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Einwanderung von Langerhanszellvorläufern in die Epidermis: Entwicklung eines Modellsystems

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4. Results

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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%

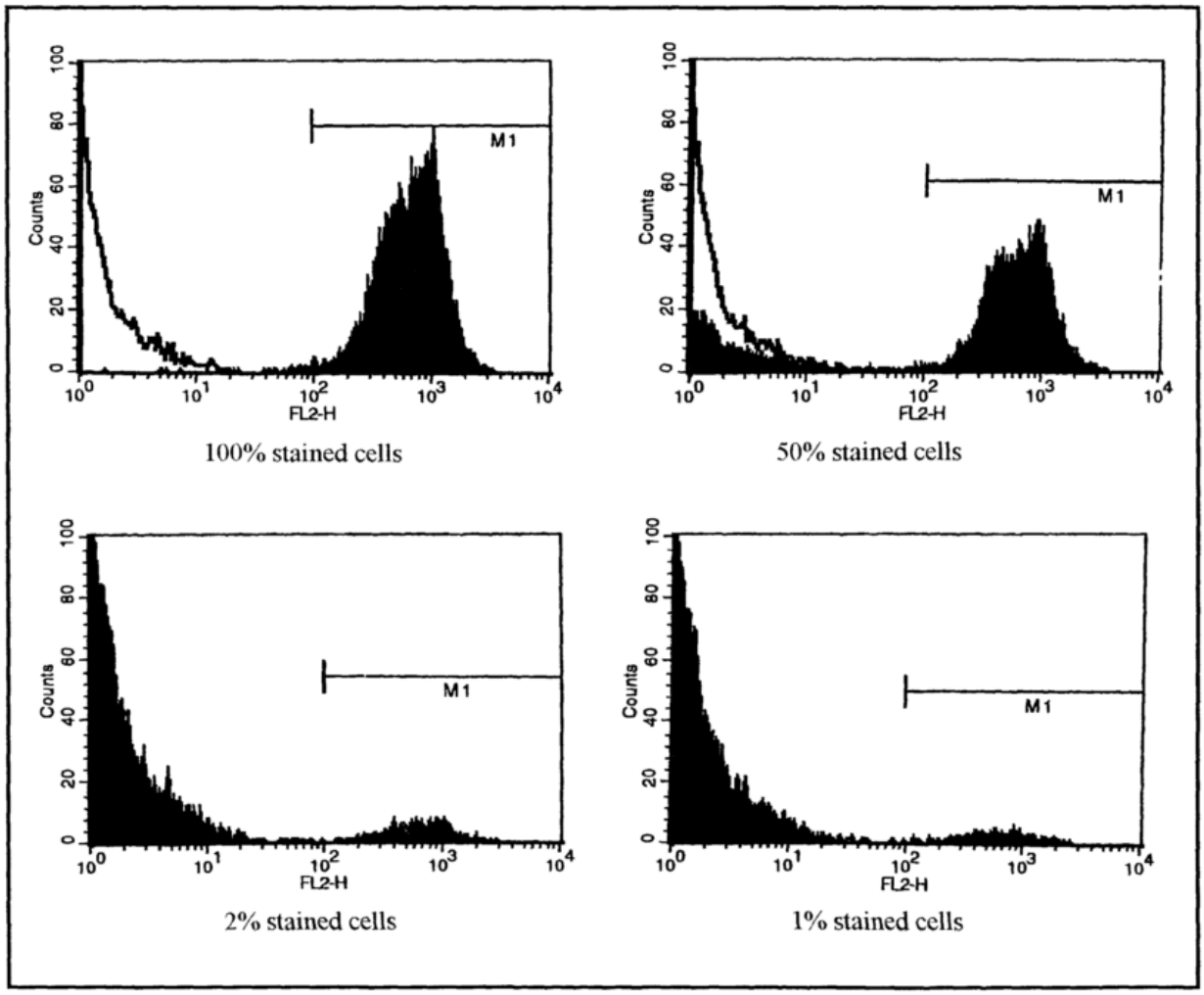


Figure 4: Sensitivity of FACS analysis of stained and unstained dendritic cell populations mixed in different proportions was tested on the day of PKH26 staining. Cells stained with PKH26 can be detected even at low levels, i.e. if only 1% of all cells are positive. 20.000 events were analysed. Open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

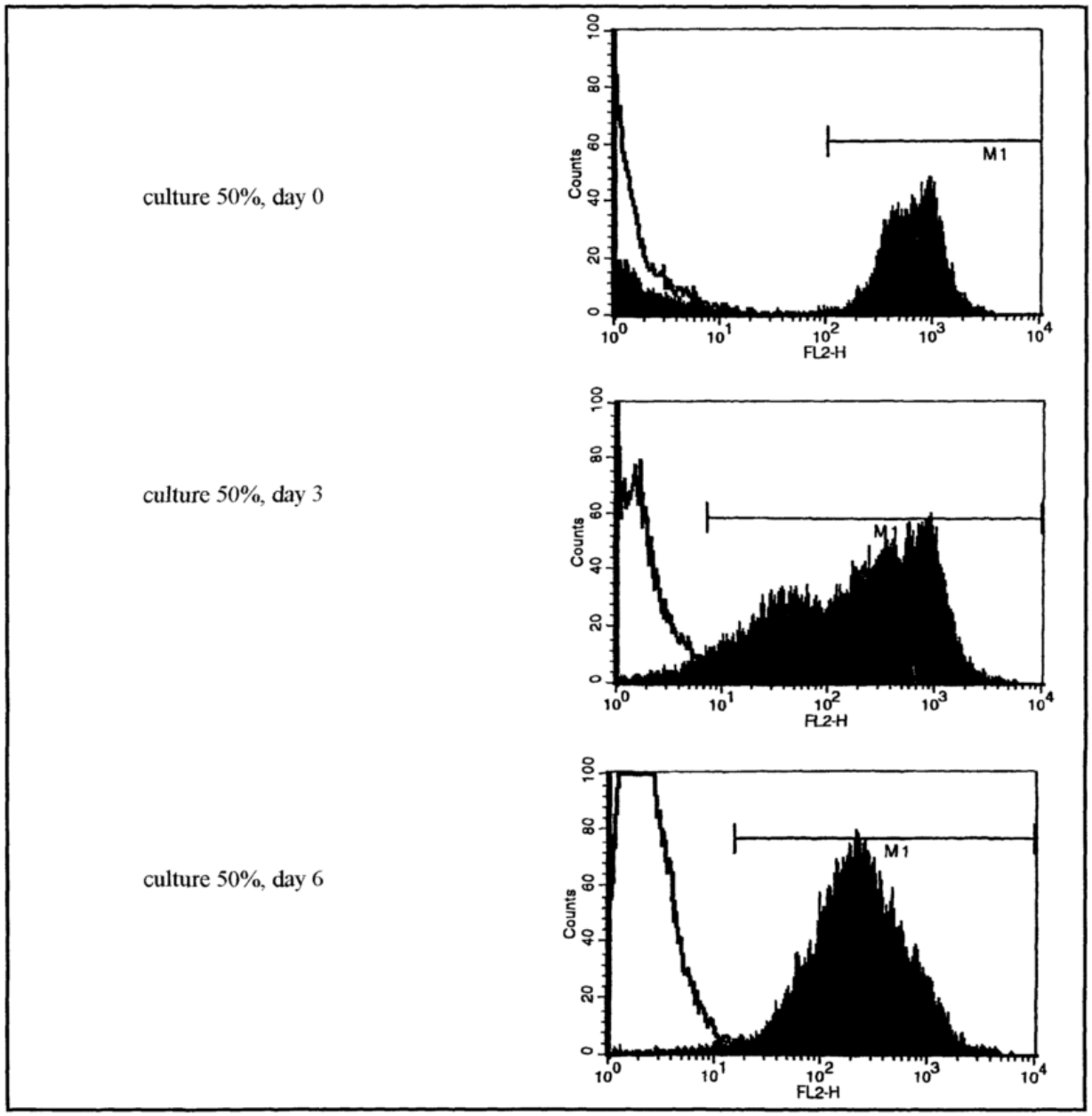


Figure 5: Analysis of co-cultures consisting of 50% PKH26-stained cells and 50% unstained cells with time (same data as in table 4). Note that the percentage of PKH26-positive cells increases to >90% on days 3 (middle panel) and 6 (bottom panel) of co-culture. 10 – 20.000 cells were analysed. Open histogram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

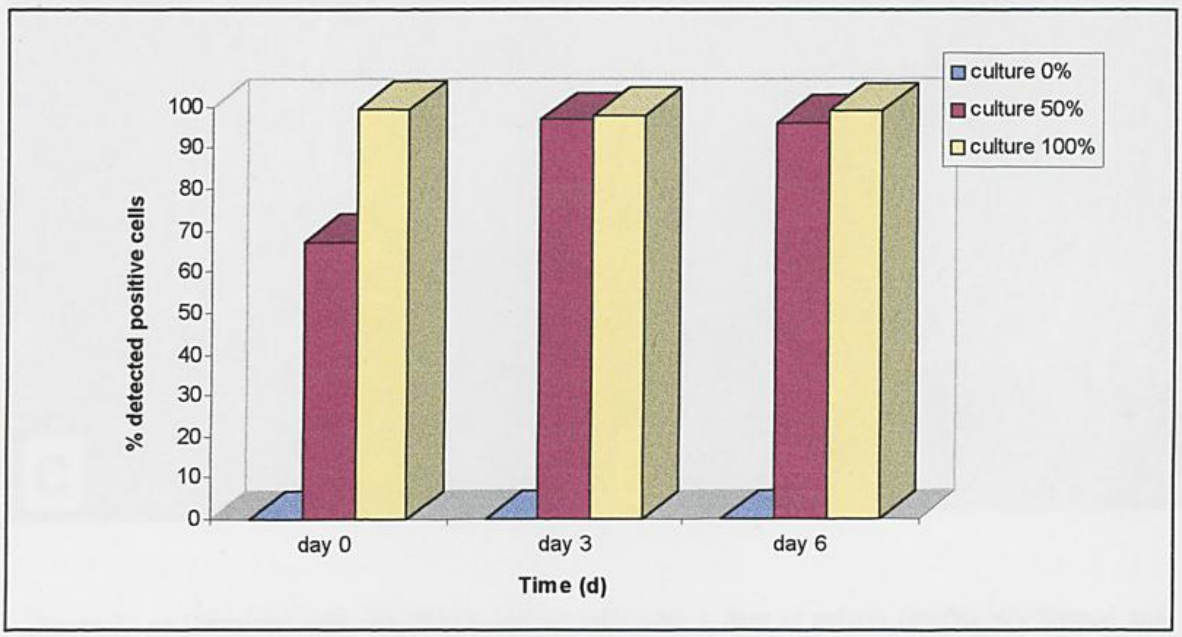


Figure 6: Change of the percentages of PKH26-positive cells during co-culture with unstained cells (same data as in table 4). Note that in the co-culture experiment on days 3 and 6 nearly all dendritic cells are PKH26-positive.

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.

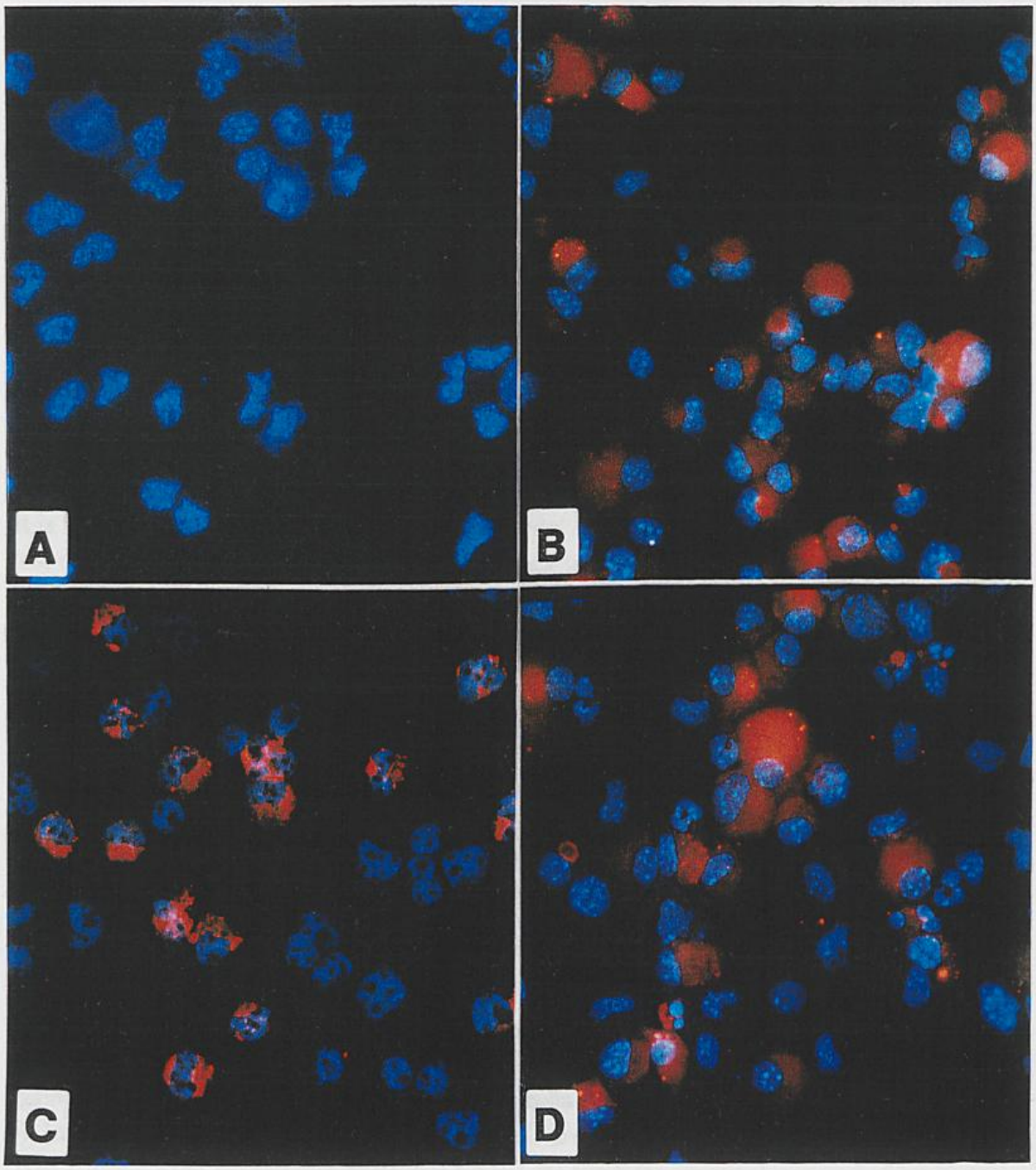


Figure 7: **A)** Unstained cells, **B)** PKH26-stained cells after 3 days of culture (100%), **C)** Stained and unstained dendritic cells the day of staining (=day 0), **D)** Stained and unstained dendritic cells after 3 days of co-culture (400x). It can be seen, that the vast majority of cells is PKH26-positive.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

