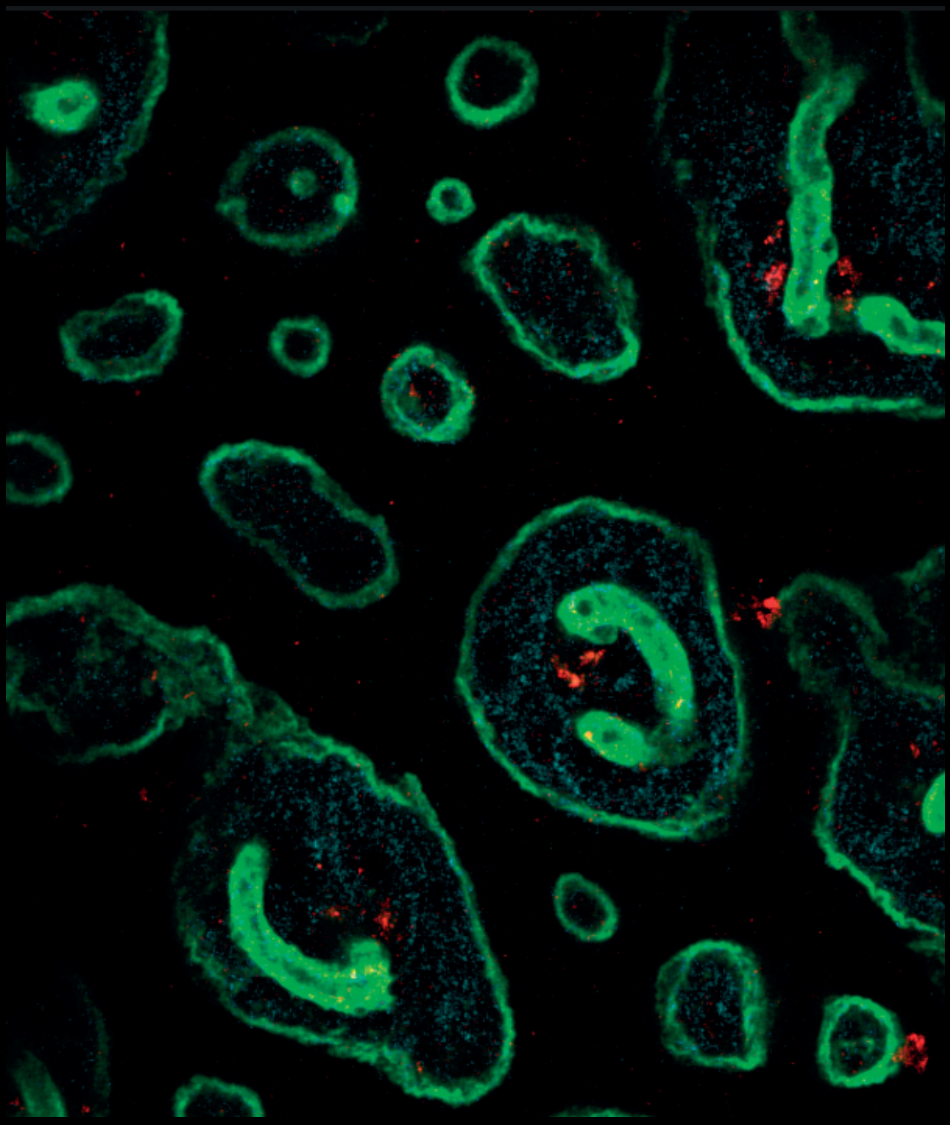

Chapter 7

Labeling and tracking of immune cells in *ex vivo* human skin

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ABSTRACT

Human skin harbors various immune cells that are crucial for control of injury and infection. However, the current understanding of immune cell function within viable human skin tissue is limited. We developed an *ex vivo* imaging approach in which fresh skin biopsies are mounted, and are then labeled with nanobodies or antibodies against cell surface markers on tissue-resident memory $CD8^+$ T cells, other immune cells of interest, or extracellular tissue components. Subsequent longitudinal imaging allows one to describe the dynamic behavior of human skin-resident cells *in situ.* In addition, this strategy can be used to study immune cell function in murine skin. The ability to follow the spatiotemporal behavior of $CD8^+$ T cells and other immune cells in skin, including their response to immune stimuli, provides a platform to investigate physiological immune cell behavior and immune cell behavior in skin diseases. The mounting, staining and imaging of skin samples requires about 1.5 days and subsequent tracking analysis requires minimally 1 day. The optional production of fluorescently-labeled nanobodies takes about 5 days.

