encounter Langerhans cells mature (70) and the number of Langerhans cells in the epidermis decreases, for example in the experimental setting of application of contact sensitizers (213,214). Langerhans cells *in situ* are immature dendritic cells (52). One of the typical features of immature dendritic cells is the ability to migrate to the draining lymph node after antigen encounter via the afferent lymphatics and to mature during this migration (215). Larsen et al. (1990) established an organ culture model in which they could show migration of epidermal Langerhans cells from the epidermis through the dermis into the culture medium (82). This was confirmed for Langerhans cells *in situ* (85).

We wanted to assess if this migration and maturation also happened in our tape stripping model *in vivo*. After tape stripping the production of epidermal proinflammatory cytokines and cellular adhesion molecules increases (216,217). Northern blot analysis of hairless mouse

epidermis has shown, that the levels of TNF-α and GM-CSF mRNAs peak within 1h following acute barrier disruption by tape stripping (216). mRNAs encoding IL-1α, IL-1β, and IL-1 receptor antagonist peak coordinately at 4 h after treatment (216,218). A similar increase in cytokines has been observed in human skin following barrier disruption by tape stripping (217). So, there are quick reactions in skin to tape stripping, that lead to inflammation.
In our experimental setting a moderate inflammation could be seen histologically in routine H&E-sections 4 days after stripping.
Four and six days after tape stripping cords filled with emigrating Langerhans cells and dermal dendritic cells were visible in the dermis. The emigrating cells were all strongly MHC class II-positive, B7-2/CD86 – positive, which is consistent with literature (219), and most of them expressed CD86, a co-stimulatory molecule necessary for T-cell-activation (220,221). Thereby, they can be considered as mature. So, also *in vivo* after tape stripping the changes described for *in vitro* and *in situ* take place.
Langerhans cells remaining in the epidermis appear to be enlarged and visibly express more MHC class II. Our data suggest that the cells remaining in the epidermis try to keep up the barrier and defence function in the epidermis. In a contact sensitizer model ten days after application antigen bearing Langerhans cells were still present in the epidermis; application of another unrelated contact sensitizer to the epidermis at this time did not lead to migration of these residual Langerhans cells (222). Taken together these findings would indicate that even if the Langerhans cell has encountered antigen and an inflammation is ongoing, there is a density of Langerhans cells that is not fallen short of.

Conclusion

We can therefore conclude, that moderate tape stripping leads to an inflammation of the skin. During this process Langerhans cells are activated and start to express higher levels of MHC class II and to mature. Maybe components of glue serve as antigen. After antigen-uptake they leave the epidermis and migrate towards the draining lymph node via afferent lymphatic vessels ("cords") and further mature during this migration. Despite this emigration at no time point the epidermis is absolutely devoid of Langerhans cells.
After 6 days the numbers of Ia^d-positive cells increases and steady state densities are reached about 3 weeks after tape stripping. Therefore, there must be signals recruiting Langerhans cells or precursors to the epidermis.
This finding leads to the hypothesis, that a demand for Langerhans cells could be created in the epidermis and that injected precursor, or Langerhans cells would preferentially immigrate in the emptied epidermis of tape stripped sites.

5.3 Migration of intravenously injected dendritic cells

Currently, dendritic cells loaded with tumour-derived peptide as antigen are used in clinical studies as a vaccine for cancer patients (223-227). Still, among other criteria, the route of