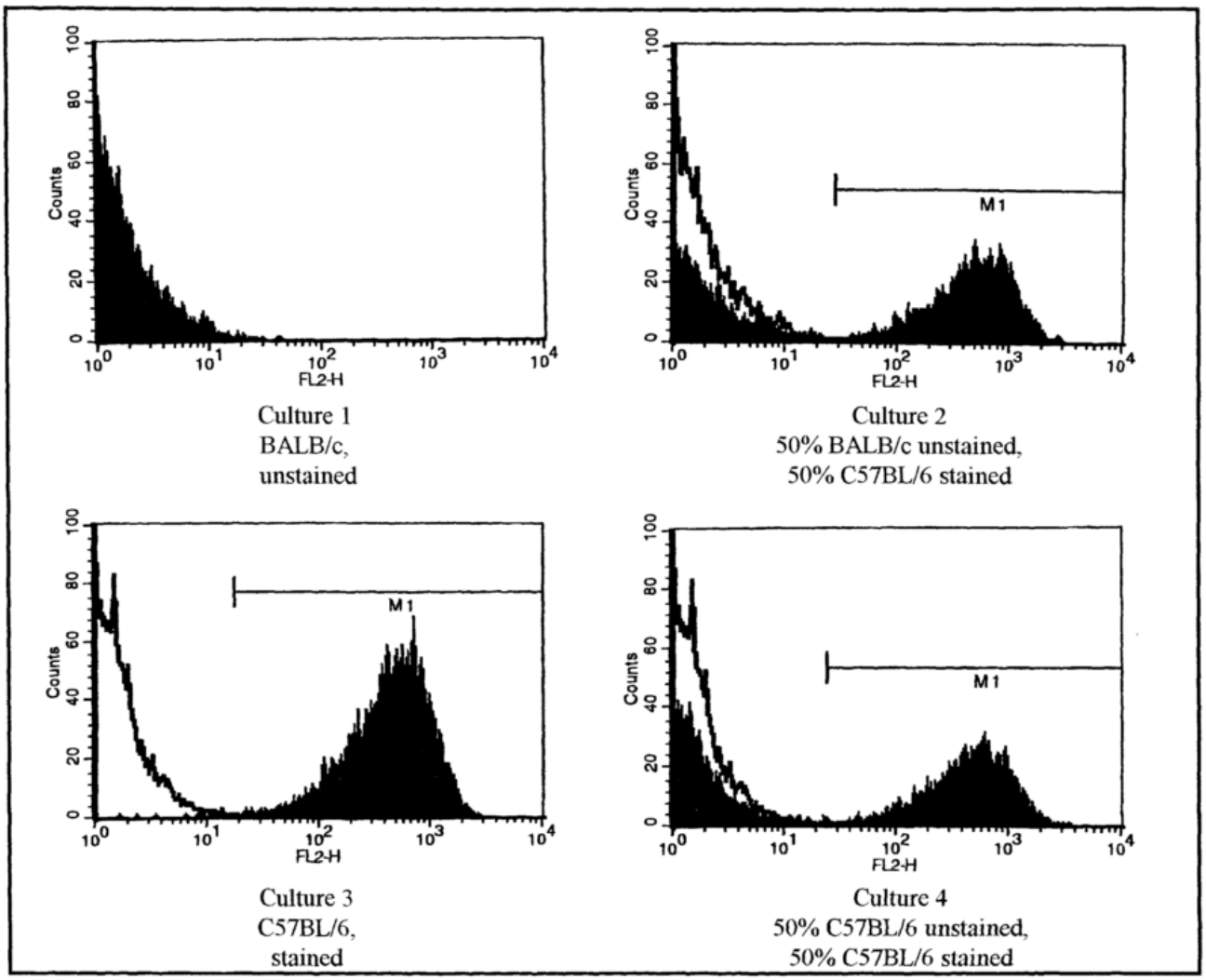
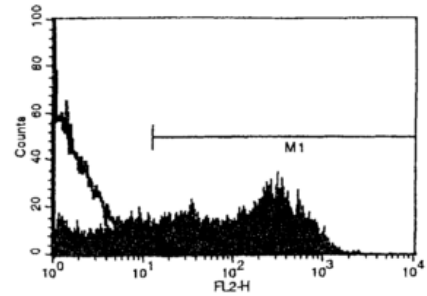
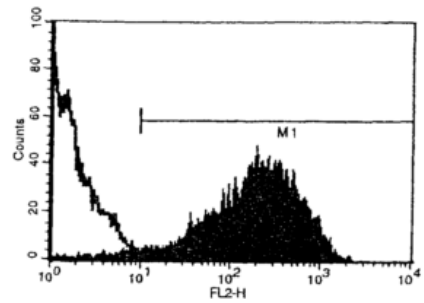


Figure 8: Flow cytometric analysis to confirm the correct mixing of cell populations. Analysis is performed before the onset of co-culture on day 6, the day of staining with PKH26. In each dish a total number of $5x10^6$ dendritic cells was plated. Open histogram represents unstained cells, 10.000 events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

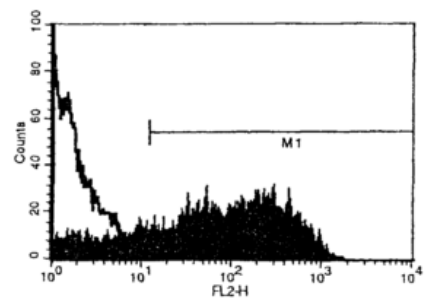
culture 2
50% BALB/c unstained,
50% C57BL/6 stained
$5x10^6$ cells per culture dish



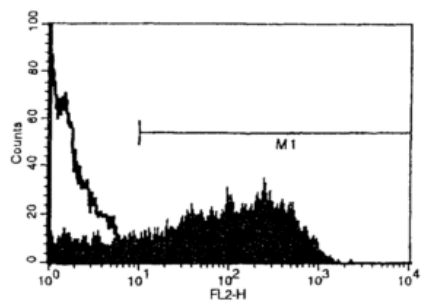
culture 3
C57BL/6, stained
$5x10^6$ cells per culture dish



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

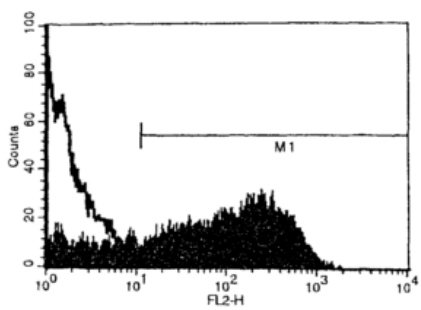


Figure 9: Flow cytometric analysis of the cultures listed in table 5 on day 9, after 3 days of co-culture. A prominent increase of PKH26-positive cells in cultures 2, 4, 5, and 6 can be seen. This increase is neither dependent on the mouse strain (compare 2 and 4), nor on cell density in the culture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

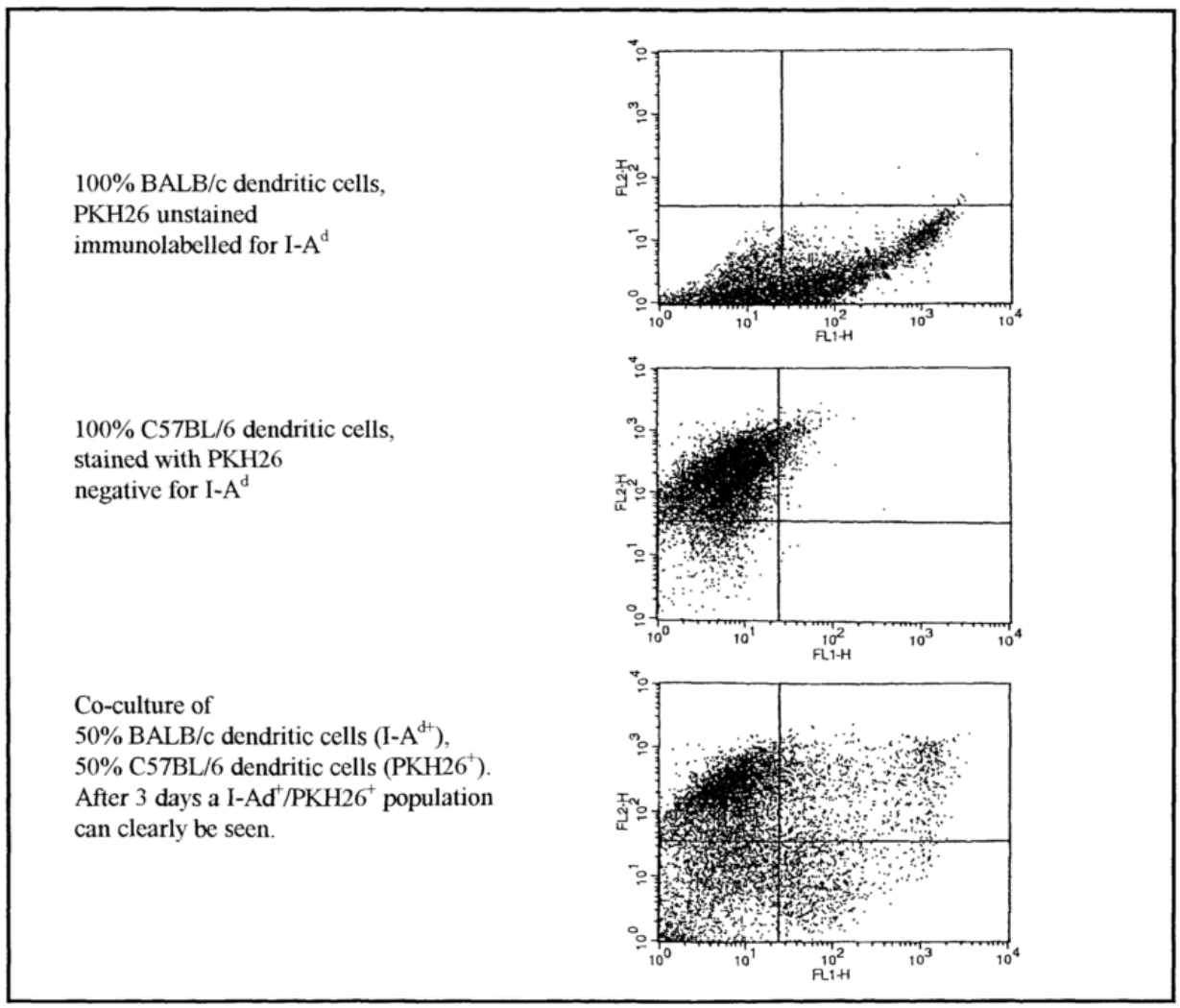
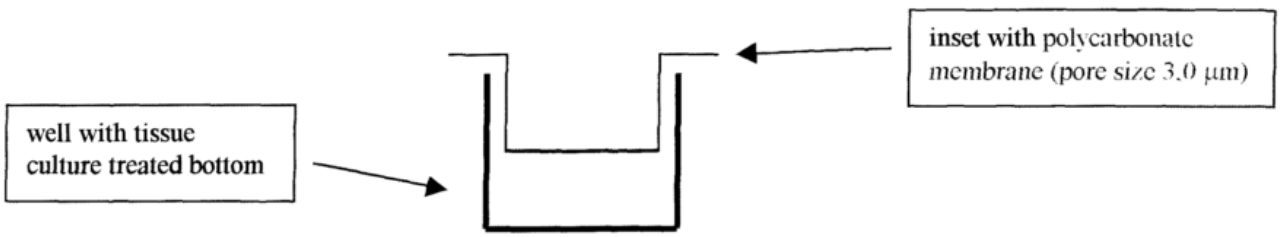


Figure 10: Transfer of PKH26 to the unstained cell population after 3 days of co-culture. **Upper panel**: distribution of BALB/c dendritic cells selectively immunolabelled for I-A^d after culture (FL1-H). These cells are PKH26$^-$ (FL2-H). **Middle panel:** C57BL/6 dendritic cells stained with PKH26 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).