INTRODUCTION

Background

The skin forms a tight barrier between the internal and external environment that is constantly exposed to external stimuli such as infectious pathogens, microbiota and allergens[1]. In line with this barrier function, the skin is populated by a variety of immune cell types, including Langerhans cells, macrophages, and dermal dendritic cells, that all contribute to skin homeostasis[2]. Over the past years, it has become evident that, next to these immune cell subsets, tissue-resident memory T cells (T_{RM}) also play a critical role in skin immunity. Tissue-resident memory T cells have been shown to arise after local immune challenges and persist up to years in mice and decades in human skin tissue[3,4]. T_{RM} are generally characterized by cell-surface expression of CD69 and CD103 (αE integrin)[5,6], which both contribute to their tissue-residency and maintenance[7,8]. $CD8^+$ skin-T_{RM} provide rapid control of viral reinfections in mouse models[9,10] and are also associated with a protective function in human skin tissue[11,12]. In addition, the presence of $CD8^+$ tumor infiltrating lymphocytes that express CD103 has been correlated with improved survival in melanoma patients and such cells were shown to expand in patients that received anti-PD-1 treatment[13]. Because of the protective role of $CD8^+$ T_{RM} in these different settings, a number of efforts to enhance local $CD8^+$ T_{RM} numbers or function has been initiated[12,14]. Conversely, the aberrant activation of $CD8^+$ T_{RM} can also lead to pathogenic skin responses, such as the IFNγ-mediated cytotoxicity of T_{RM} against melanocytes in vitiligo, and the production of the pro-inflammatory cytokine IL-17 by T_{RM} in psoriasis[15]. In these settings, therapies are aimed at limiting the density or function of existing T_{RM} pools[16].

Over recent years, intravital imaging in mice has provided tremendous insight into the spatiotemporal aspects of $CD8^+$ T cell behavior in the skin[17-20]. As an example, $CD8^+$ T_{RM} in murine skin were shown to interact with the surrounding cell compartment by migrating through the epidermis and sampling keratinocytes for possible renewed infection[17]. However, technologies to study the functional behavior of cells within human skin have been limited, an issue that is of particular relevance as the number and composition of the local immune cell pool differs between (laboratory) mice and humans[2,21]. To allow an improved understanding of immune cell behavior in human skin tissue, we developed an *ex vivo* imaging technology that can be used to follow $CD8^+$ T cells and other immune cell types in fresh skin samples[22]. Following validation on murine skin tissue, this method was applied to analyze immune cell behavior in healthy human skin samples. With this approach, we revealed that human $CD8^+$ T_{RM} patrol the papillary dermis and the epidermis below a sessile population of Langerhans cells[22]. Here, we describe a detailed protocol to label and track $CD8^+$ T cells in murine and human *ex vivo* skin tissue, and provide recommendations for the labeling of other immune cell types and structural components within human skin.

Development of the *ex vivo* imaging system of human skin

Traditionally, $CD8^+$ T cells within the skin have been studied by their isolation from the tissue and subsequent analysis upon in vitro culture. However, these methods neglect the important role that the local environment plays in immune cell function[1]. Conversely, immunohistochemistry and fluorescence-based techniques have been valuable to map the spatial arrangement of T cells and other immune cell types in tissues, but do not allow the analysis of the dynamic behavior of these cells in either the steady state or upon administration of immune stimuli. More recently, technologies for *in vivo* imaging of immune cells in human skin have been developed, but these label-free imaging methods identify cells based on physical parameters (e.g. size and shape) and thus do not reliably detect different cell types[23].