administration as well as the maturational state of the injected dendritic cells are a hot spot of controversy.
In these two experiments the distribution of intravenously injected, PKH26-stained BMDC in spleen, mesenterial lymph nodes, bone marrow, epidermis and dermis was to be examined. Different stages of maturation are injected to assess possible differences: immature day 6 cells, an in-between-stage as day 8, a more mature stage (day 10 GM-CSF) and fully matured dendritic cells (day 10 TNF-α). One limitation for the interpretation of the performed experiments is, that there were only two of them. This reduces the meaningfulness of all conclusions that can be drawn from the obtained results.
The method of choice for tracking the injected dendritic cells in the various lymphoid and non lymphoid tissues was labelling them with the fluorescent cell linker PKH26. Samlowski et al. extensively investigated the effects of labelling with PKH26 on murine lymphocytes (184). Questions addressed were the effects on viability, expression of homing receptors, homing *in vivo*, *ex vivo* frozen section binding assay, cytotoxicity and mitogen responsiveness. All tests showed, that among other labelling methods PKH26 the least changed all the assessed parameters (184). This was also shown for the migration behaviour of murine hematopoietic stem cells (197). In our *in vitro* experiments PKH26-stained dendritic cells behaved normally (morphology, phenotype, cell growth) as compared to unstained cells (see also 5.1 PKH26). We therefore conclude that the migration behaviour of dendritic cells stained with PKH26 is not changed either.

5.3.1 Migration to lymphoid organs

Evaluation method and experimental setting

First it was necessary to find a suitable evaluation method by which the immigration of dendritic cells into lymphoid organs could be quantified. In literature the migration of intravenously injected lymphocytes (176,196) or spleen cells (177) labelled with PKH26 has often been studied and the method for evaluation was flow cytometric analysis of cell suspensions of the examined organs.
In our experimental setting the use of FACS analysis was not possible. The number of dendritic cells immigrated in the single organs is so small as not to form a distinct, measurable population in a dot plot (see figure 21). Even if in literature similar scatter plots of dendritic cells in skin draining lymph nodes can be found [(228) Fig.6, (91) Fig.2] the desired quantitative evaluation is not possible and the numbers of $PKH26^+$ dendritic cells in our experiments are very low.
Other evaluation methods to determine the immigration of dendritic cells would be immunohistochemistry (229) or measuring of radio-label (230) of the tissues, that should be examined, but then again no direct quantification would be possible.
Therefore we thought up a semi-quantitative system (described in the results, page 65) that evaluates Cytospin preparations of cell suspensions of the examined organs. In my personal opinion in this experimental setting this semi-quantitative system was the best evaluation method available. The obtained data seem to reflect the actual immigration. Nevertheless, they must be interpreted carefully. The changes described range from 0,02% to 1,2%, so it is

a point in favour of this semi-quantitative evaluation, that in this scale we can see constant differences between the single organs examined. A point to be aware of is, that differences during time course in one organ need to be relatively substantial to be trusted.
In the first performed experiment I compared immature day 6 dendritic cells and an in-between stage as day 8. There were no striking differences in the migration behaviour of these cells. The maturation stage of the population of more mature day 8-dendritic cells was not perfectly homogenous, because newly "born" daughter cells in the culture dishes are still immature. The so obtained population contains different levels of maturation. This fact could falsify the image of the homing behaviour. Therefore, in the second experiment a separation-step was performed on day 6 of the culture (see 4.3.2) and dendritic cells were cultured until day 10 with or without TNF-α, that pushes maturation. The so obtained populations of dendritic cells should be more homogeneous and differ more from each other regarding their maturation stage. Nevertheless, again no differences could be found. More experiments would be needed to assess, whether the impression gained from the performed experiments is definitely substantial, or not.

Bone marrow

It was quite surprising that in the bone marrow a discrete number of injected dendritic cells of any maturation stage could be found. Morse *et al.* also found mature dendritic cells (PBMC cultured for seven days with GM-CSF and IL-4; phenotype: $CD86^+$, $HLA\text{-}DR^+$, $CD14^-$) in the bone marrow of patients with metastatic malignancies 24 h after intravenous injection (231). The number of dendritic cells immigrated into the bone marrow in our experiments ranged between 0,09% - 0,8% of all bone marrow cells and was on a "medium level" as compared to spleen and lymph nodes. During the first six days this number was quite variable. Around day 18 numbers increased and even exceeded the values measured one day after injection (in one experiment). A similar increase of PKH26-positive cells can be seen in all the examined lymphoid organs in the second, and for spleen also in the first experiment.
A possible explanation could be, that some of the injected cells retained their ability to home to the bone marrow in a way that is independent from the predominant maturation degree of the injected dendritic cells, and, that they are in the position to develop daughter cells. These daughter cells then home to spleen and lymph node, whereas the putative stem cells stay in the bone marrow and continue to divide, sharing the fluorescence intensity between the daughter cells. On the Cytospin preparations, though, a decrease in the intensity of PKH26-fluorescence was not noticed. The same phenomenon was observed even in populations of mature dendritic cells, where residual stem cells are less likely to occur.