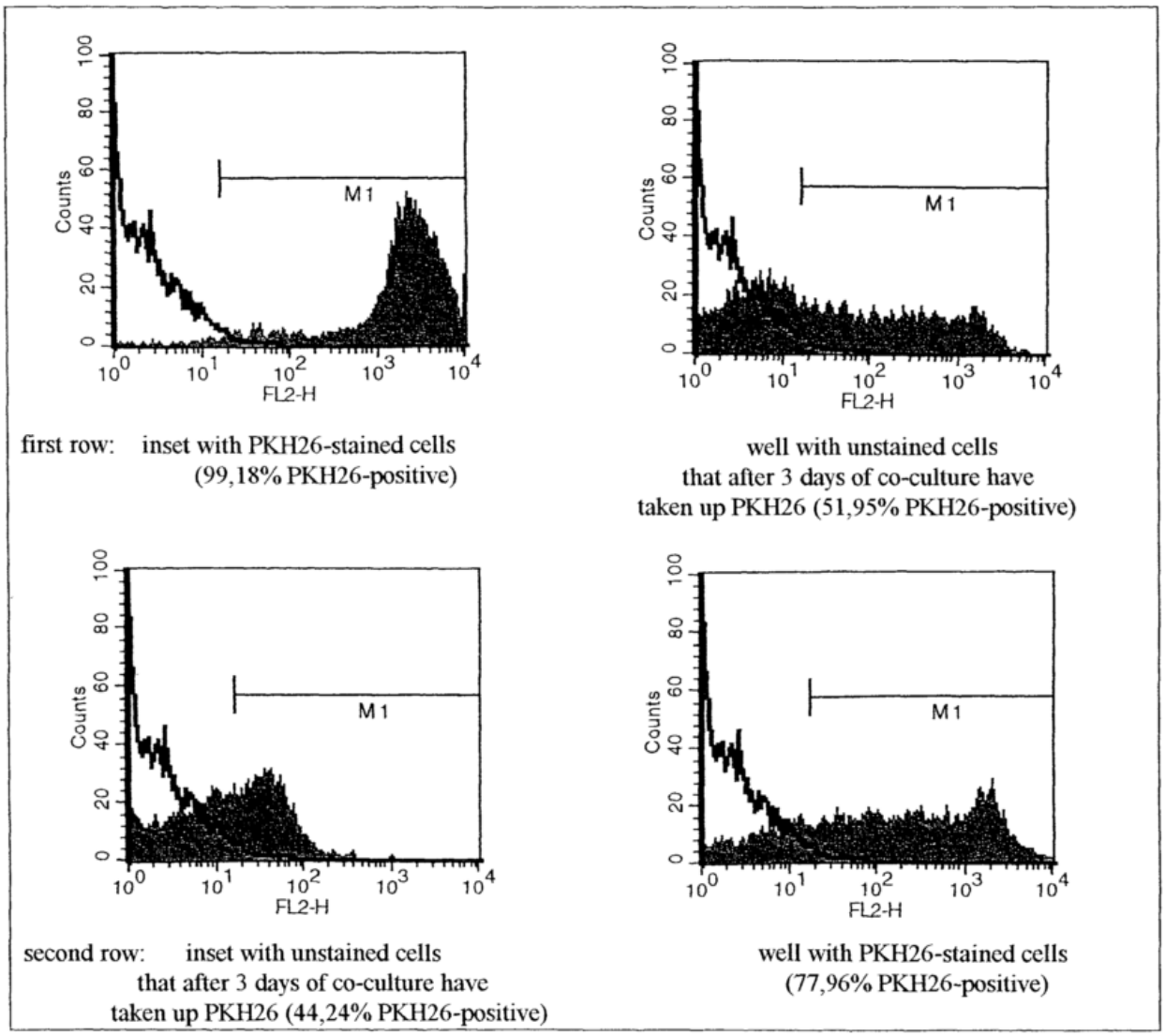


Figure 11: Transfer of PKH26 from stained to unstained dendritic cells separated by an inset with a polycarbonate membrane (pore size 3,0 µm). After 3 days of co-culture unstained dendritic cells, independently of the compartment in which they were plated, have become PKH26-positive (first row, right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

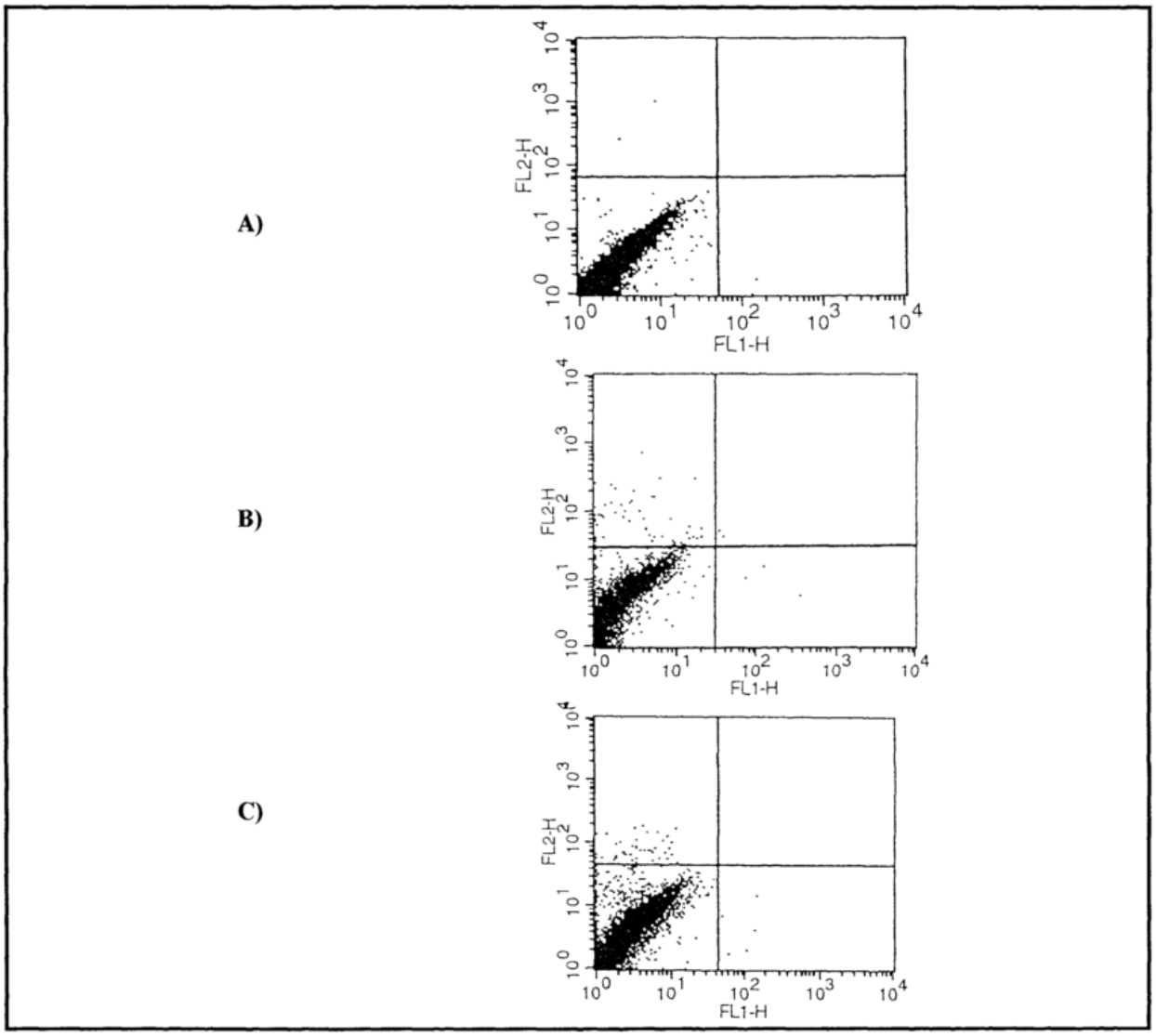


Figure 12: FACS analysis of bone marrow-derived dendritic cells incubated with sterile filtered supernatnat of PKH26-stained dendritic cells after 9 (**A**), 13 (**B**) and 15 days (**C**). Hardly any PKH26-positive cells (FL2-H) can be detected.

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.

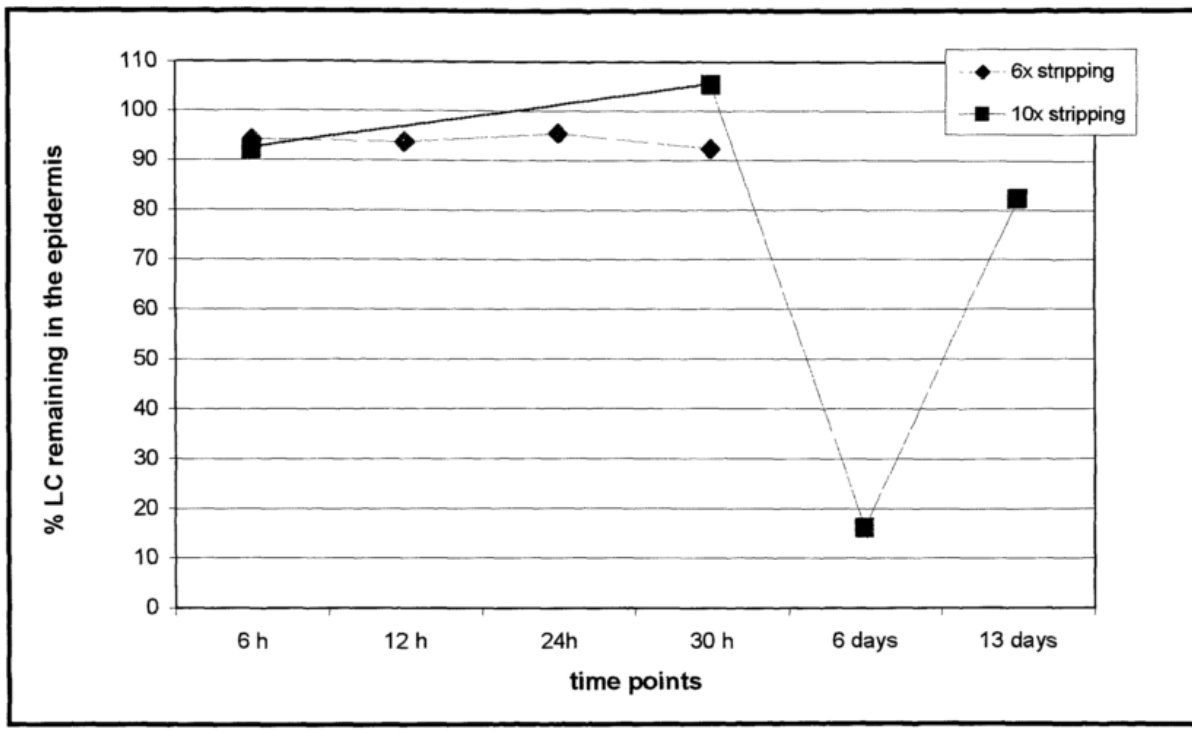


Figure 13: Emigration of Langerhans cells from the epidermis detected in a preliminary experiment. Two different intensities of tape stripping (6 strippings vs. 10 strippings) were tested for their effect on the emigration of Langerhans cells from the epidermis. Apparently only a higher number of strippings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

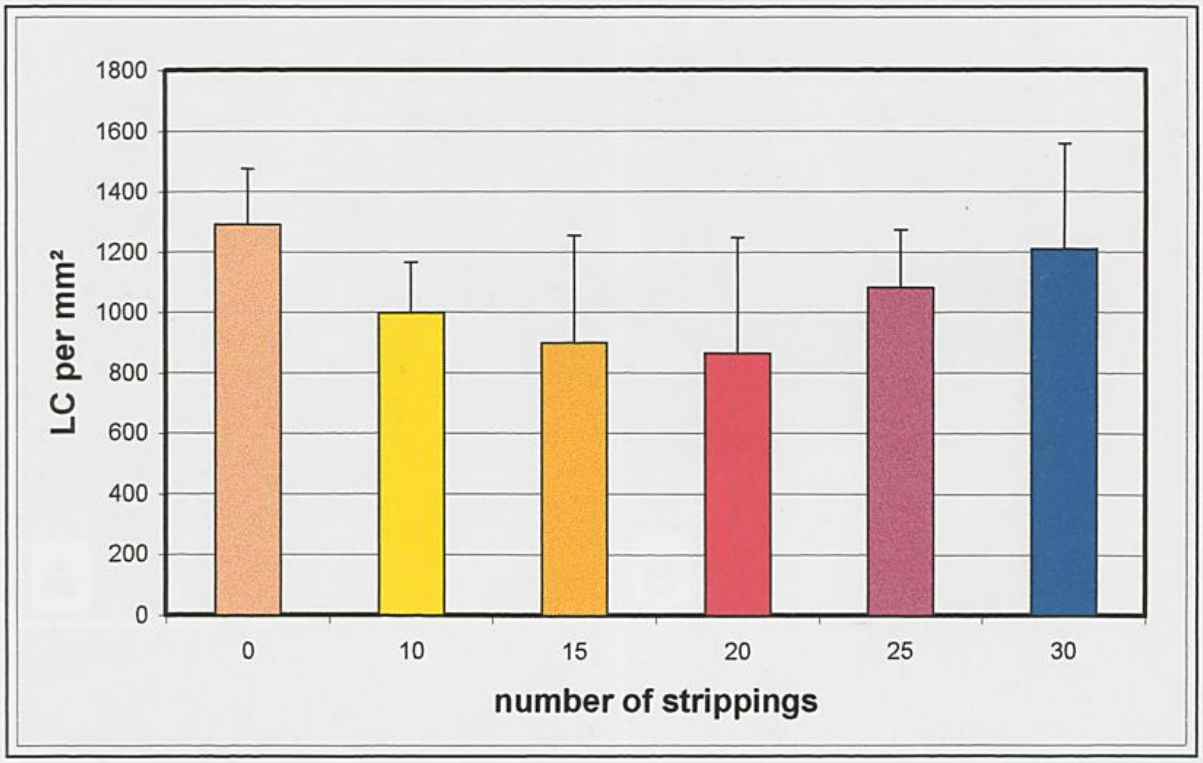


Figure 14: Density of Langerhans cells in the epidermis detected immediately after tape stripping. Even if the number of strippings is raised up to 30 Langerhans cell numbers are not substantially changed. Thereby it can be concluded that Langerhans cells are not pulled out of the epidermis.

