Chapter 5

Ultraviolet Radiation-Induced Immunosuppression: Induction of Regulatory T Cells

A. Bruhs and T. Schwarz

Abstract

Solar/ultraviolet (UV) radiation exerts a variety of biological effects, including suppression of the immune system. UV-induced immunosuppression is induced by suberythemogenic/physiological UV doses, and it affects primarily T-cell driven immune reactions. Another characteristic feature of UV-induced immunosuppression is its antigen-specificity. This is due to the induction of T cells with suppressive features, called regulatory T cells. Since UV-induced regulatory T cells may harbor therapeutic potential phenotypic and functional characterization of these cells is ongoing. Most of these studies have been performed in the murine model of contact hypersensitivity. In this protocol we describe a method for the UV-induced suppression of the induction of contact hypersensitivity and the adoptive transfer of immune response.

Key words Contact hypersensitivity, Immunosuppression, Regulatory T cells, Tolerance, Ultraviolet radiation

1 Introduction

It is well known for decades that ultraviolet (UV) radiation in particular the mid wave range (UVB, 290–320 nm) suppresses the immune system. UV-induced immunosuppression exerts unique features: (1) It is caused by low suberythemogenic doses. (2) It is antigen-specific and thus differs from immunosuppression caused by drugs. (3) Primarily T-cell-driven reactions are affected [1]. Most of the relevant studies have been performed in the model of contact hypersensitivity (CHS) in mice. Topical application of contact allergens (haptens) on the skin results in obligatory sensitization. Upon successful sensitization a specific ear swelling response can be induced by applying much lower concentrations of the same hapten on one ear (ear challenge; Fig. 1a). This concentration does not induce swelling in non-sensitized mice, thus excluding a toxic reaction. In addition, other unrelated haptens do not induce ear challenge in sensitized mice, indicating the antigen-specificity of this immune response.

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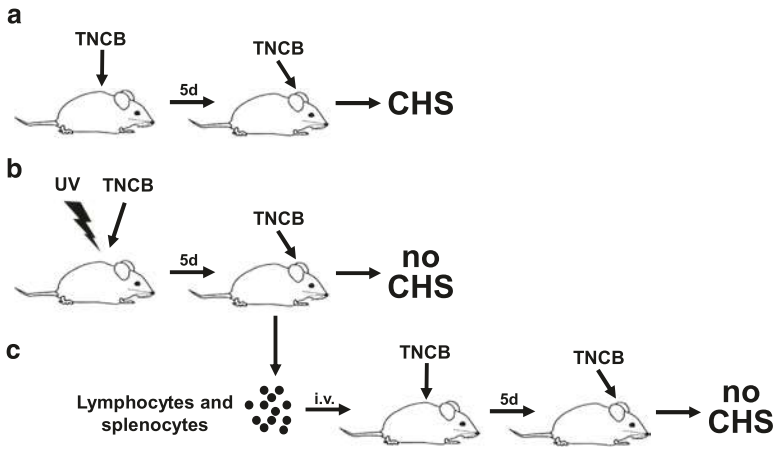

Fig. 1 UV radiation induces antigen-specific immunotolerance. (**a**) Epicutaneous application of a contact allergen (e.g., 2,4,6-trinitrochlorobenzene [TNCB]) on the back of the mice followed by a challenge 5 days later on the left ear results in contact hypersensitivity (CHS) indicated by an ear swelling response. (**b**) In contrast, application of the contact allergen onto UV-exposed skin does not result in CHS. (**c**) Lymphocytes and splenocytes obtained from UV-tolerized mice suppress sensitization against the same hapten in recipient mice after adoptive transfer

Application of haptens onto skin which has been exposed to suberythemogenic doses of UVB radiation does not result in sensitization [2]. Since irradiation and hapten application take place in the same area, this phenomenon is called local immunosuppression. More importantly, application of haptens onto UV-exposed skin results in long term immunosuppression since these mice cannot be resensitized at later time points even without being further UV exposed. However, all other immune responses are unaffected, indicating the antigen specificity of UVR-induced immunosuppression.