Spleen

The highest numbers of PKH26-positive cells could be found in the spleen. This is not surprising as it is the first lymphoid tissue to be encountered after intravenous injection and dendritic cells are able to enter the spleen from the blood circulation (232). Furthermore the indications for such a distribution are numerous in literature (233-235). After intravenous injection spleen dendritic cells are sequestered in the lungs, but they actively migrate into the liver and spleen and become interdigitating cells in the white pulp of the spleen within 24h

(230,232). It can be speculated, that in our bulk bone marrow-derived dendritic cell population there were many spleen dendritic cell precursors.
During the first 6 days there was a decrease in $PKH26^+$ dendritic cells detected in the spleen (immature DC: from 1,2% one day after injection to 0.8% 6 days after injection; fully mature DC: from 1,1% to 0,48%). This decrease probably reflects the loss of dead cells. Having not encountered any antigen-specific T cells for presentation these cells are "useless" in the spleen and it can be speculated, that they are induced to undergo apoptosis.
After 15 to 20 days, though, the level of $PKH26^+$ cells has risen to values found one day after injection. This course could reflect be the time needed by the stem cells that have migrated into the bone marrow to produce daughter cells, that then reach the spleen around day 15 to day 20. Again no decrease in fluorescence intensity could be noticed on the Cytospin preparations, as might be expected when stained cells divide and the dye dilutes out. On the other hand, staining of cytospins may not be sensitive enough to pick up such subtle changes.

Mesenterial lymph nodes

In the mesenterial lymph nodes only very few PKH26-positive cells could be found (0% - 0,1%). This would be consistent with our expectations and literature (229,230,236). Spleen and bone marrow derived dendritic cells are unable to enter mesenteric lymph nodes from the bloodstream (230,234). So maybe those dendritic cells, that homed to the mesenterial lymph nodes during the first week, had taken the route through the spleen and from there through the lymphatic system. Or, as described by Matsuno *et al.* and Kudo *et al.*, dendritic cells translocate from the blood through the hepatic sinusoids to the liver-draining lymph, travel to the celiac lymph nodes, and maybe from there continue to the mesenterial lymph nodes (66,237).
Once they have entered a lymph node lymph borne dendritic cells are unable to recirculate (233). For mature and fully mature dendritic cells in the mesenterial lymph nodes a 5- to 10-fold increase as compared to other time points could be seen 18-20 days after intravenous injection. This increase was not so striking for immature dendritic cells. This is probably due to the homing of daughter cells produced in the bone marrow from PKH26-positive stem cells, rather than to a varied migration behaviour of the different maturation stages. To prove this statement though, more experiments need to be performed.

Conclusion

To conclude, in both experiments different levels of immigrated cells for the examined lymphoid organs could be detected, that mostly reflect the accessibility of these organs for dendritic cells in the blood stream. The increase of PKH26-positive cells in the examined organs after 15 to 20 days is probably due to the proliferation of stem cells that were still among the injected dendritic cells, independently of their maturation stage.

5.3.2 Immigration in the epidermis

In the epidermis PKH26-positive cells could be identified at no time point. Langerhans cells can not only be found in the epidermis (28), but also in other stratified epithelia such as mouth, esophagus, and lung (37,39-41). As after intravenous injection dendritic cells are

sequestered in the lungs (230,234) it can be assumed, that precursors committed to epithelium are retained there and do not circulate any further.
Furthermore it is not yet clear, whether Langerhans cell precursors repopulate a depleted epidermis "directly" form the bloodstream or if they first immigrate in the dermis, where they reside as Langerhans cell precursors or maybe as dendritic cells, that retain their potential to differentiate into Langerhans cells when required, awaiting a moment of demand. So another point, but surely one of minor importance, is, that injected precursors could be retained in the dermis.