Ex vivo tissue cultures, or tissue explants, are a valuable alternative to *in vivo* models for monitoring immune responses within the tissue microenvironment[24,25]. Such culture systems where the tissue is embedded in agarose, sectioned using a vibratome, immobilized and submerged in medium for imaging, have been established for tissues such as brain[26], lymph nodes[27] and tumors[28]. While this approach works well for the indicated tissue types, we reasoned that such a closed system would be less suited for a surface-exposed tissue such as the skin, as atmospheric oxygen has been shown to substantially contribute to the oxygen supply of murine and human epidermis[29,30]. Therefore, in order to track the dynamic behavior of immune cells in skin tissue, we set out to develop an *ex vivo* culture system in which the epidermal side is exposed to air. To this end, we adapted an *ex vivo* imaging setup that was originally described to study melanoblast migration in embryonic mouse tissue[22,30]. In this setup (**Fig. 1**), a fresh skin sample is placed in a Lumox dish (Sarstedt), with the epidermal side facing the air-exposed gas-permeable bottom, and the dermal side facing upwards, covered by a filter and Geltrex to allow for passive diffusion of nutrients from the

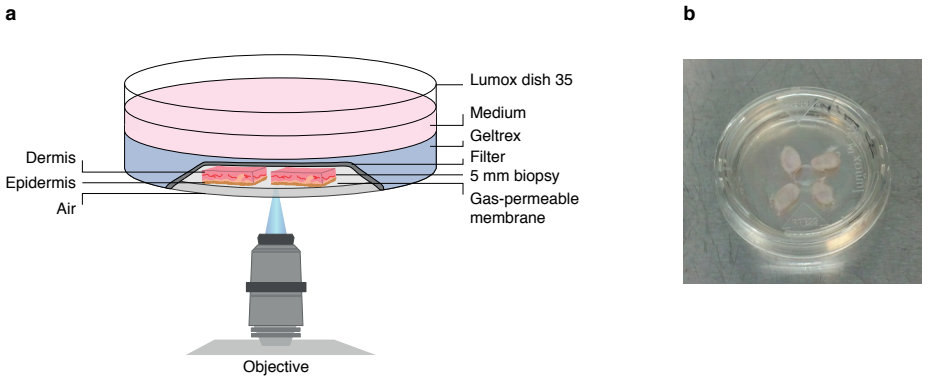

Figure 1 | Setup of the *ex vivo* imaging system for skin biopsies. a, Schematic overview of the *ex vivo* imaging setup. Note that a cross-section of the dish is shown to illustrate how the biopsies are placed on the gas-permeable membrane. In reality, the filter, Geltrex and medium enclose the biopsies. **b**, Representative image of healthy human skin biopsies mounted in the *ex vivo* imaging dish.

medium. Notably, instead of using vibratome-cut sections, we opted for whole skin biopsies to maximally preserve skin anatomy and to be able to readily use skin biopsy material from healthy or diseased individuals. In order to trace $CD8^+$ cells within *ex vivo* skin tissue over time, we developed Alexa Fluor (AF)-594 labeled anti-CD8 nanobodies[22]. In addition, the capacity to label other cell populations and extracellular skin components was demonstrated. Importantly, the *ex vivo* system developed here was validated by comparison of the *ex vivo* behavior of both unlabeled and nanobody-labeled $CD8^+$ T cells with the *in vivo* behavior of unlabeled $CD8^+$ T cells in murine skin by confocal microscopy. Based on this validation, it is plausible that data generated using the *ex vivo* system on human skin will recapitulate *in vivo* human skin cell behaviors, with the caveats that the validation performed was restricted to $CD8^+$ T cells and may not extend to aspects of skin biology that differ between mice and humans. In addition to the above-mentioned validation of the technology using murine skin, several parameters of tissue integrity were monitored in *ex vivo* cultured human skin. Specifically, the viability of $CD8^+$ T cells extracted from *ex vivo*-cultured biopsies was analyzed, and the migratory behavior of $CD8^+$ T cells was monitored over time, demonstrating that the migration speeds of $CD8^+$ T cells remain constant throughout 4-hour recordings. Furthermore, the continued presence of sessile Langerhans cells in *ex vivo* human skin was analyzed as additional indicator for skin integrity[22]. Combined, these data show that the *ex vivo* imaging system described here can be used to label and track $CD8^+$ T cells in both the papillary dermis and epidermal compartment, but also allows the visualization of other immune cell types such as Langerhans cells[22].