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).

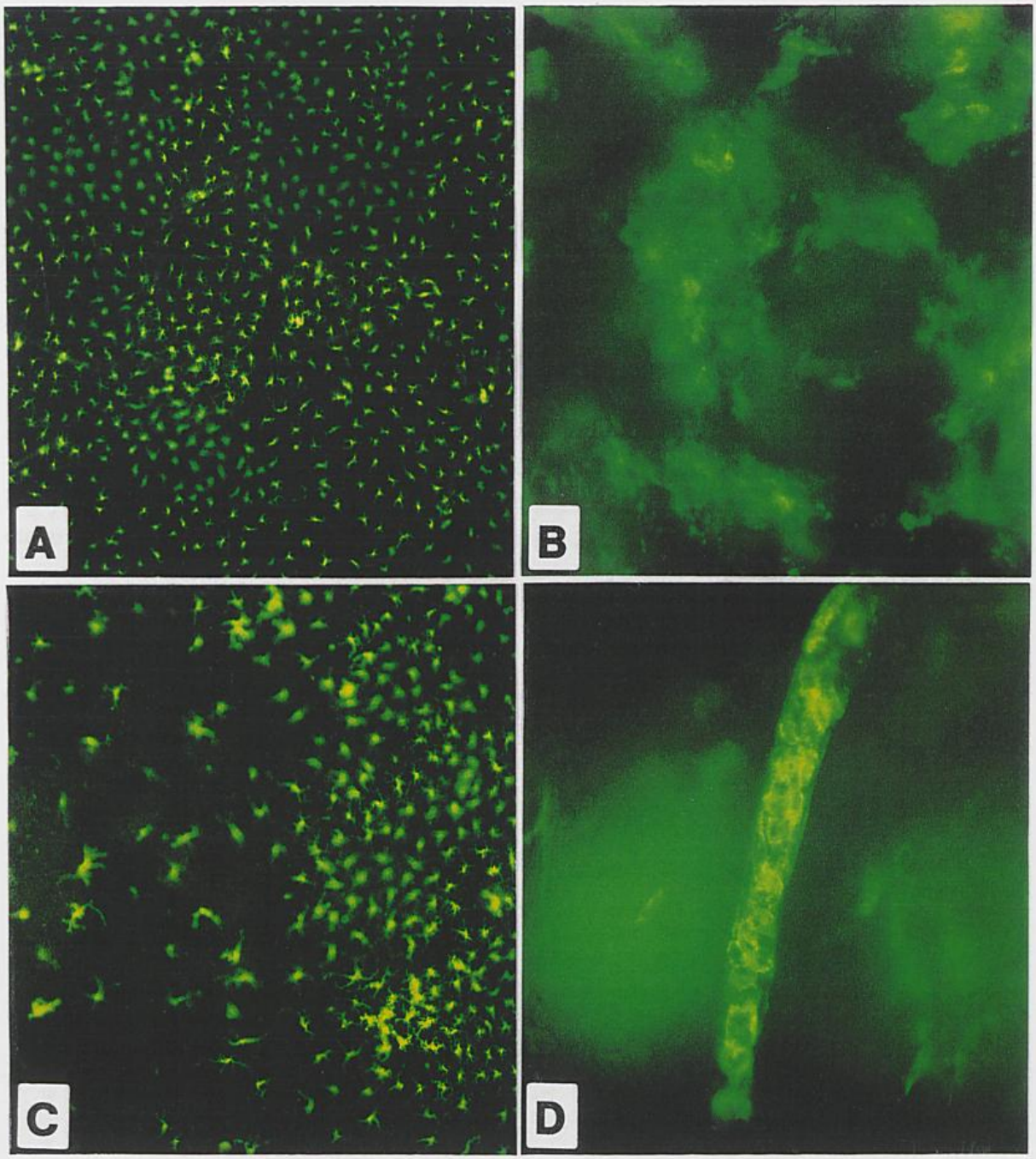


Figure 15: Overview of untreated epidermis (**A**, 100x) and untreated dermis (**B**, 400x). Epidermis 4 days after tape stripping, with visible patchy emigration (**C**, 100x). Dermis, 4 days after tape stripping, with a lymphatic vessel filled with emigrating Langerhans and dendritic cells, a so called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

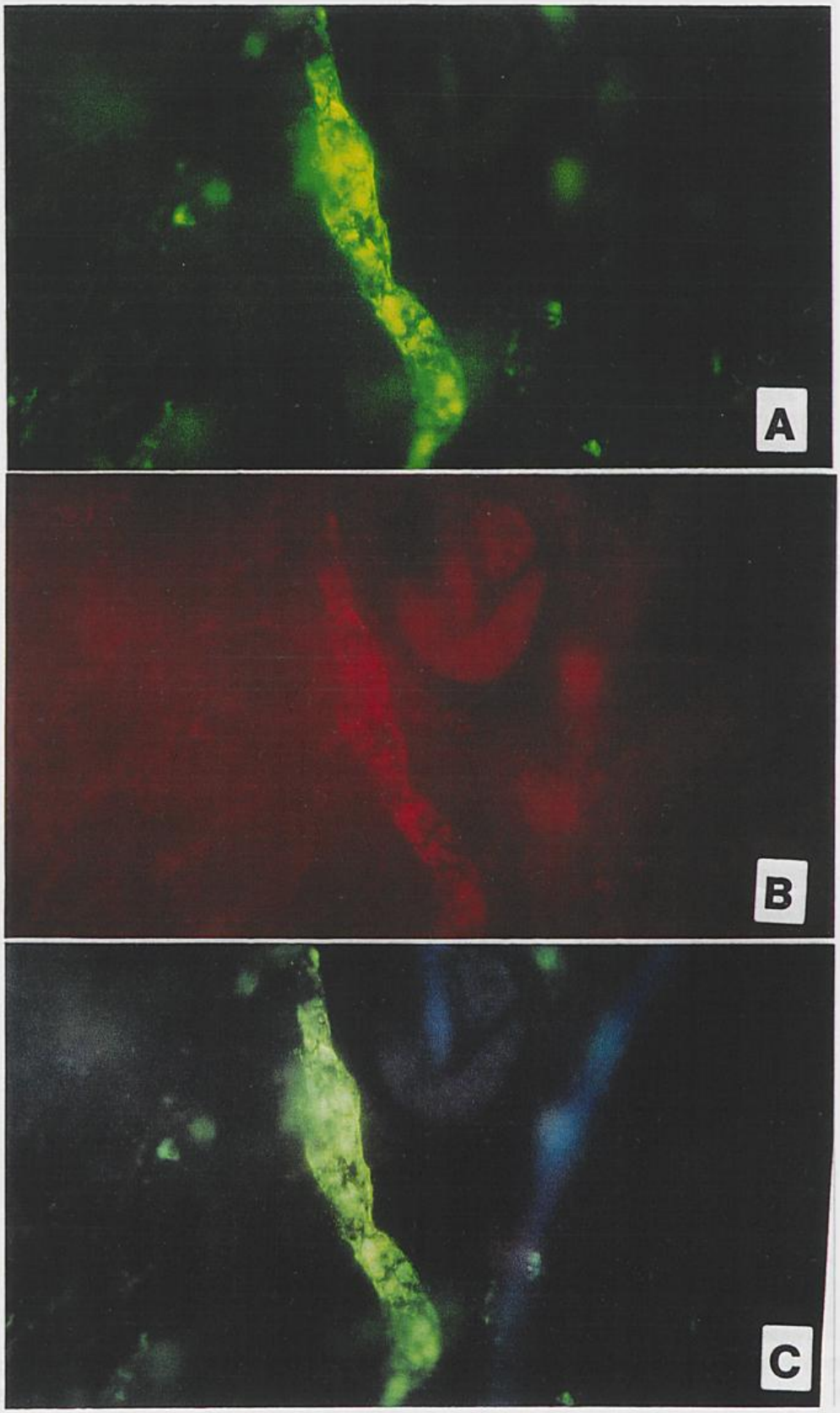


Figure 16: Maturing Langerhans and dendritic cells can be found emigrating the skin of tape stripped ears (4 days after stripping, 400x). Cells were stained for MHC class II (green fluorescence, **A**) and the costimulatory molecule CD86 (red fluorescence, **B**). **C**: double exposure.

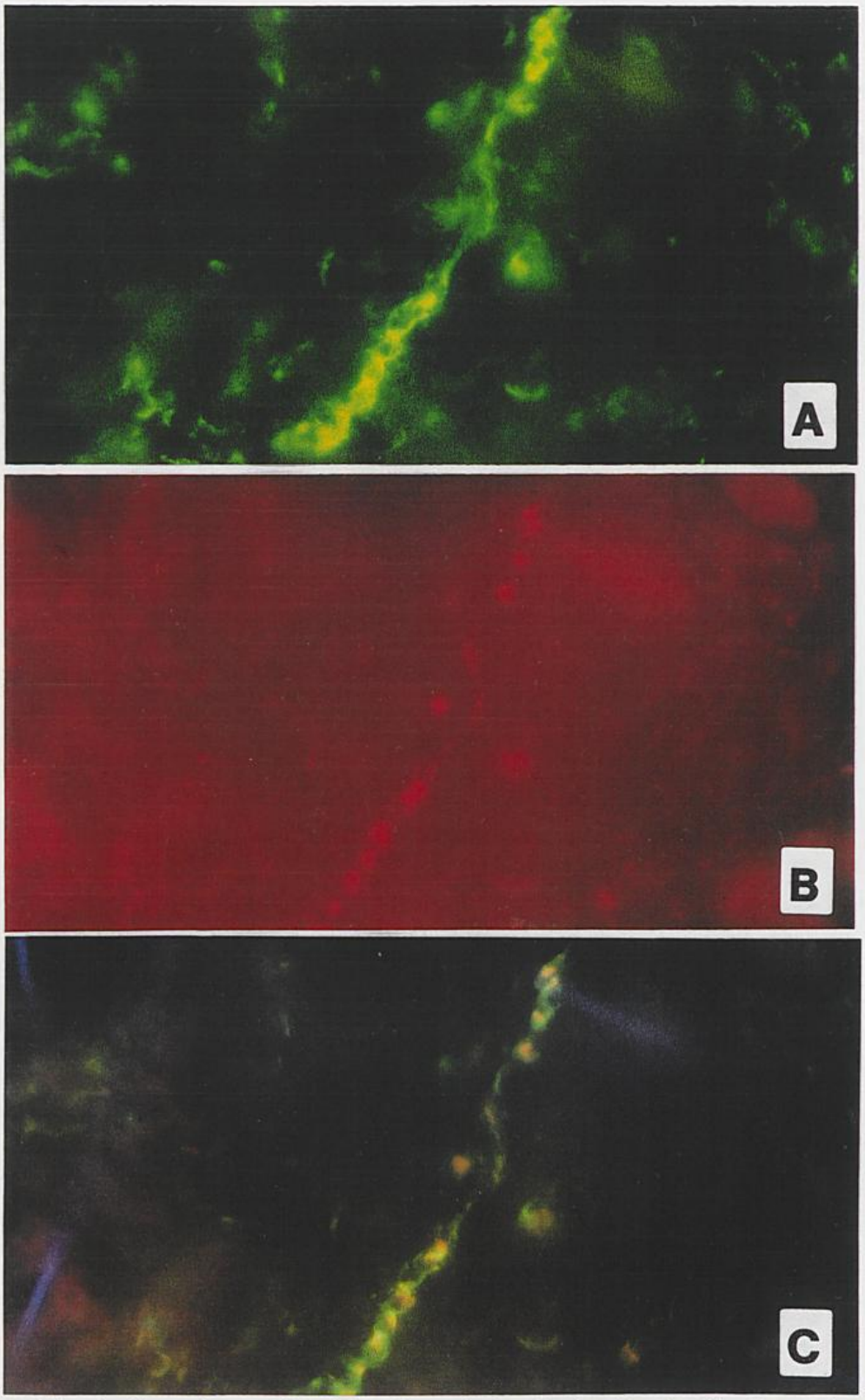


Figure 17: Maturing Langerhans and dendritic cells can be found emigrating the skin of tape stripped ears 6 days after stripping (400x). Cells were stained for MHC class II (green fluorescence, **A**) and an intracellular organelle typical for mature dendritic cells with the primary antibody 2A1 (red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