Culture and treatment previous to injection

We hypothesised, that a keratinocyte-monolayer must synthesise the signal(s) necessary for Langerhans cell recruitment into the epidermis. Therefore bone marrow-derived dendritic cells were cultured until day 6 with 200 units per ml GM-CSF and 30 Vol% keratinocyte-supernatant, stained with PKH26 and injected intravenously into tape stripped mice. Epidermal and dermal sheets were prepared.
At no time point PKH26- positive cells were detected in dermis or epidermis. Most probably precursor cells destined to home to epithelia have been retained in the lung of the injected mice and therefore could not reach the epidermis. As also in the dermis no precursors could be detected it plays no role whether Langerhans cells are replaced from the bloodstream or from precursors residing in the dermis. In this experimental setting this facet has no relevance.

5.5 Immigration of intradermally injected bone marrow derived Langerhans cells into the epidermis

The migration of Langerhans cells to lymph nodes draining an examined area of skin has closely been studied (86,238), specially in the context of contact hypersensitivity (239-245). In contrast the mechanisms leading to tissue-specific homing of Langerhans cells/Langerhans cell precursors are poorly understood, and the exact maturational stage at which Langerhans cells enter the skin *in vivo* is still unknown.
The method of application of dendritic cells was changed, because when injected intravenously no immigration into the epidermis could be detected. In these experiments immature bone marrow-derived dendritic cells were injected intradermally into tape-stripped and untreated control ears. At defined time points thereafter epidermal and dermal sheets were prepared.
First of all the most exciting result is, that Langerhans cells were found in the epidermis after injection of immature bone marrow-derived dendritic cells (culture with GM-CSF for 6 days). This shows that in this bulk population some of the arising cells have the ability to differentiate into Langerhans cells and to migrate through the basement membrane into the epidermis *in vivo*. That cells were directly injected into the epidermis can be excluded. This is not possible as for the handling. Only when cells are injected between dermis and cartilage the injection is successful, what could clearly be seen for each of the injected ears (see also: 3.6.5 Intradermal injection, experimental procedure, point 4 and 5).

These Langerhans cells must have been present also in the cultures of immature dendritic cells, that were injected intravenously. Most probably this time these cells could reach the epidermis, because they were not retained in the epithelia of the lung.
The evaluation system adopted in the performed experiments is very definite. Injected Langerhans cells that immigrated into the epidermis could clearly be distinguished from resident cells. This method though is very labour-intensive, in particular the immunohistochemical evaluation. To perform more experiments and to test different kinds of cytokines or culture methods, as mentioned in the outlooks below, it needs to be simplified. It could be tried to inject syngeneic PKH26-stained dendritic intradermally. Or to inject allogeneic dendritic cells and to selectively stain MHC class II molecules (167,246). These changes would simplify the experimental procedure because detection of immigrated cells would be made easier. As to the quantification of immigrated Langerhans cells one could try to make epidermal cell suspensions followed by flow cytometric analysis either for PKH26 or MHC class II. With regard to the exactness of the data – in a sense of absolute data – for immigrated cells it must be mentioned, that for each ear the entire area available after epidermal sheet preparation was examined. This area was not always of the same size and it could happen, that some sheets could not be evaluated because the double-staining for I-A^b and I-A^d was not successful. Nevertheless the differences between tape-stripped and untreated ears and in time course are striking.
Two days after injection peaks for immigrated cells were detected in tape stripped epidermis (experiment 1: 59 Langerhans cells; experiment 2: 718 Langerhans cells). In the second experiment the incredible number of 681 injected Langerhans cells was detected in one single epidermal sheet. As described in chapter 5.2 the emigration after tape stripping is patchy. So this local concentration maybe shows a site of perfect interplay between the created demand with emigrated recipient cells and the site of injection with new donor Langerhans cells, that are ready to immigrate. Already 3 days after intradermal injection the number of Langerhans cells detected in the epidermis decreased to levels that could still be detected on day 10 (4 to 18 Langerhans cells/ear), but were still higher than numbers in control ears. The reason for this decrease still needs to be examined, as one would expect the Langerhans cells to stay in the epidermis, as it is known, that the turnover in skin is 21days (50). To establish a time course more experiments need to be performed.
It must be noticed, that an immigration of Langerhans cells into the epidermis could be detected in both tape-stripped and untreated ears. Nevertheless, the assumption that with tape stripping a demand for Langerhans cells in the epidermis must be created, before immigration of precursor cells can take place to a certain degree, was confirmed in an impressive way. Immigration into tape-stripped ears was up to 300-fold higher than into untreated ears Those few cells, that immigrated into untreated ears probably filled a gap in the net of defence in the epidermis, too. As we are working in a biologic system, that is furthermore alive, this is not surprising. It would be sufficient that the mouse scratches over its ear, irritated by the injection, and a patch depleted of Langerhans cells would occur.
The injected Langerhans cells detected in untreated epidermis perfectly resembled resident Langerhans cells by shape, showing a cell body surrounded by long dendrites. The shape of