Antigen specificity is due to the induction of antigen-specific regulatory T cells (Treg) [3] Injection of T cells obtained from UV- and hapten-treated mice into naïve syngeneic mice renders the recipients resistant to sensitization (adoptive transfer; Fig. 1b). Again suppression after adoptive transfer is antigen-specific since all other immune responses against unrelated haptens are not affected in the recipient mice [3]. Utilizing adoptive transfer experiments it was shown that UV-induced Treg belong to the $CD4^+CD25^+$ subtype and express Foxp3, CTLA-4, GITR and neuropilin [4–7]. Upon antigen-specific stimulation UV-induced Treg release interleukin-10 which is crucially involved in UV-induced immunosuppression [5, 8].

Phototherapy is utilized for various inflammatory dermatoses which equally well respond to immunosuppressive drugs [9]. Hence, one relevant mode of action of phototherapy may be

immunosuppression. In contrast to immunosuppressive drugs, phototherapy is not associated with the typical long term side effects including superinfections. This might be due to the antigen-specificity caused by Treg. Hence studies are ongoing to further characterize UV-induced Treg phenotypically and functionally with the ultimate aim to utilize these cells in a therapeutic setting but not only for dermatoses but also for other inflammatory and (auto)immune diseases.

2 Materials

2.1 UV-Irradiation of Mice

1. Adult female mice (6–7 weeks old) that have been bred and housed under specific and pathogen-free conditions (*see* **Note 1**).
2. Shaving device (e.g., Oster A-2).
3. UV-source (e.g., TL12 fluorescent lamps, Philips, Eindhoven, The Netherlands).
4. Stopwatch.

2.2 Induction of Contact Hypersensitivity

1. Adult female mice (6–7 weeks old) that have been bred and housed under specific and pathogen-free conditions.
2. 1% (w/v) 2,4,6-trinitrochlorobenzene (TNCB) in 4:1 acetone–olive oil (*see* **Note 2**).
3. 0.5% (w/v) TNCB in 4:1 acetone–olive oil (*see* **Note 2**).
4. Spring loaded micrometer.

2.3 Isolation of Lymphocytes and Splenocytes

1. 70% (v/v) ethanol.
2. Sterile surgical instruments including forceps and pairs of scissors (*see* **Note 3**).
3. Dissecting board and needles.
4. RPMI complete medium: RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine and 100 U/ml Penicillin/Streptomycin.
5. Tubes of appropriate size for lymph nodes and spleen.
6. Metal cell strainers (mesh size 100 μm).
7. 60 mm Petri dishes.
8. 5 ml syringes.
9. Disposable nylon cell strainers (mesh size 70 μm).
10. Erythrocyte lysing buffer: 150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM NaEDTA, pH 7.5 (*see* **Note 4**).
11. Hemocytometer.

2.3.1 Separation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T Cells from Lymphocytes and Splenocytes

1. Magnetic cell sorter (e.g., autoMACS® Pro Separator, Miltenyi Biotec, or equivalent) including required equipment as columns and buffers for the separation procedure.
2. $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse (Miltenyi Biotec, or equivalent).
3. MACS running buffer: DPBS supplemented with 2 mM EDTA and 0.5 % (w/v) BSA.
4. Allophycocyanin (APC)-conjugated CD4 antibody for flow cytometry.
5. APC-conjugated Ig antibody for flow cytometry.
6. Phycoerythrin (PE)-conjugated Ig antibody for flow cytometry.
7. Sterile FACS staining buffer: DPBS supplemented with 1 % (w/v) BSA.
8. Sterile FACS blocking buffer: DPBS supplemented with 3 % (w/v) BSA.
9. Flow cytometer and appropriate analysis software.

2.4 Adoptive Transfer of Immune Response

1. 70 % (v/v) ethanol.
2. 0.9 % sodium chloride injection solution.
3. 1 ml syringes and needles (30G × ½ inch).
4. Red heat lamp.
5. Mouse restrainer usable for tail vein injection.
6. Adult female mice (6–7 weeks old) that have been bred and housed under specific and pathogen-free conditions as recipients.

3 Methods

3.1 UV-Induced Suppression of the Induction of CHS in Mice

The contact hypersensitivity (CHS) reaction is a common in vivo assay to study cell-mediated host immune responses to contact allergens. The vast majority of contact allergens used in this model are chemically reactive substances of low molecular weight which have to bind to proteins of the host to exert their antigenic properties. Thus, these substances are also called haptens. Topical application of haptens onto razor-shaved skin of mice results in sensitization in almost all animals treated (Fig. 1a). In contrast, if the hapten is painted on skin that was immediately before exposed to rather low doses of UVB radiation, CHS is not induced (Fig. 1b).