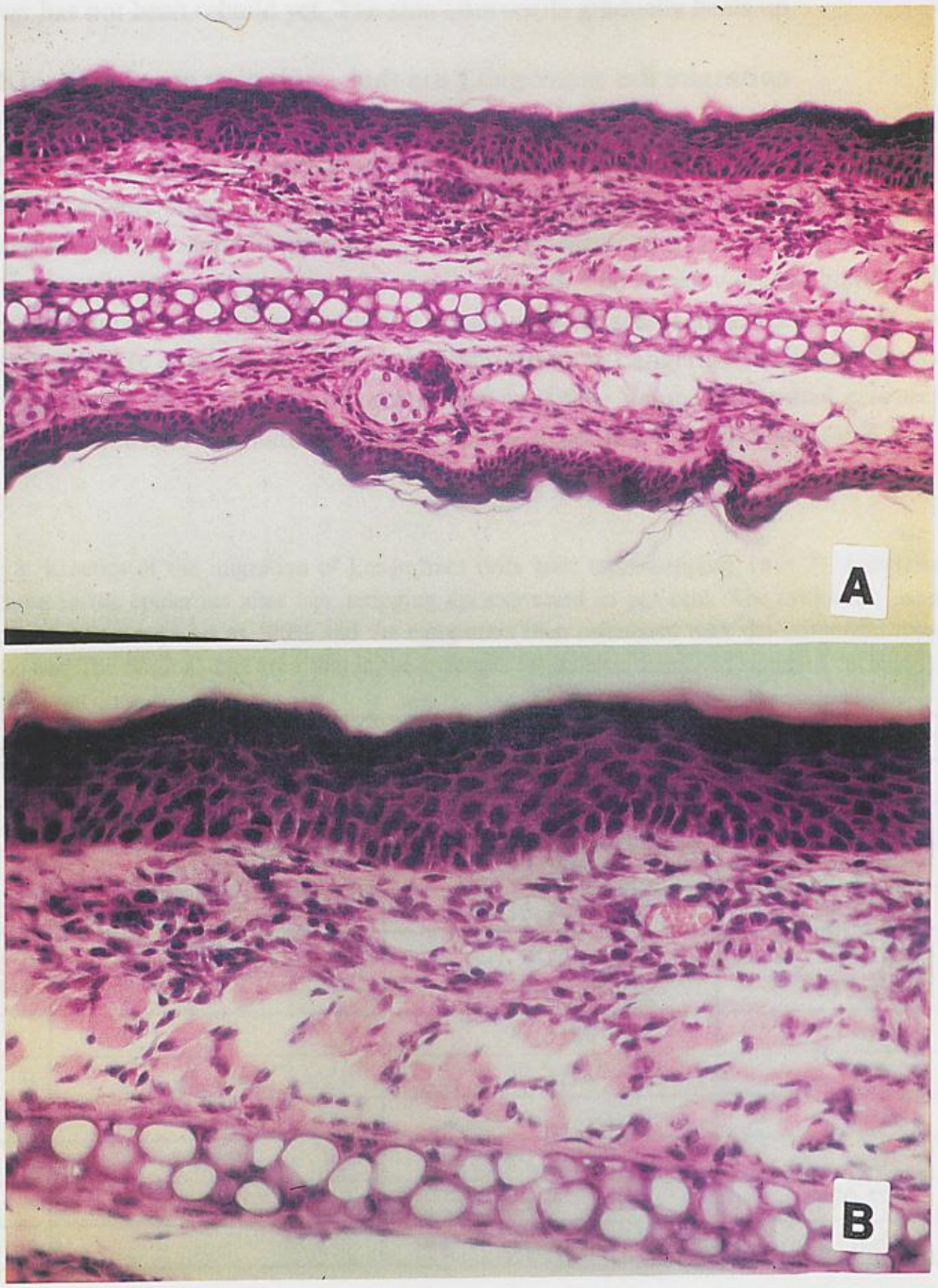


Figure 18: Routine H&E-stained sections of a mouse ear 4 days after tape stripping. **A)** The epidermis of the tape stripped dorsal half is swollen compared to the untreated ventral half (100x). **B)** Lymphocytes and macrophages infiltrate the moderately inflamed skin (400x).

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.

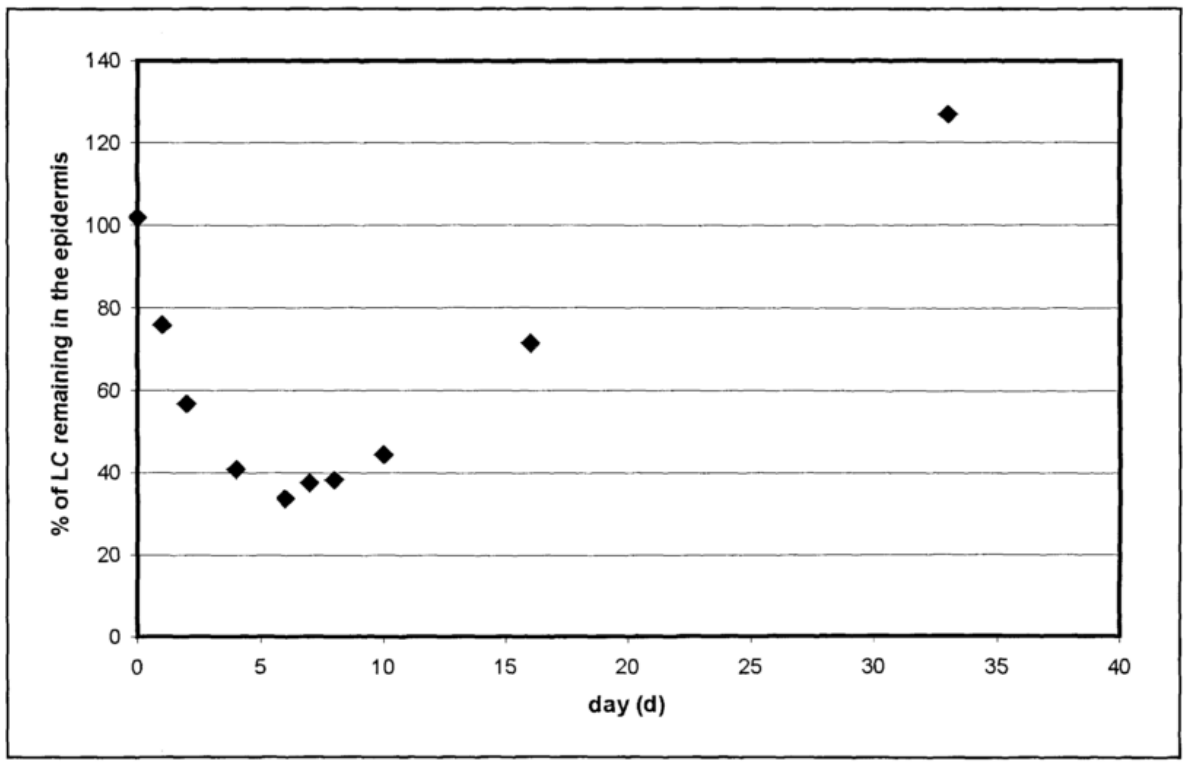


Figure 19: Time course of the migration of Langerhans cells after mechanical irritation of the skin by tape stripping (n = 3). The number of Langerhans cells remaining in the epidermis decreases continually after tape stripping and reaches a nadir on day 6 (33,74% LC still found in the epidermis). Afterwards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

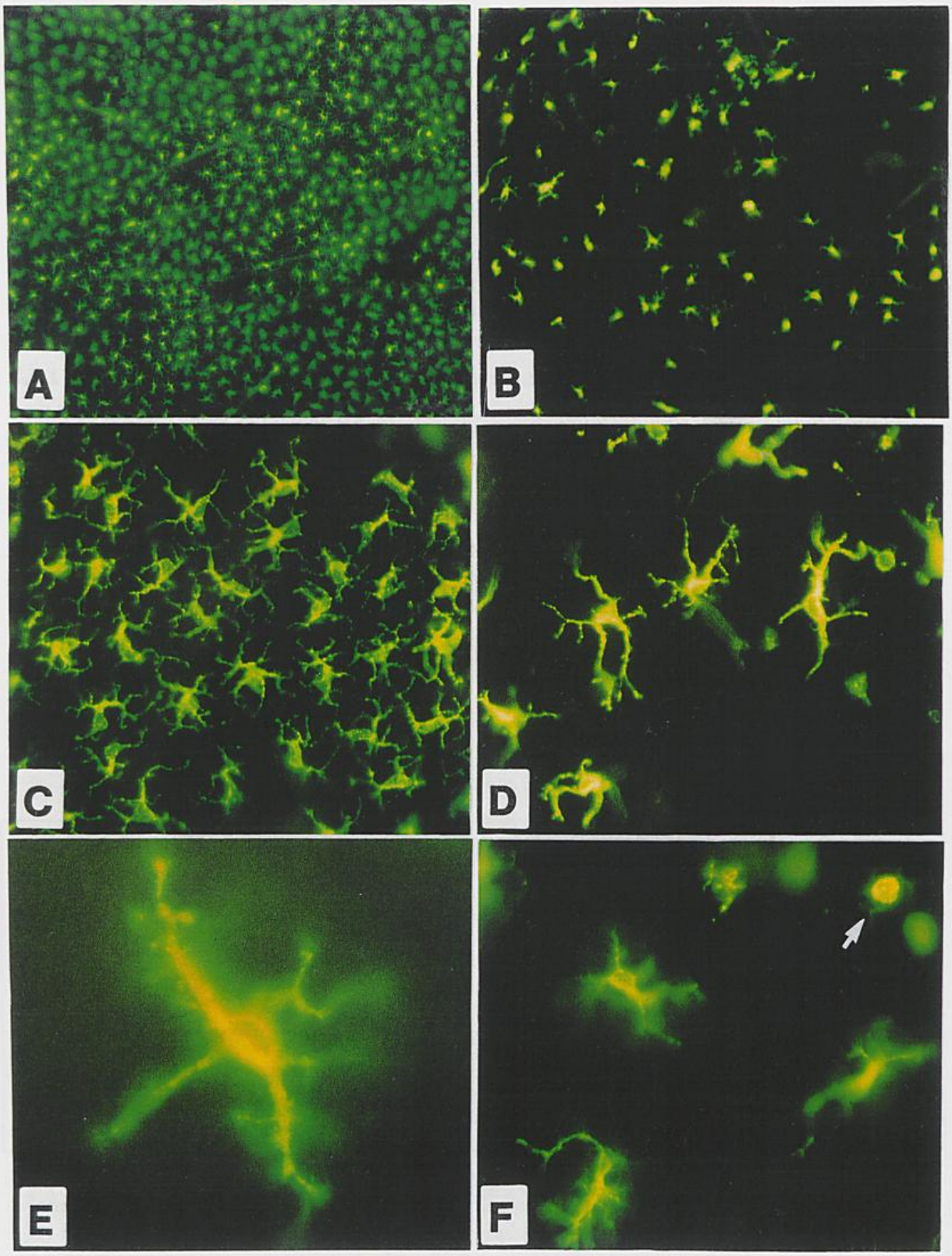


Figure 20: Comparison between untreated epidermis (**A**, **C**) and tape stripped epidermis (6 days after stripping, **B**, **D**, **E**, **F**). Langerhans cells in untreated epidermis seem to form a regular net of defence against foreign antigens (**A**, 100x; **C**, 400x). In tape stripped epidermis 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).