Langerhans cells immigrated in tape-stripped epidermis was elongated with no or one dendrite. They seemed to just have slipped in. A possible explanation could be, that tape-stripped epidermis is "in motion" after a stimulus that has caused an inflammation. The rearrangement to a steady state apparently is more complex and the single forces on dendritic and Langerhans cells are more interlocked, than previously assumed.

5.6 Outlook

In the experiments described I have established a model which may be useful to study the conditions for the immigration of Langerhans cell precursors into the epidermis. Different reagents such as cytokines, chemokines, blocking antibodies, etc. may be co-administered with mature or immature dendritic cells. Some interesting candidates will be discussed below.

- **Transforming growth factor-β1 (TGF-β1)**

There is strong evidence both in the murine and in the human system that TGF-β1 plays an essential role in the development of Langerhans cells and their recruitment to the epidermis. At first it was seen as a mediator that keeps human Langerhans cells in an immature state (247,248) and suppresses an up-regulation of MHC class II after stimulation (249). A few years later, in 1996, Borkowski et al. published the finding, that the skin of TGF-β1 -/- mice is devoid of epidermal Langerhans cells (150). Langerhans cells were detected neither in epidermal cell suspensions, nor in epidermal sheets. Other skin associated cells of the immune system, like dendritic epidermal T cells and $CD11c^+$ dendritic cells in the lymph nodes, though, were present in normal numbers. Further characterisation of the TGF-β1 -/- phenotype revealed, that bone marrow from -/- mice gave rise to Langerhans cells after transfer into lethally irradiated recipients, and that the skin of TGF-β1 -/- mice was repopulated with Langerhans cells after engraftment onto BALB/c nu/nu recipients (151). Taken together these studies indicate, that TGF-β1 is essential for the normal murine Langerhans cells development and their epidermal localisation. On the other hand, in experiments of Dr. Franz Koch TGF-β1 had no effect on the differentiation of bone marrow-derived progenitors into Langerhans cells (unpublished data).
In the human system different progenitor cells and many different markers for sorting populations, were used to culture Langerhans cells, but in each of these systems TGF-β1 played an important role (148,149,155,156,250,251), even though it was found not to be absolutely essential (148). flt3 ligand potentiates the effect of TGF-β1 and further enhances the development of progenitors into Langerhans cells *in vitro* (147,252). So in the human system again there is a strong evidence for the necessity of TGF-β1 for Langerhans cells development.
In our experimental setting it would be interesting to try to rise the number of Langerhans cells immigrated into the epidermis by co-culturing the progenitors with TGF-β1, or to paint TGF-β1 onto tape stripped epidermis or to co-inject the bulk population of bone marrow derived, immature dendritic cells and TGF-β1 into the dermis.