1. On the first day of UV-treatment a large area of the back of the animals is shaved thoroughly (*see* **Note 5**).
2. The shaved backs are exposed to UV radiation (*see* **Note 6**) delivered from a bank of four TL12 fluorescent lamps (Philips,

Eindhoven, The Netherlands), which emit most of their energy within the UVB range (290–320 nm) with an emission peak at 313 nm. Mice (*see* **Note 7**) are exposed to UV daily for 4 consecutive days (1000 J/m^2/exposure).

3. *Sensitization phase:* 24 h after the last UV exposure mice are sensitized by the epicutaneous application of 50 µl of 1% TNCB solution to the surface of the irradiated area.
4. *Elicitation phase:* 5 days after sensitization each side of the left ear is treated with 10 µl of 0.5% TNCB solution (20 µl total). The vehicle acetone–olive oil is applied to the right ear. Naïve mice that were not exposed to the sensitizing dose of TNCB (**step 3**) serve as negative controls to exclude nonspecific ear swelling induced by the irritating effects of the chemical.
5. After 24 h ear thickness is measured with a spring-loaded micrometer (*see* **Note 8**). CHS response is determined as the amount of swelling of the hapten-challenged ear compared with the thickness of the vehicle-treated ear in sensitized animals and is expressed as centimeters $\times 10^{-3}$ (mean ± SD).

3.2 Adoptive Transfer of Immune Response

UV-induced tolerance can be adoptively transferred into naïve recipients via injection of T cells. As described in several publications [10–12] lymphocytes and splenocytes obtained from UV- and hapten-treated mice are sufficient to suppress immune responses in recipient mice upon adoptive transfer (Fig. 1c). Isolation and injection of enriched CD4$^+$CD25$^+$ Treg from bulk cells will increase the suppressive efficacy in recipients (*see* **Note 9**).

3.2.1 Dissection of the Spleen and Lymph Nodes and Preparation of a Single-Cell Suspension

1. Mice are killed by cervical dislocation and placed with the left side up on a dissecting board.
2. Wet fur with 70% ethanol and cut away about 1 cm^2 of the skin, about half-way between the front and back legs (*see* **Note 10**). The spleen should now be visible under the peritoneum. The spleen is dissected and placed into RPMI complete medium.
3. The mouse is now extended on the back, with the limbs spread and fixed with needles in the four paws. Using forceps and a pair of scissors, the skin is cut along the ventral midline from the groin to the chin, being careful to only cut the skin and not the peritoneal wall. Four further incisions are made from the start of the first incision downward to the knees and elbows on both sides. Pull the skin back on the sides and fix the skin with needles.
4. The inguinal, the axillary and the brachial lymph nodes are dissected and placed into RPMI complete medium.
5. Spleens and lymph nodes can be pooled (*see* **Note 11**).
6. Spleens and lymph nodes are put into a sterile metal cell-strainer, the remaining medium is discarded. The strainer containing organs is placed into a 60 mm Petri dish and 4 ml of fresh RPMI complete medium are added.

7. Using the barrel of a 5 ml syringe, spleens and lymph nodes are pressed through the strainer until only fibrous tissue remains in the strainer. The strainer is washed with fresh RPMI complete medium.
8. The cells are resuspended using a serological pipette and sieved through a 70 µm nylon mesh cell strainer into a 50 ml conical tube. Cells are centrifuged at 300 × *g* for 5 min at 4 °C and resuspended thoroughly in 0.5–3 ml erythrocytes lysis buffer (*see* **Note 12**) and incubated on ice for not more than 90 s (*see* **Note 13**).
9. The reaction is stopped by adding at least twice the volume of RPMI complete medium. To get rid of precipitates that may form after adding the medium, the cell suspension is sieved through a 70 µm nylon mesh cell strainer into a new 50 ml conical tube. Cells are washed with fresh medium and centrifuged at 300 × *g* for 5 min at 4 °C and resuspended in 10–20 ml RPMI complete medium, depending on cell density.
10. Using a hemocytometer cells are counted and the absolute cell number is calculated.