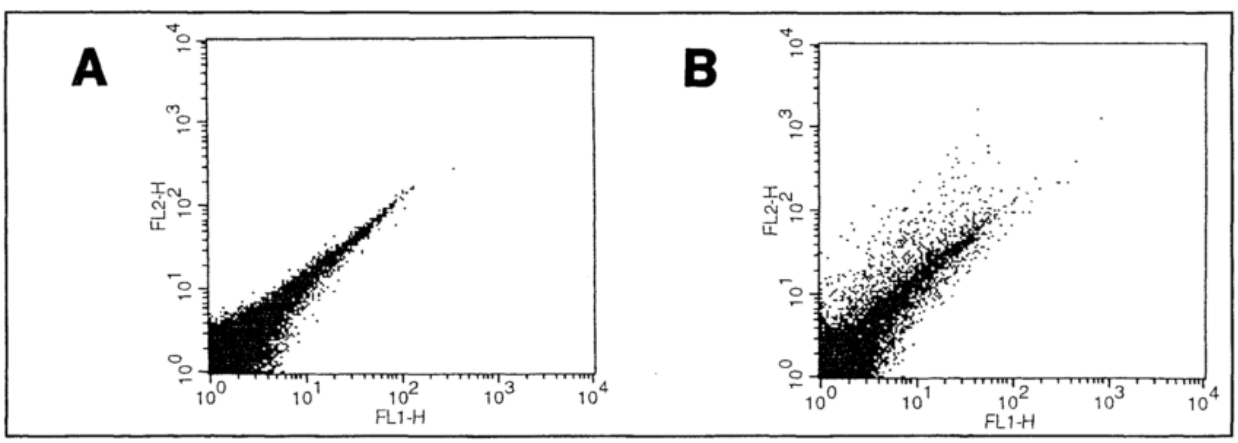


Figure 21: Flow cytometric analysis of single cell suspensions of lymphoid organ taken from mice injected intravenously with PKH26-stained dendritic cells. **A**) Suspension of spleen cells, negative control. **B**) Suspension of spleen cells 7 days after intravenous injection of PKH26-stained dendritic 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.

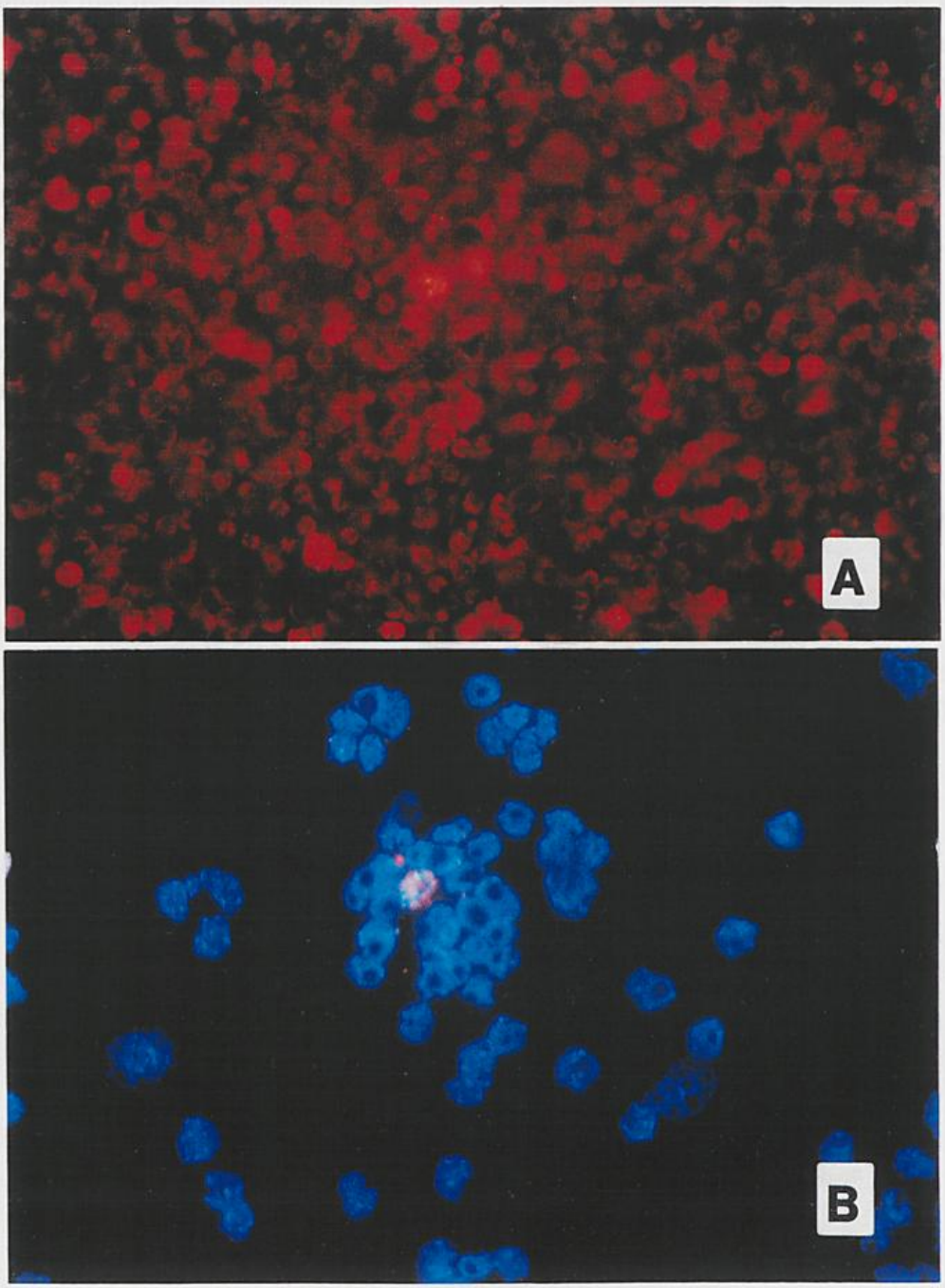


Figure 22: **A**) Cell suspension of bone marrow containing PKH26-stained immature dendritic cells injected 8 days before. Cells are as densely packed as possible (100x). **B**) Cytospin preparation of spleen cell suspension, showing one PKH26-positive cell (red fluorescence) amongst spleen cells stained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

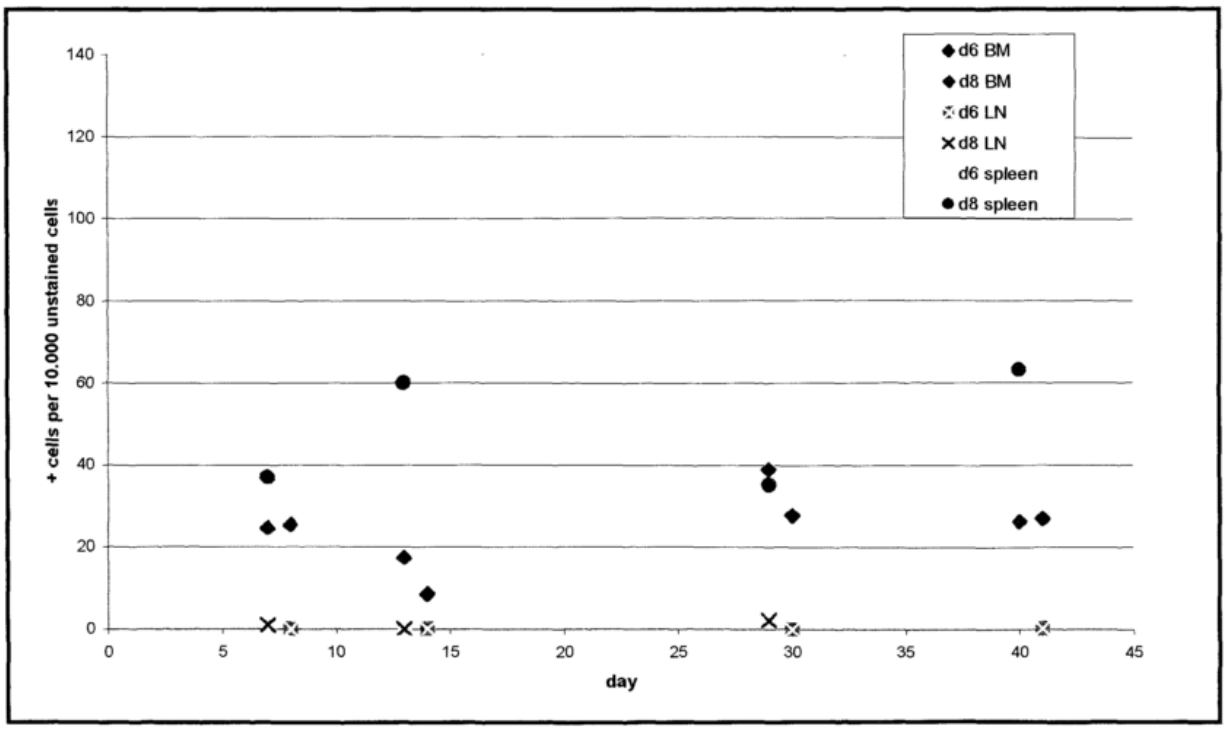


Figure 23: Time course for PKH26-positive cells found in single cell suspensions of lymph node (LN), bone marrow (BM) and spleen after intravenous injection of PKH26-stained immature (d6...6 days of standard culture) and intermediate (d8...8 days of standard culture) bone marrow-derived dendritic cells. 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.

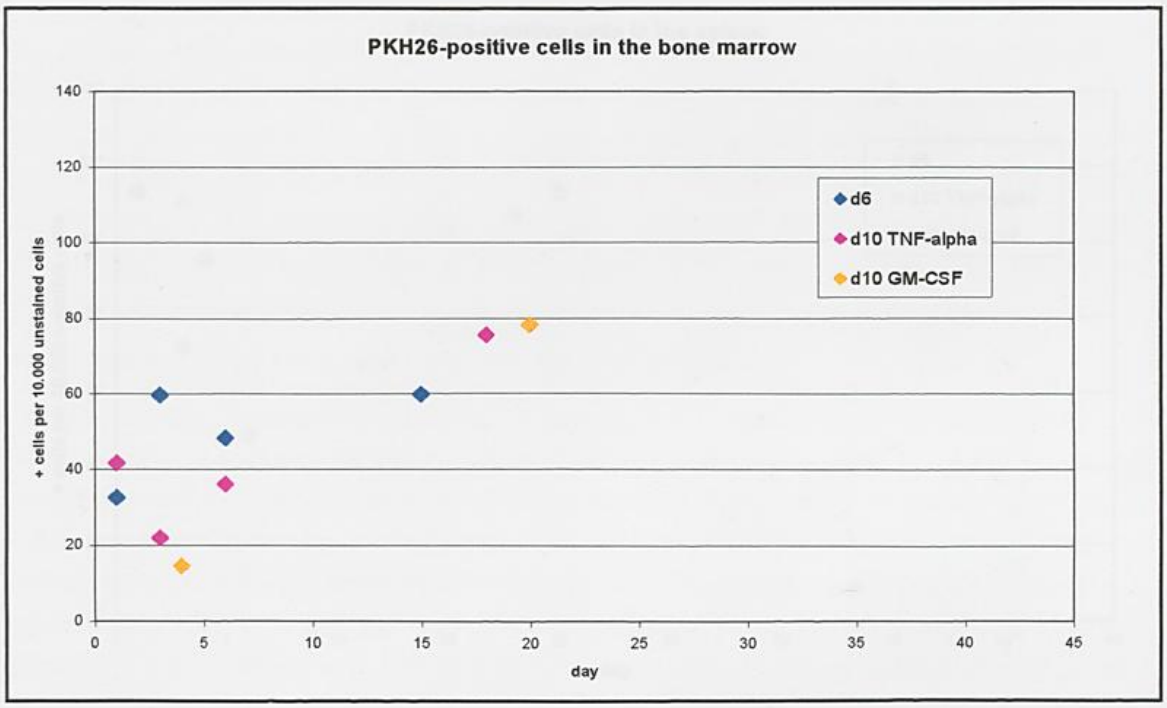
4.3.2 Comparison between dendritic cells enriched for mature and immature cells

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)

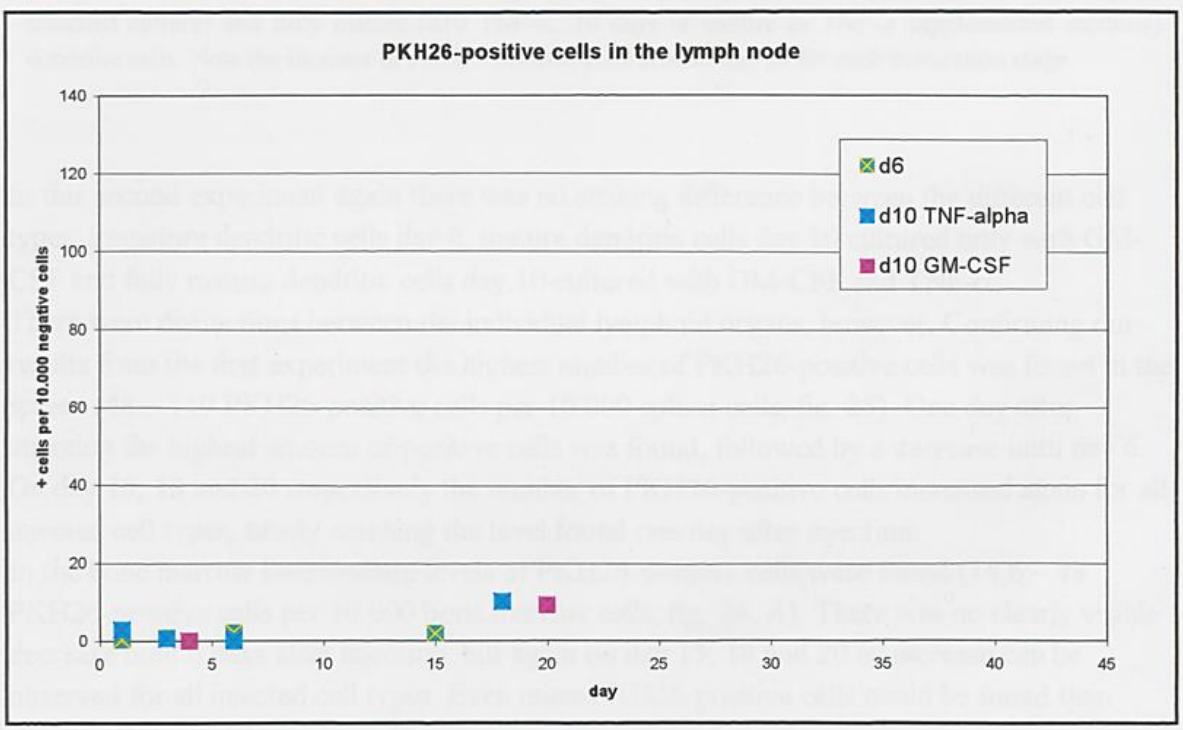


Figure 24: Time course for PKH26-positive cells found in single cell suspensions of bone marrow (**A**) and mesenterial lymph nodes (**B**) after intravenous injection of immature (d6...6 days of standard culture), mature (d10 GM-CSF...10 days of standard culture) and fully mature (d10 TNF-α...10 days 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.