Applications of the method

This protocol allows for the successful staining of different cell surface markers on immune cell types in human skin, as demonstrated by nanobody staining of CD8 and antibody labeling of CD1a, and CD103[22]. In addition, nuclei and the extracellular skin component collagen type IV can be visualized by simple addition of conventional staining reagents to the *ex vivo* medium. Moreover, label-free second harmonic generation (SHG) imaging can be used to visualize collagen type I[22]. While we have not tested this, label-free third harmonic generation (THG) imaging may allow for the visualization of cell membranes and additional structural components, such as elastin fibers[31]. While the protocol described here is developed for healthy human skin tissue, biopsies from diseased skin are also suitable for *ex vivo* labeling and imaging. For instance, skin samples from patients with vitiligo, psoriasis, early-stage skin neoplasms or viral infections such as herpes simplex virus infections may be studied in this setting. Please note though that in all these settings, epidermal thickness, biopsy size, edema and pigmentation can form factors that adversely affect imaging quality (see also ‘Experimental design’). Air exchange at the epidermal side is vital for the physiological behavior of $CD8^+$ T_{RM} in murine skin[22]. However, since exposure to air may cause dehydra-

tion in other tissue types, we expect that the *ex vivo* imaging technology described here can only be used for other tissues when air exchange is avoided.

Although not investigated, it is reasonable to assume that the human *ex vivo* imaging platform also allows for the testing of $CD8^+$ T_{RM}-related and other skin cell therapeutics or other immune stimuli. As previously shown, addition of peptide antigen to the medium of murine skin mounted *ex vivo* leads to a rapid (~30 min) response by antigen-specific GFP^+ $CD8^+$ T_{RM}[22]. However, as human skin is thicker than mouse skin[2], and as therapeutics and other immune stimuli are likely to vary in tissue penetration, optimization of drug incubation time is recommended. As an alternative to the addition of therapeutics to the *ex vivo* medium, molecules may potentially be delivered by intradermal injection or DNA vaccination[32]. Please bear in mind that these treatments may induce skin damage that potentially affects the quality of the imaging data (as discussed in 'Experimental design'). Finally, skin immune responses may also be monitored after topical treatments. In view of the potential effect of the immersion medium (i.e. the cream) on the imaging refractive index[33], such an application will require the use of an objective with a suitable numerical aperture, or may require pre-treatment with the drug, prior to the mounting of skin samples.

Additional applications of the *ex vivo* imaging setup that may be considered concern the monitoring of *in situ* T cell activation, e.g. by staining with fluorescent reporters of T cell activation such as calcium dyes[34]. Additionally, labeling of *ex vivo* skin biopsies with photo-convertible staining reagents[35,36] could enable the *in situ* marking of skin regions or cell populations of interest, which subsequently may be analyzed by e.g. single cell sequencing, in order to couple spatiotemporal data to information about cell state.

Experimental design

Below, we highlight aspects of the protocol that are important to consider before exploiting the *ex vivo* imaging system for the labeling and tracking of $CD8^+$ T cells and other immune cells in human skin.

