Einwanderung von Langerhanszellvorläufern in die Epidermis: Entwicklung eines Modellsystems

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3. Materials and methods

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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).

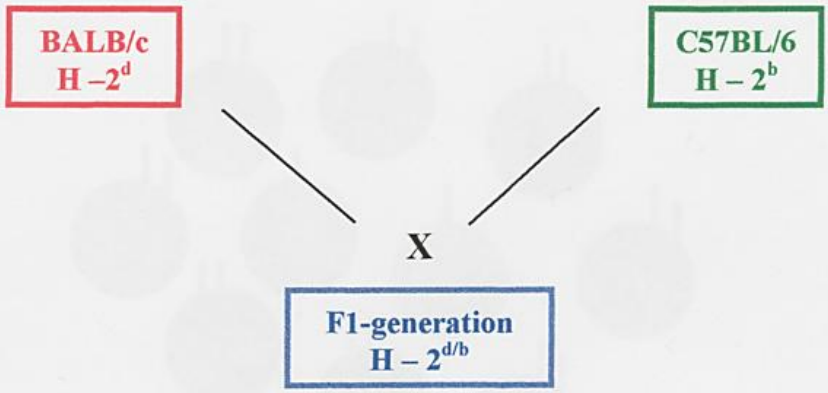


Figure 1: Concept and experimental setting for the intradermal injection of bone marrow derived dendritic cells. Following the Mendel rules for genetics the F1 generation possesses both alleles for the MHC class II haplotype.

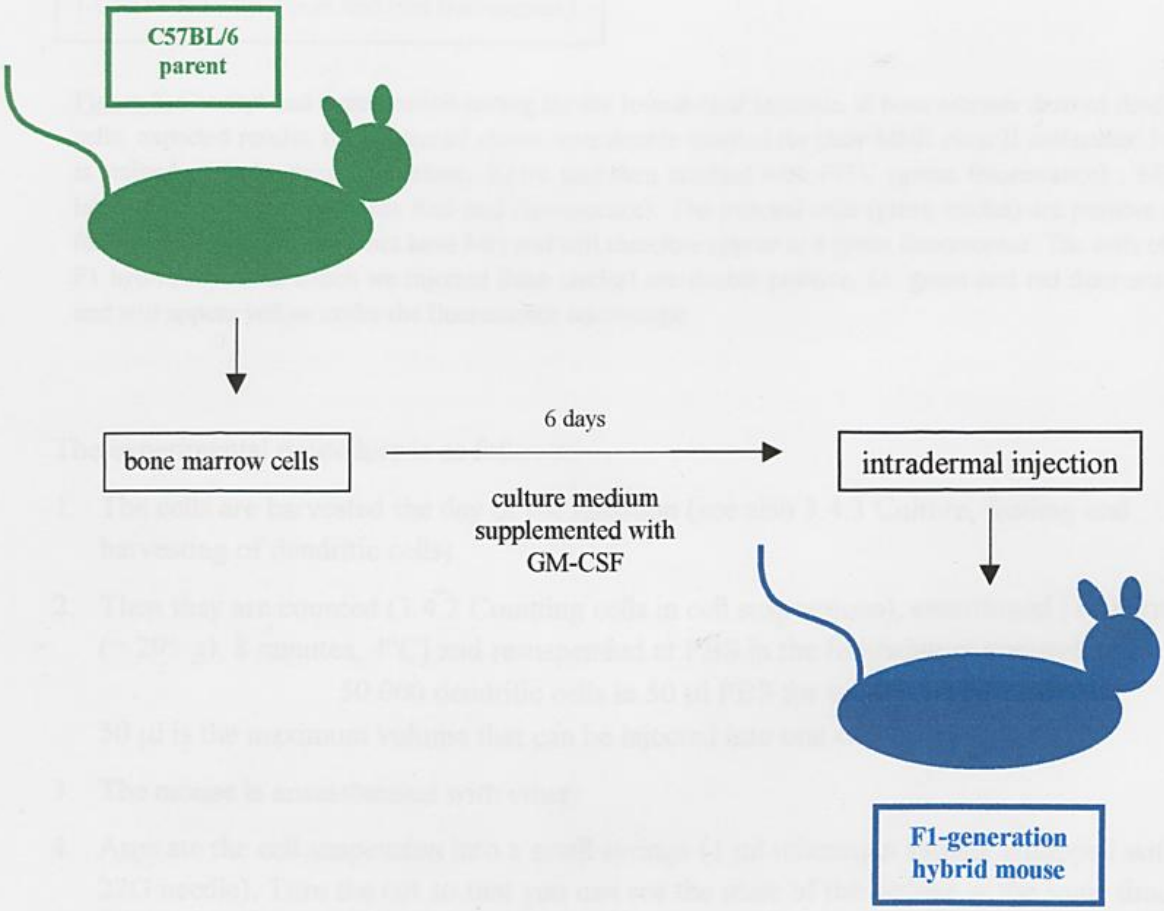
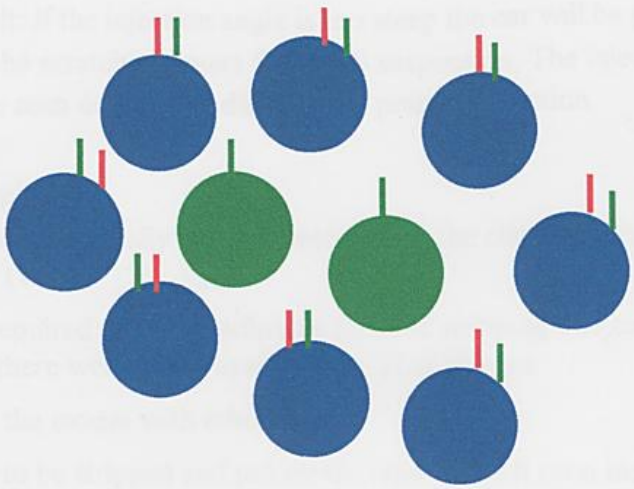


Figure 2: Experimental setting for the intradermal injection of bone marrow derived dendritic cells. The bone marrow of a C57BL/6 parent mouse is cultured for 6 days in our routine fashion and is then injected in the dermis of a F1-generation hybrid mouse. The ears of the F1-generation hybrid mouse were 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.