For adoptive transfer experiments we usually inject 5×10^7 lymphocytes in 200 µl of a 0.9 % sodium chloride injection solution per mouse. This amount is sufficient to suppress immune responses in recipient mice, but it can be scaled up to 1×10^8 lymphocytes. Alternatively pure Treg can be isolated as described in the following section.

For adoptive transfer of bulk lymphocytes directly proceed to Subheading 3.2.3.

3.2.2 Isolation of CD4⁺CD25⁺ Treg from a Single-Cell Suspension

The methodology described here is for using the Miltenyi MACS isolation and autoMACS Pro Separator usage. One could also use other magnetic separation techniques. Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^7 lymphocytes. For larger initial cell numbers, scale up volumes accordingly.

1. The lymphocytes from Subheading 3.2.1 are counted and centrifuged at 300 × *g* for 10 min. The supernatant is aspirated completely and the cells are resuspended in 40 µl MACS Running Buffer.
2. 10 µl of Biotin-Antibody Cocktail (*see* **Note 14**) are added and the mixture is incubated for 10 min at 4–8 °C.
3. 30 µl MACS Running Buffer, 20 µl of Anti-Biotin MicroBeads, and 10 µl of CD25-PE antibody are added to the cells, mixed well and refrigerated for additional 15 min in the dark at 4–8 °C.
4. Cells are washed by adding 1–2 ml of MACS Running Buffer per 10^7 total cells and centrifuged at 300 × *g* for 10 min. The supernatant is removed completely and the cells are

resuspended in 500 µl MACS Running Buffer for up to 10^8 cells. For larger cell numbers the appropriate amounts of buffer have to be used.

5. After preparing and priming the autoMACS Pro Separator, the magnetically labeled cells are fractionated using the separation program "Depl025". During this procedure $CD4^+$ T cells are enriched by depletion of unwanted cells. Both fractions are collected and the CD4 positive cells are counted. CD4 negative cells are discarded (*see* **Note 15**).
6. $CD4^+$ T cells are centrifuged at $300 \times g$ for 10 min and resuspended in 90 µl of MACS Running Buffer per 10^7 initially used lymphocytes.
7. 10 µl of Anti-PE MicroBeads are added to cells, mixed and the suspension is refrigerated for 15 min in the dark at 4–8 °C.
8. Cells are washed by adding 1–2 ml of MACS Running Buffer per 10^7 total cells and centrifuged at $300 \times g$ for 10 min. The supernatant is removed completely and the cells are resuspended in 500 µl MACS Running Buffer for up to 10^8 cells. For larger cell numbers the appropriate amounts of buffer have to be used.
9. The magnetically labeled $CD4^+$ T cells are applied to the autoMACS Pro Separator and fractionated using the separation program "Possld2". Both fractions are collected: The negative fraction contains $CD4^+CD25^-$ T cells and the positive fraction is the enriched $CD4^+CD25^+$ Treg fraction.

For adoptive transfer experiments, we usually inject 5×10^5 enriched Treg in 200 µl of a 0.9% sodium chloride solution per mouse into the tail veins.

Flow Cytometry Analysis of Fractionated $CD4^+$ T-Cells

The enrichment of Treg is proved by double staining of CD4 and CD25 followed by FACS analysis. As T cells are already labeled with a CD25-PE antibody during the MACS isolation procedure (Subheading 3.2.2, **step 3**), cells have to be stained only with a fluorochrome conjugated anti-CD4 antibody.

1. About $2–5 \times 10^5$ cells in 100 µl of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells are prepared in a 96-well U-bottom microplate. One well of T cells for IgG control should be also included. Preparation of two wells with $CD4^+CD25^-$ Treg for single staining is recommended for proper compensation of the FACS device.
2. Cells are centrifuged at $300 \times g$ for 5 min, resuspended in 100 µl FACS blocking buffer and incubated for 30 min at 4–8 °C.
3. Cells are centrifuged at $300 \times g$ for 5 min and resuspended in 100 µl FACS staining buffer.
4. An appropriate amount of an APC-conjugated CD4 antibody or IgG antibody is added to the respective wells and cells are incubated for 60 min at 4–8 °C.

5. Cells are centrifuged at $300 \times g$ for 5 min, washed once with FACS staining buffer, resuspended in 400 µl FACS staining buffer and subjected to FACS analysis (Fig. 2).