Considerations for the mounting of human skin samples

Several tissue characteristics can affect the *ex vivo* labeling and tracking of immune cells in human skin and should therefore be taken into consideration when obtaining tissue material. First, highly autofluorescent objects such as hairs reduce the quality of the multiphoton (MP) imaging data (see Troubleshooting step 24)[19]. For this reason, we generally recommend obtaining biopsies from hairless skin areas. Note though that hair follicles are important anatomical niches for immune cell trafficking and maintenance[1,37], and a focus on hairless skin may thus skew the results obtained. Second, melanin present in the epidermis absorbs light, thereby reducing the signal of fluorescent markers and increasing the chance of cell damage upon prolonged and/or high intensity laser exposure[19,38]. For this reason, it is advised to obtain biopsies from lowly pigmented areas, for instance from sun-protected

regions, such as the abdomen or breast from people with light skin[39]. Third, as opposed to murine epidermis that consists of only a few cell layers, human epidermis contains up to 10 cell layers depending on the body site[2,40]. As $CD8^+$ T cells are located in the bottom of the epidermis and in the dermal compartment[22], we recommend avoiding thicker skin types such as the elbow to allow visualization of these cells by MP microscopy (range ~200 μm). In addition, when obtaining diseased skin samples, please consider that, for example, psoriatic lesions display a thickened epidermis, hyperparakeratosis and edema[41], thereby limiting the ability to image immune cells deeper in the tissue. This protocol describes the use of 5 mm biopsies. However, in case only smaller size biopsy material is available, please note that, while biopsies as small as 1.5 mm can be mounted and imaged successfully, smaller biopsies are more prone to dehydration.

Despite the fact that healthy human skin contains relatively high amounts of memory T cells [39,42], the number of $CD8^+$ T cells varies between individuals and biopsies[22] and the cells may be present in clusters. Therefore, we advise to mount 4 biopsies per *ex vivo* imaging dish, to increase the chance of successful imaging of $CD8^+$ T cell infiltrates.

An important practical consideration is that, while the freshly obtained skin material should be mounted the same day (see **Table 1** for the planning of all steps), subsequent imaging should be initiated following an overnight incubation. Specifically, as established using *ex vivo* cultures of mouse skin, the process of isolation and mounting of skin induces a transient stress response in $CD8^+$ T_{RM} that makes the cells lose the mobility and dendritic morphology that they display *in vivo*. However, during overnight incubation T_{RM} regain their typical dendritic morphology and patrolling behavior[22] (**Supplementary Video 1**). While the occurrence of such a transient stress response was not directly established for human material, imaging of $CD8^+$ T cells in human *ex vivo* skin tissue is therefore also performed

Table 1 | Timing of labeling and tracking of $CD8^+$ T cells in *ex vivo* skin. Schematic overview of the timing of the various phases of the protocol. First column indicates the relevant protocol steps, the second column indicates the action and the third column indicates the time the action requires. ▲ CRITICAL STEP Please note that time-lapse imaging should be performed after an overnight resting period and no later than 24 h of *ex vivo* culture.

Step	Action	Required time
Optional (Box 1)	Production, purification and labeling of nanobodies	5 days
1	Obtain fresh skin material	variable
2	Obtain punch biopsies	0.5 h
3-13	Mount biopsies in *ex vivo* setup	1.5 h
14-15	Prepare staining	0.5 h
16	Rest and stain overnight	12 h
17-23	Wash and prepare for imaging	0.5 h
24	Time-lapse imaging	4 h
25	Tracking analysis	> 8 h

after an overnight recovery period. With respect to the maximum duration of *ex vivo* skin cultures, histopathological analysis of mouse *ex vivo* skin harvested at different time points after culturing indicates that tissue slowly deteriorates over time, with cultures lasting over 24 hours being qualified as unreliable to include in the analysis[22]. Thus, MP imaging should be performed after an overnight recovery period, but before the 24 hours in culture (i.e. between ~12 and 24 hours). We note that the composition of the *ex vivo* culture medium has not been optimized with respect to long-term tissue viability, and this may offer a route towards the reliable imaging of human skin tissue for longer time periods, something that may be of particular relevance when testing drug candidates.