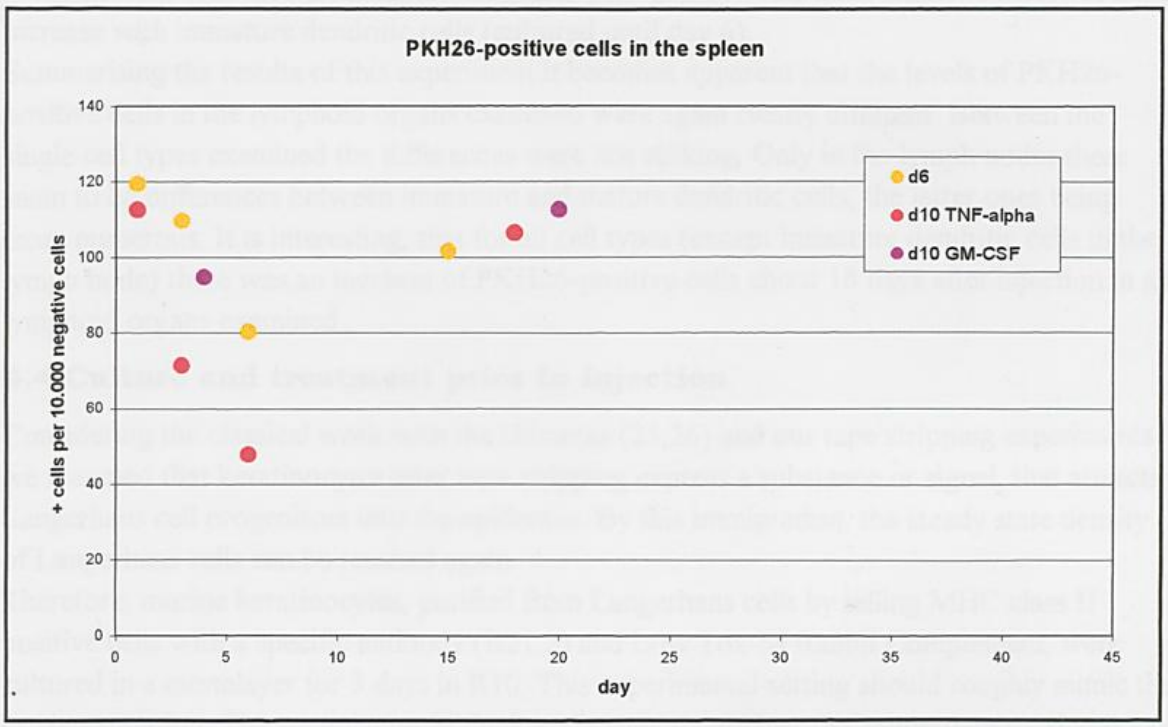


Figure 25: Time course for PKH26-positive cells found in single cell suspensions of the spleen after intravenous injection of immature (d6...6 days of standard culture), mature (d10 GM-CSF...10 days of standard culture) and fully mature (d10 TNF-α...10 days of culture in TNF-α supplemented medium) dendritic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

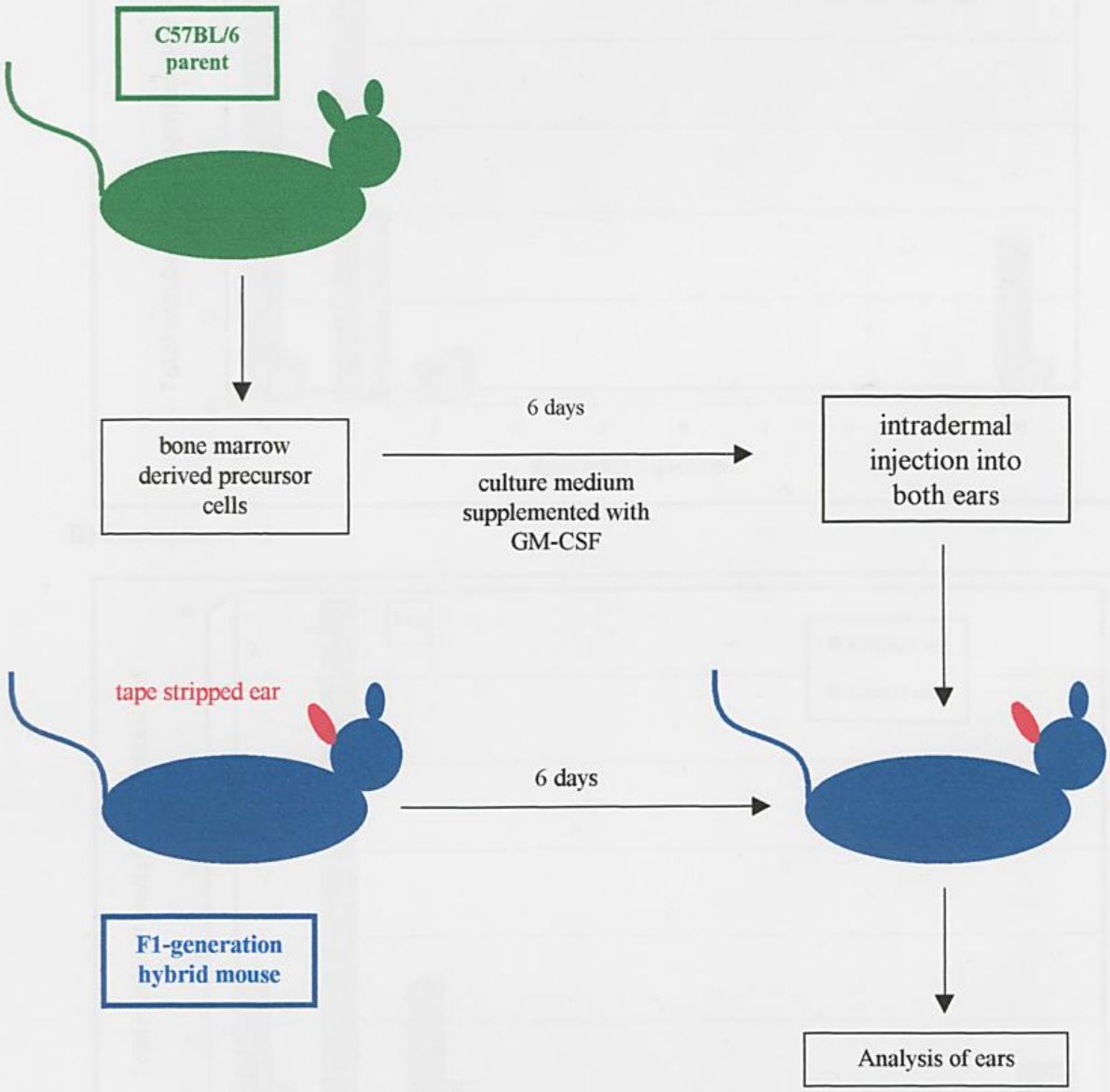
Considering the classical work with the chimeras (25,26) and our tape stripping experiments we assumed that keratinocytes after tape stripping express a substance or signal, that attracts Langerhans cell progenitors into the epidermis. By this immigration, the steady state density of Langerhans cells 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.

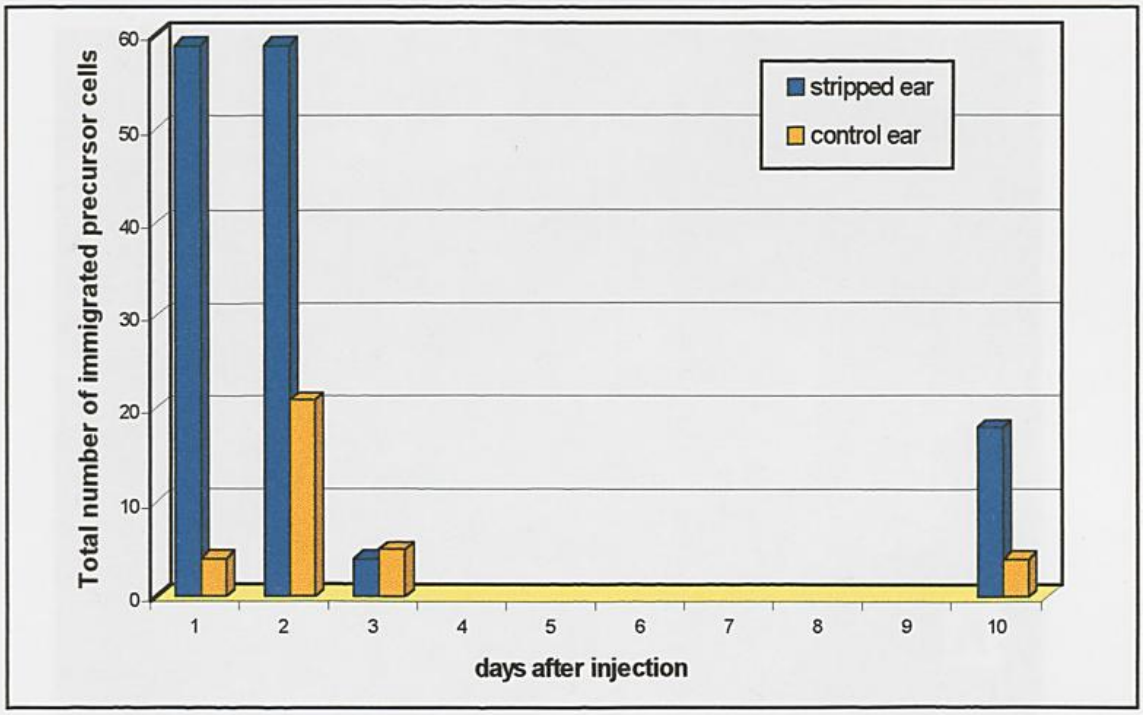
4.5 Immigration of intradermally injected bone marrow derived Langerhans cells into the epidermis

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

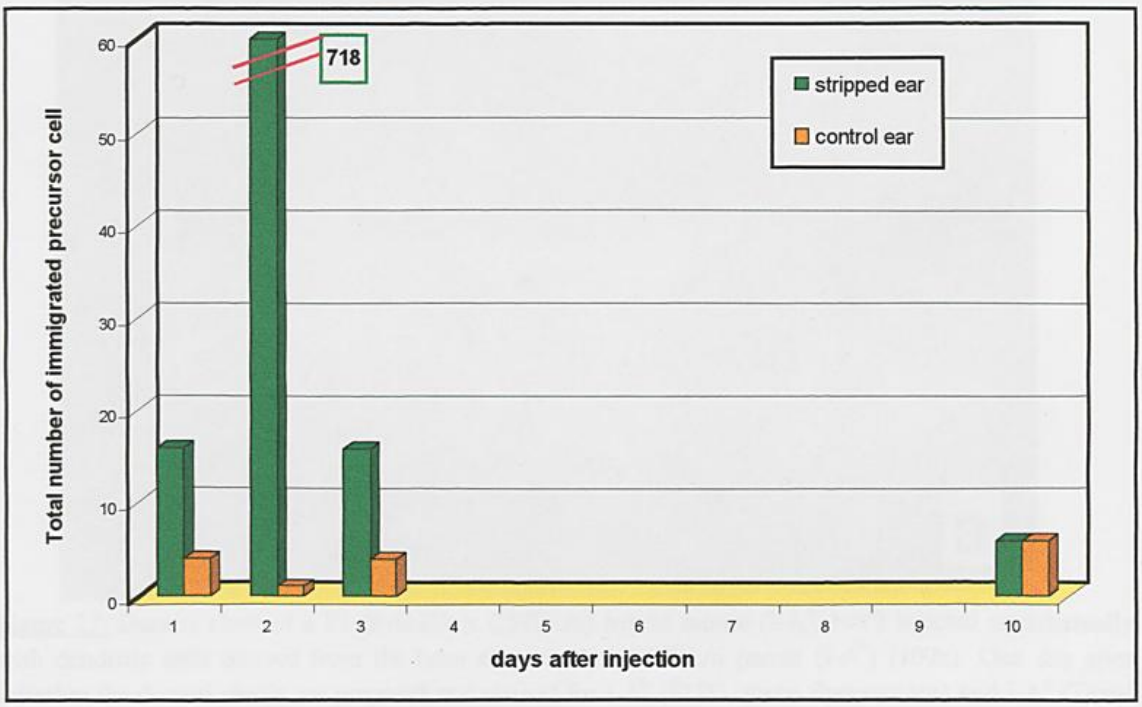


Figure 26: Time course of the immigration of Langerhans precursor cells into the epidermis. The left ear was treated with adhesive tape 6 days before intradermal injection of immature bone marrow-derived dendritic cells (stripped ear). The right ear of the same mouse remained untreated before injection (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).