3.2.3 *Intravenous Injection of Isolated Cells into Naïve Mice*

For adoptive transfer experiments we usually inject 5×10^7 lymphocytes or 5×10^5 enriched Treg in 200 µl of a 0.9% sodium chloride injection solution per mouse.

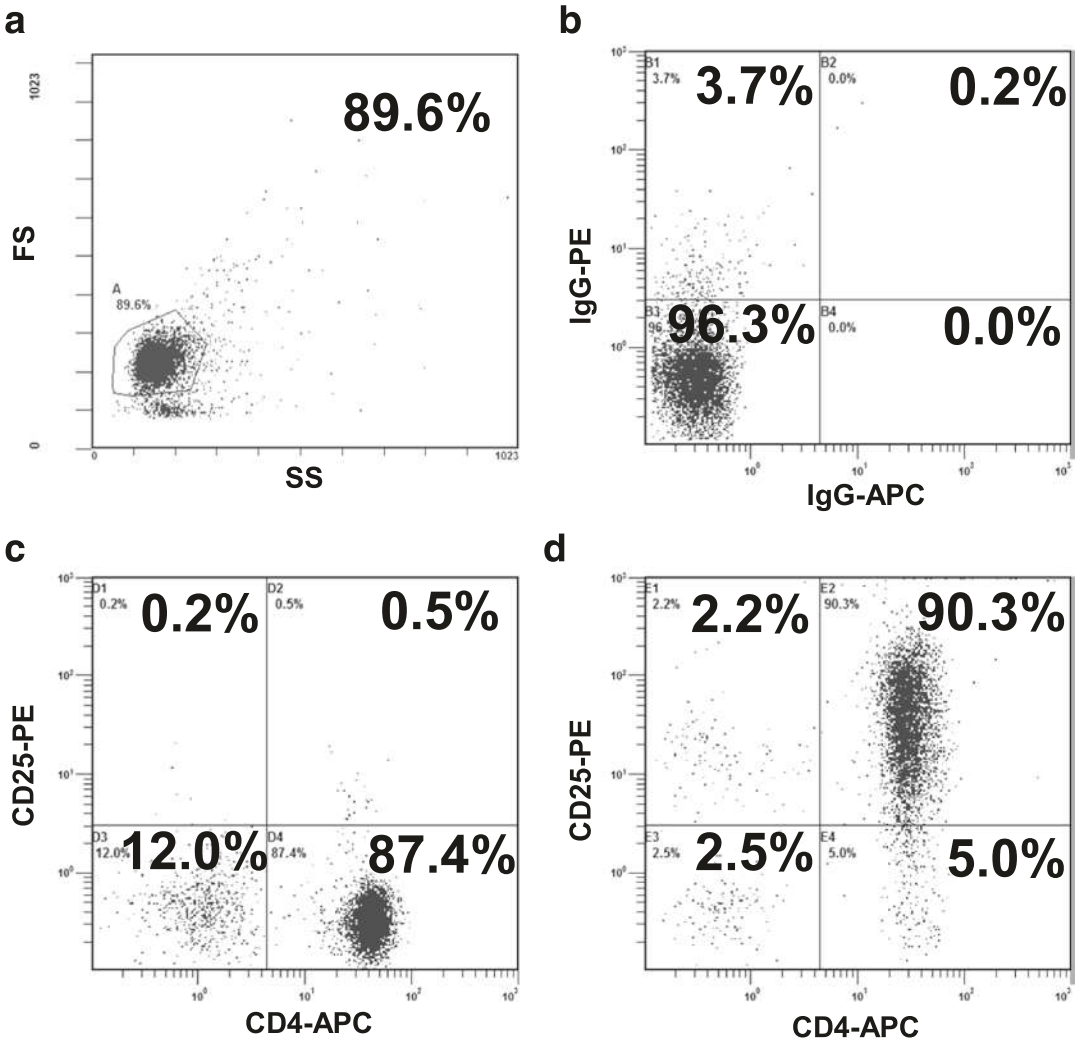

Fig. 2 Separation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from lymphocytes. Lymphocytes from UV-radiated donors were isolated, separated in $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells by magnetobead separation, stained with CD4 and CD25 antibodies, and subjected to FACS analysis. (**a**) Gating strategy to identify lymphocytes. (**b**) $CD4^+CD25^+$ Treg stained with PE and APC-conjugated IgG antibodies. (**c**) $CD4^+CD25^-$ T cells stained with CD4-APC and CD25-PE antibodies. (**d**) $CD4^+CD25^+$ Treg stained with CD4-APC and CD25-PE antibodies