Considerations for labeling

Nanobodies are single domain antibodies that are about 10-fold smaller than conventional antibodies (~15 kDa and ~150 kDa, respectively)[43]. As the relatively large size of antibodies may potentially affect tissue penetration, we conjugated a nanobody against human CD8 to the small molecule (~1 kDa) fluorescent dye AF594 (see **Box 1**). Incubation of *ex vivo* human skin material with anti-hCD8-AF594 nanobody results in labeling of nearly all $CD8^+$ cells in both the dermal and epidermal skin compartment[22]. As an alternative to nanobody-based reagents, staining with more widely available antibody reagents against immune cell markers (i.e. CD1a and CD103) or structural skin components (i.e. collagen type IV) has however also been shown to be successful in the *ex vivo* system described in Ref. [22] and in the 'Anticipated results'. For this reason, we expect that labeling and tracking of $CD8^+$ cells with conventional anti-CD8 antibodies should also be feasible, although this has not been formally established. Please note that, while overnight staining with the same batch of anti-hCD8 nanobody results in reproducible labeling, staining with Hoechst dye and with conventional antibodies against CD1a, CD103 or collagen type IV is more sensitive to differences in skin permeability and thickness, resulting in variation in fluorescence intensity between individuals and within individual biopsies (unpublished observations, F.E.D. and M.H.). To accommodate for differences between individuals, we recommend titration of Hoechst dye and of antibodies against markers such as CD1a, CD103 and collagen type IV for each individual (i.e. one concentration per dish, several dishes per individual/staining reagent). In addition, to compensate for differences between individual biopsies, we recommend mounting multiple biopsies per dish (e.g. 4 biopsies of 5 mm per dish, see also **Fig. 1b**). As a positive control for successful *in situ* staining, a relevant nanobody or antibody labeled with a different fluorochrome may be used for double staining of the population of interest. Alternatively, *in situ* staining may be validated after imaging by digestion of biopsies followed by counterstaining and flow cytometry[22], or by fixating tissues for subsequent immunofluorescent counterstaining and imaging[44]. As a control for autofluorescence, a dish with unstained biopsies can be taken along. Additionally, the level of background signal may be determined by staining a dish with an irrelevant (preferably isotype-matched) staining

reagent labeled with the same fluorochrome as used in the other analyses. Note that staining of cells with antibody or nanobody reagents may potentially influence cellular function[22], and we recommend an analysis of the effect of labeling on cell behavior if relevant for the research question of interest.

Considerations for multiphoton imaging

The MP imaging system used to develop the *ex vivo* imaging technology was an upright system and permitted imaging of single *ex vivo* imaging dishes that were sealed and inverted such that the epidermal side was facing the objective[22]. If available, an inverted multiphoton microscope is preferred over this upright setting, as this reduces the chance of biopsies detaching from the gas-permeable membrane and opens up the possibility to work in a multiwell format (e.g. Lumox multiwell 24, cat. no: 94.6110.024, Sarstedt).

The *ex vivo* culture setup allows for the longitudinal imaging of $CD8^+$ T cells and other cells of interest within the skin. In addition, the *ex vivo* imaging system makes it possible to re-analyze areas of interest in subsequent imaging sessions (e.g. before and after addition of an immune stimulus), through the use of physical reference points on the dish, recording the x, y, z-coordinates within the tissue in the acquisition software, and/or by the capturing of identifiable collagen type I structures using the SHG signal. Acquisition of SHG signal is also recommended to provide anatomical context and to facilitate discrimination between autofluorescent and labeled objects. In our experience, the migration parameters of $CD8^+$ T cells can be traced reliably for at least 4 hours of imaging with a 3-minute interval[22]. Please bear in mind that an increase in exposure time increases the chance of photo-bleaching and photo-toxicity (see also 'Troubleshooting – step 24').

Considerations for imaging analysis

The migratory behavior of immune cells may be traced using various imaging analysis programs such as Fiji[45] (NIH), Huygens (Scientific Volume Imaging) or Imaris (Bitplane). We prefer analysis in Imaris, because of its capacity to allow 4D data visualization and the fast rendering of large datasets. As longitudinal 3D MP-recordings produce datasets of up to 35 gigabytes, the availability of sufficient computational power to process such large data sets is required (for details, see 'Equipment').