- **Monocyte chemoattractant protein 1 (MCP-1)**

The chemokine monocyte chemoattractant protein-1 (MCP-1) is a potent in vitro monocyte activator that has been associated with monocytic infiltration in several inflammatory diseases (253-256). MCP-1 induces chemotaxis, calcium flux, regulation of adhesion molecule expression and cytokine production in human monocytes (257-260). The *in vitro* responses to MCP-1 in the murine system are mediated by CCR2 (261,262). In the human system two related receptors, CCR2A and CCR2B, were identified (263).
Transgenic mouse models have demonstrated monocyte/macrophage recruitment to sites of human MCP-1 or murine MCP-1 analogue (JE) expression (264-266), and neutralising antibody studies have implicated MCP-1 as a major mediator of macrophage recruitment in several inflammatory models (267-269).
MCP-1 can be induced a broad variety of cells (270,271); for example it is secreted by cytokine-activated endothelial cells upon stimulation by IL-4 (272-274), or by proliferating basal keratinocytes in psoriatic lesions (275). It further acts as an activator on human basophils (276,277) and as chemoattractant of $CD4^+$ and $CD8^+$ T cells (278-281), macrophages (282) and, last but not least, dendritic cells (283-285).
In 1995 Nakamura *et al.* generated transgenic mice, that expressed MCP-1 in the basal layer of the epidermis (286). Despite the production of high levels of functional MCP-1 by basal keratinocytes, documented *in vivo* and *in vitro*, these mice did not exhibit spontaneous cutaneous inflammation or any other discernible skin pathology. Additional phenotypic characterisation of the normal appearing skin in these mice showed, that the only spontaneous change was a dramatic increase and redistribution of CD45+, I-A+ cells in the dermis, that assumed a dendritic morphology *in situ*, including a subset, that expressed markers characteristic of Langerhans cells (CD11c, F4/80). These cells were often clustered near the dermal epidermal junction and around hair follicles; that is in apposition to epithelial cells producing transgenic MCP-1.
MCP-1 is another chemokine that could be involved in the recruitment of Langerhans cells to the epidermis. Maybe an increase of immigration in our tape stripping model could be observed, when MCP-1 is injected intradermally together with immature dendritic cells, or painted on the skin of tape stripped, injected mice.

- **Macrophage inflammatory protein-3α (MIP-3α)**

Immature dendritic cells, derived from $CD34^+$ hematopoietic progenitor cells or from monocytes in the presence of TGF-β1, respond to many CC- and CXC-chemokines, and in particular to MIP-3α/liver and activation-regulated chemokine (LARC) (287,288). MIP-3α acts through the receptor CCR6, that is mainly found on dendritic cells and lymphocytes (289-291). Like most other chemokines acting on immature dendritic cells, MIP-3α is inducible upon inflammatory stimuli (292,293). It could be found in inflamed epithelial crypts of tonsils (287) and in the epidermis after tape stripping (Dr. Matthias Schmuth, unpublished data). Differential expression of CCR6, though, revealed heterogeneity among dendritic cell populations (250,294).
In a recently published paper it could be shown, that $CD1a^+$ Langerhans cell precursors selectively and specifically respond to MIP-3α in Transwell Insert Chemotaxis Assay and

Boyden-type Microchamber Chemotaxis Assay (93). Langerhans cells lose the migratory responsiveness to MIP-3α during their maturation and acquire responsiveness to MIP-3β (93), like monocyte derived dendritic cells and $CD34^+$ hematopoietic precursor cell-derived dendritic cells. Langerhans cells become responsive to MIP-3β during their final maturation because of the de novo expression of CCR7, a specific receptor for MIP-3β (287,295-298). Briefly, the model, that can be discussed from these findings paints the following life-cycle for Langerhans cells: the precursors committed to become Langerhans cells express CCR6 and migrate to the epidermis attracted by MIP-3α. Upon antigen encounter and appropriate inflammatory stimuli Langerhans cells mature and lose their responsiveness to MIP-3α (downregulation of CCR6, de novo expression of CCR7). They leave the epidermis and can be attracted to the lymph vessels by MIP-3β. An identical picture can be drawn for immature/mature dendritic cells at an inflamed site of injury (287,293).
The injection of immature dendritic cells together with MIP-3α, MIP-3β, or with blocking antibodies against MIP-3α, MIP-3β, CCR6 or CCR7, or the epicutaneous application of MIP-3α on tape stripped skin, would be interesting experiments in our tape stripping model.