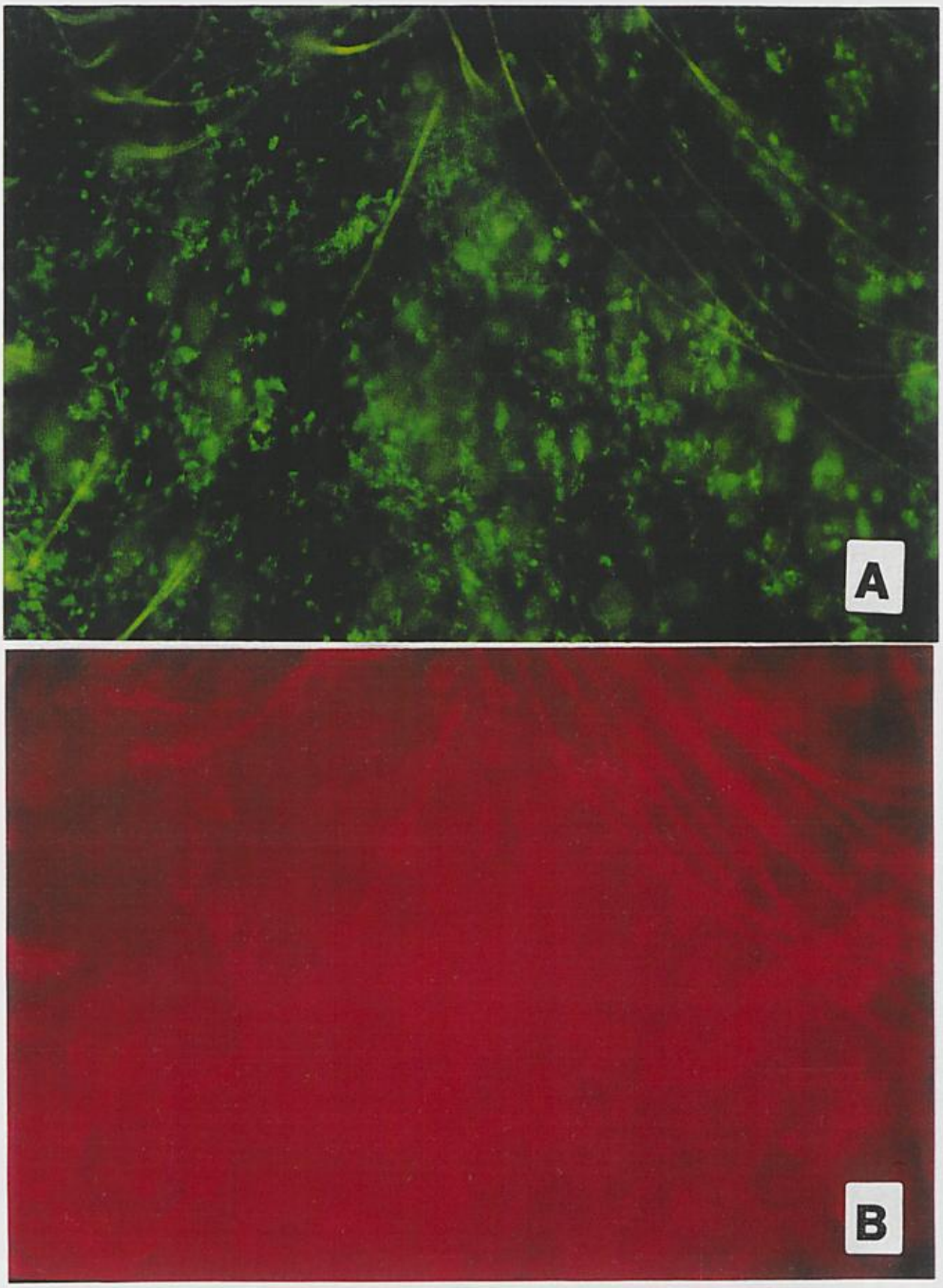


Figure 27: Dermal sheet of a F1 (BALB/c x C57BL/6) hybrid mouse (I-A^d, I-A^b) injected intradermally with dendritic cells derived from the bone marrow of a C57BL/6 parent (I-A^b) (100x). One day after injection the dermal sheets are prepared and stained for I-A^b (FITC, green fluorescence) and I-A^d (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.

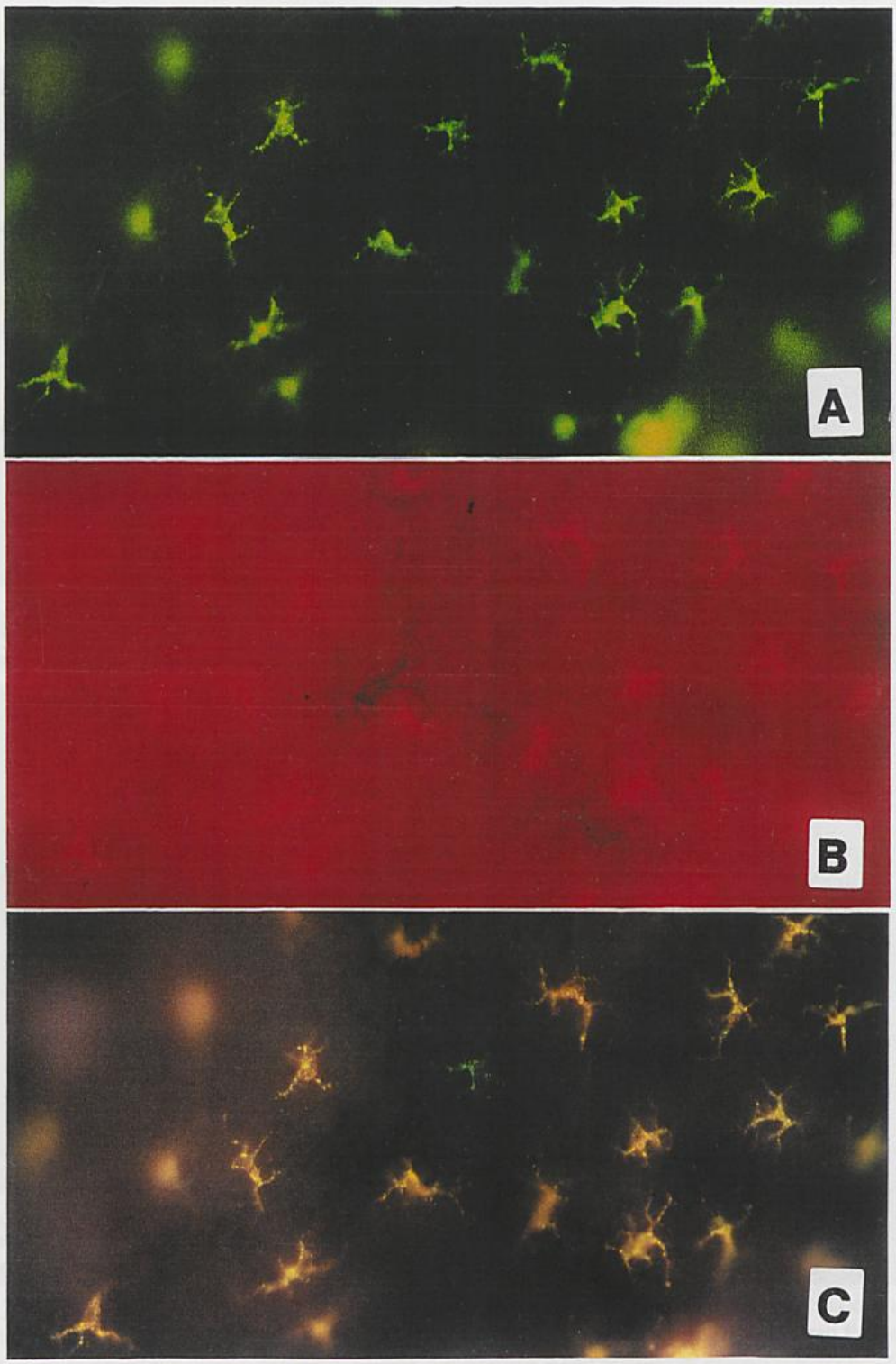


Figure 28: Single Langerhans cell immigrated into untreated epidermis 1 day after injection (400x). C57BL/6 immature dendritic cells (I-A^b) were injected intradermally into F1 (BALB/c x C57BL/6) hybrid mice (I-A^b, I-A^d). The immigrated Langerhans cell, focused in the centre, is positive for I-A^b (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**).

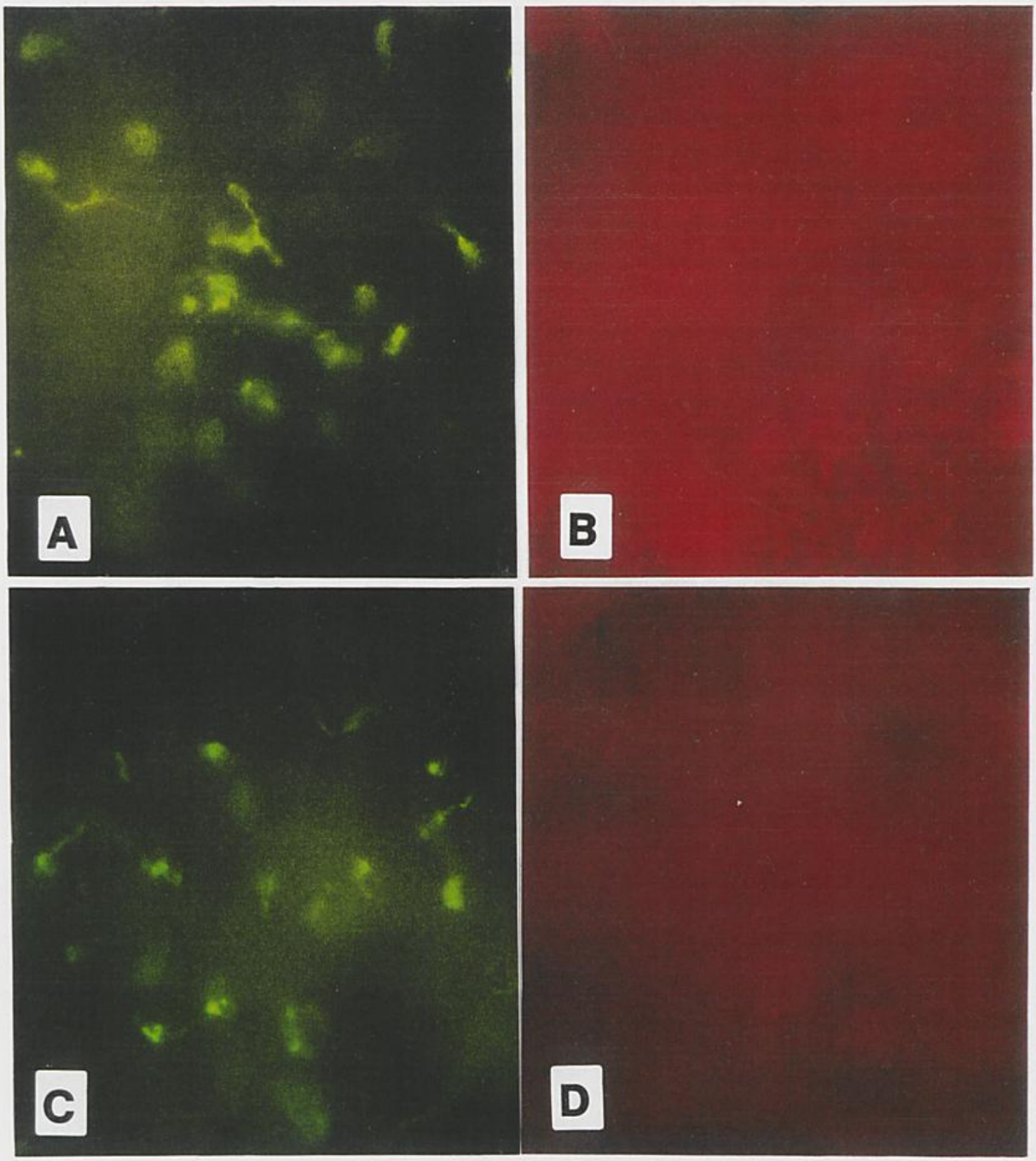


Figure 29: Groups of Langerhans cells immigrated into tape stripped epidermis 2 days after injection (400x). The staining procedure is detailed in the legend of figure (I-A^b...green fluorescence, I-A^d...red fluorescence). Groups of immigrated I-A^{b+}/I-A^{d-} Langerhans cells are positive when elicited 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.