1. Cells are adjusted to the desired concentrations with 0.9% sodium chloride and taken up by a syringe. Air bubbles are completely removed.
2. Using a red heat lamp, the tail of the mouse is warmed up in order to dilate the veins (*see* **Note 16**).
3. The mouse is placed into a restraining device. The tail is grasped and gentle pressure is applied in order to stabilize the tail and raise the veins. The lateral veins are located superficially and can be visualized by slight rotation of the tail.
4. Alcohol is applied to the injection site. By maintaining the needle and the syringe parallel to the tail, the vein is punctured and the cells are injected by gentle pressure.
5. Once the cells are injected, the needle is removed and pressure applied with a clean gauze to the injection site to stop bleeding.
6. Continue with **step 3**, Subheading 3.1 for sensitization and challenging.

4 Notes

1. We prefer using female mice. But the model also works fine with male mice. An age of at least 6 weeks ensures a fully developed immune system [13].
2. Be careful when working with TNCB! Always wear safety glasses and gloves. As a potent sensitizer it is an irritant to eyes, skin, digestive tract, and respiratory tract and very toxic by inhalation, ingestion, and skin contact. There is also a potential fire and explosion hazard.
3. Use two sets of surgical instruments consisting of two forceps and one pair of scissors: one for making the incisions through the skin and a different one for the section of the inner part and dissection of the organs of the mouse. This lowers the risk of contamination.
4. Erythrocyte lysis buffer can be prepared as a 10× solution and stored in 1 ml aliquots at −20 ° C. Once thawed and diluted, the 1× solution is stored at 4 °C and used within 4 weeks.
5. Ensure no mice get hurt by shaving the back. Do not irradiate or sensitize mice with injured skin! You can also shave the mice one day prior to irradiation or sensitization. Potential irritations from shaving might disappear in the meantime.
6. Prior to radiation, the intensity (mW/cm^2) of the UV output has to be measured in a constant distance (we use 30 cm) using a UV-Meter. Based on this measurement, the time of UV exposure is calculated so that the UV dose of 1000 J/m^2/exposure

will be delivered to the mice placed in the same distance. You can use a mouse cage base placed in this distance from the fluorescent lamp to ensure delivery of the correct UV dose.

7. Up to three mice can be irradiated at the same time. It is not necessary to protect their eyes.
8. For experienced persons it is possible to measure ear thickness without anesthesia of the mice. The mouse has to be properly restrained with pressure behind the neck and immobilized on a tabletop. We prefer using an isofluran vaporizer for very short procedures like ear challenge and ear swelling measurements. The use of isofluran inhalant anesthesia for rodent procedures is recommended, due to its wide safety margin, reliability, ease of administration, and rapid return to consciousness for animals after end of exposure.
9. The $CD4^+CD25^-$ fraction can also be used in further experiments. The cells can be stimulated with various substances or molecules in vitro and injected in mice to test the suppressive capacity of in vitro stimulated $CD4^+CD25^-$ T cells in different models. We have shown this for example for the antimicrobial peptide mBD14 [14].
10. Do not cut away too much of the skin as it would impede or even preclude the subsequent dissection of the inguinal lymph node.
11. Spleens and lymph nodes from up to six mice can be pooled and processed at the same time.
12. The amount of lysis buffer used depends on the pellet size. You can approximately calculate 0.5 ml lysis buffer per mouse.
13. The erythrocyte lysis step can be skipped when the suspension is subjected to magnetic separation as in the first separation step the erythocytes will be depleted.
14. Non-$CD4^+$ cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies. The cocktail contains antibodies against CD8a, CD11b, CD45R, CD49b, and Ter-119.
15. $CD4^+$ T cells are pre-enriched by depletion of unwanted cells. The antibody cocktail described in **Note 14** labels all cells excluding CD4 positive cells. Keep in mind that when using the MACS program "Depl025" the so-called "positive fraction" contains the labeled, CD4 negative cells. The CD4 positive cells are unlabeled and thus collected in the so-called "negative fraction."
16. Warm up the tail for about 30–60 s using the red heat lamp. By holding mouse tail in front of the red heat lamp you prevent the mouse from over warming as your fingers also became warm.

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