- **Matrix metalloproteinases (MMPs)**

Matrix metalloproteinases (MMPs) are enzymes that degrade extracellular matrix, including basement membrane components (299). They are implicated in the complex, integrated events underlying cell migration, but throughout the research of the past years no definitive single mechanism has emerged (300,301). MMPs also play an important role in wound healing (302), or were found to mediate an alternative pathway for the creation of biologically active IL-1β *in vitro* (303). They are secreted by macrophages (304), T cells (305-307), dendritic cells (308) and monocytes (309). MMPs are involved in the migration of these cells through the basement membrane, and, as to the monocytes, are believed to cause destruction of connective tissue in chronic inflammatory lesions (309). The overexpression of MMPs seems to be a crucial factor in some diseases, for example high amounts of MMP-12 mRNA could be detected in granulomatous skin lesions (304). In knock-out mice with deficiencies for MMPs the outbreak of allergen-induced airway inflammation (310), or bullous pemphigoid, an autoimmune subepidermal blistering disease (311), could be prevented.
As to Langerhans cells it could be shown, that pre-treatment of skin explants with MMP inhibitors can block their emigration after exposure to a contact sensitizer (312), and that Langerhans cells express MMP-9 in the human epidermis after application of haptens (313). It could be interesting to co-inject MMP-inhibitors together with immature dendritic cells in our tape stripping model and to evaluate an eventual impairment of passing the basement membrane and thereby immigration.

- **Macrophage inflammatory protein-1α (MIP-1α)**

The chemokine MIP-1α is released by macrophages and monocytes, and it functions as a potent chemoattractant for activated T cells (279,314,315) and eosinophils (281). Also immature dendritic cells respond to MIP-1α by chemotaxis and transendothelial migration, and thus may be attracted to sites of inflammation (287,293,296). In a murine tumour vaccination model genetically engineered tumour cells released GM-CSF, and thereby

induced the production of MIP-1α in the surrounding tissue and the local accumulation of dendritic cells (316), what suggests an interrelation between GM-CSF and MIP-1α. Evidence exists, that the expression of MIP-1α is down-regulated by IL-10, and thus an exaggerated infiltration may be prevented (314).
MIP-1α furthermore plays important roles in the immune response to viral infections. It is necessary for the induction of an inflammatory response to viral infection (317). Together with RANTES (regulated on activation, T cell expressed and secreted) and MIP-1β it is one of the major HIV-suppressive factors for macrophage-tropic virus strains produced by $CD8^+$ T cells (318-321).
MIP-1α was also found to raise the emigration rate of Langerhans cells from the epidermis in an organculture model by 10 – 20% in a dose dependent manner (322). It can be speculated, that by painting MIP-1α on tape stripped ears the immigration of Langerhans cell precursors into the epidermis can be raised. Maybe a synergistic effect between MIP-3α and MIP-1α exists.

- **CD44**

CD44 is an acidic sulphated integral membrane glycoprotein and is expressed by lymphocytes and other hematopoietic or mesodermal cells (323-326). It is implicated in lymphocyte homing (327,328), lymphohemopoiesis (329-331) and T cell activation (332,333). Recent studies have proved that CD44 is the principal cell-surface receptor for hyaluronate and is involved in its degradation (334-336). CD44 has been suggested to be closely associated to actin filaments through its cytoplasmic domain and to be involved in cell migration (326,337-339). Like other hematopoietic cells, Langerhans cells and dendritic cells express CD44 on their cell surface (340-342). On Langerhans cells in *in vitro* studies it is up-regulated by TNF-α and down-regulated by IL-10 (341,343). It could be shown, that CD44 is essential for Langerhans cells migration to the skin draining lymph node (96,344,345).
It could be interesting whether in our tape stripping model the emigration of Langerhans cells from the epidermis is impaired by anti-CD44 antibody.

- **E-cadherin**

E-cadherin is a homophilic, Ca^{++}-dependent cell adhesion molecule, that is expressed on human and murine Langerhans cells (346-348). It could be shown, that E-cadherin is a differentiation antigen, that is characteristic for Langerhans cells and lineage related cells, i.e. skin-associated lymph node dendritic cells (349). E-cadherin mediates the adhesion of Langerhans cells to keratinocytes *in vitro* and *in vivo* (347,348,350,351). Therefore it plays a crucial role in the controlled exit of Langerhans cells after antigen exposure. In fact E-cadherin expression on Langerhans cells is down-regulated during maturation (94), by inflammatory mediators such as IL-1, TNF-α, LPS *in vitro* (95,352) and by contact and fragrance allergens *in situ* and *in vitro* (353,354). It has also been proposed to be one of the molecules, that mediate Langerhans cells recruitment to the epidermis (353).
To verify these hypotheses in our tape stripping model intradermally co-injected Langerhans cells and anti-E-cadherin in tape stripped ears should impair the immigration into the epidermis.

In conclusion it can be said, that the tape stripping model elaborated could be a promising tool for the investigation of *in vivo* immigration of Langerhans cells. It must be emphasised again, that the experimental setting is very labour-intensive and needs to be simplified.

6. Summary

Langerhans cells are a well examined part of the dendritic cell system of professional antigen presenting cells. Together with all other dendritic cells they have the unique capacity to stimulate naive T cells and thereby are at the beginning of almost all acquired immune responses. In this work the migration behaviour of dendritic and Langerhans cells was studied in the murine system, particularly with regard to the immigration of Langerhans cell progenitors into the epidermis.

In a first approach immature and mature bone marrow-derived dendritic cells, labelled with PKH26 were injected intravenously into BALB/c mice. In preliminary experiments PKH26, an fluorescent cell linker, was extensively tested as for being suitable for dendritic cell migration experiments. At defined time points after injection lymphoid organs (spleen, mesenterial lymph nodes, bone marrow), epidermis and dermis were prepared and analysed for the presence of fluorescent cells.

Bone marrow-derived dendritic cells preferentially homed to spleen, the first lymphoid organ encountered, followed by a discrete number in the bone marrow, most probably pluripotent stem cells, and only single dendritic cells in the mesenterial lymph nodes. As long as the lymphoid organs were examined it could be observed, that dendritic cells homed to their destination site and stayed there. Experimental evidence suggested that the stem cells that homed to the bone marrow subsequently divided and gave raise to a novel $PKH26^+$ dendritic cell population that could be detected in all lymphoid organs examined around day 18 after injection. Interestingly, there were no differences between the maturation stages.

No intravenously injected $PKH26^+$ cells were found in the skin, probably because cells able to home to the epidermis were retained by the lung epithelium, that also contains dendritic cells similar to Langerhans cells.

Concentrating on the epidermis, in a second approach we established a model in which a demand for Langerhans cells was created in the epidermis by a novel gentle tape stripping-technique. This technique depleted the epidermis of Langerhans cells by mimicking the inflammation that often occurs with antigen-uptake, that led to the emigration of Langerhans cells from the epidermis. The epidermis was not severely damaged, as in previously described experimental settings (171,178,205). Intradermal injection of immature bone marrow-derived dendritic cells was chosen as administration route to circumvent the lung epithelium. Two days after injection into tape-stripped ears up to 300 times more Langerhans cells, selectively stained for their MHC class II molecule-haplotype, were detected in the epidermis, as compared to untreated ears. These results confirmed that a demand for Langerhans cells is needed to allow new immigration of precursor cells. Already on day 3 after injection the number of injected cells decreased to lower levels, still detectable on day 10.

We have established a suitable *in vivo* model for studying the immigration of Langerhans cells into the epidermis, with which chemokines and cytokines apparently involved in migration and skin homing of Langerhans cells can be tested in future experiments. Their ability to modulate Langerhans cell numbers detected in the skin and their influence on the time course would be of interest.

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