Ex vivo imaging of murine skin

While the protocol presented here was initially developed to study the behavior of $CD8^+$ T cells in *ex vivo* human skin tissue, the *ex vivo* imaging technology is also suited to investigate the migratory behavior of murine $CD8^+$ T cells *in situ*[22]. Contrary to healthy human skin[39], the skin of immune naïve mice housed under specific-pathogen-free (SPF) conditions is nearly devoid of $CD8^+$ T cells[46]. In order to generate local $CD8^+$ T_{RM} pools, adoptive transfer of (fluorescently labeled) naïve $CD8^+$ T cells, followed by local skin immunization can be

performed, as described previously[22]. Next to the potential to study $CD8^+$ skin-T_{RM} in murine *ex vivo* tissue, *ex vivo* cultures of mouse skin can serve as a validation for the real-time study of human immune cells *in situ* (see also 'Troubleshooting – step 24'). For example, the migratory parameters of anti-mCD8-AF594 nanobody-labeled or unlabeled GFP^+ T_{RM} in *ex vivo* tissue can be compared to the dynamic behavior of anti-hCD8-$AF594^+$ human skin-T_{RM}[22]. As the mouse epidermis consists of only a few cell layers[2], $CD8^+$ T cells in murine tissue can be visualized using either confocal or multiphoton microscopy[22].

In this protocol, the confocal imaging of $CD8^+$ T cells in murine skin is also described. Please note that all materials, procedures and analysis steps listed here refer to both mouse and human tissue, unless specified otherwise.

MATERIALS

Biological materials

Skin samples

- *Human skin samples.* Fresh human skin tissue with a low level of pigmentation (e.g. obtained directly from abdominoplastic or breast-reconstructing surgery);

▲ CRITICAL Healthy human skin material can be obtained from tissue locations other than abdomen or breast, however variation in skin thickness and pigmentation should be taken into consideration.

- *Mouse skin samples.* Depilated skin of mice containing $CD8^+$ T_{RM} harvested directly after sacrificing the animal (e.g. skin of hindlegs[22]).

▲ CRITICAL As hairs may hamper the quality of the imaging data (see also 'Troubleshooting – step 24'), remove fur as much as possible - preferably - by using depilation cream. Please note that this can induce tissue damage, and we therefore advise to depilate skin tissue one day prior to the harvesting of material. ! CAUTION Apply safety measures when working with biological materials, such as the use of gloves, protective clothes and working in a biological safety cabinet. Please make sure skin materials are disposed according to institutional and national guidelines. ! CAUTION All experiments should be performed in accordance with relevant institutional and national ethics regulations. For patient material, informed consent must be obtained in line with institutional and national ethics regulations.

REAGENTS

! CAUTION For all items marked with 'Caution', please wear proper personal protection equipment (i.e. proper attire, gloves and eye protection). Where appropriate, hazardous re-

agents should be handled in a fume hood. For more information, please refer to the material safety data sheets (MSDS) of each item.

Production, purification and labeling of nanobodies (**Box 1**)

- pHEN6c plasmid encoding the desired nanobody and an LPETGG-6xHis sequence (see **Supplementary File 1** for the DNA sequence of the empty vector (i.e. pHEN6c-NcoI-BstEII-LPETGG-6xHis))
- *Escherichia coli*, WK6 strain (e.g. ATCC®, cat. no: 47078)
- 5M or 7M-sortase (e.g. produced according to the protocol described in[47])
- Luria-Bertani (LB) medium (VWR, cat. no: 97064-112)
- Carbenicillin (Fisher scientific, cat. no: BP2648250)
- Terrific Broth (TB) (Fischer Scientific, cat. no: BP24682)
- Glycerol (Biosolve, cat. no: 071287)
- Isopropyl-b-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, cat. no: I5502)
- Ni-NTA beads (Qiagen, cat. no: 30230)
- DMSO (Sigma-Aldrich, cat. no: 276855)

! CAUTION DMSO is an irritant and flammable

- $NaHCO_3$ (Sigma-Aldrich, cat. no: S5761)
- GGGC peptide (kind gift from H. Ovaa and D. El Atmioui, Leiden University, in-house peptide synthesis) (the peptide can be stored in dry form or dissolved in sterile water (700 μM) and kept at -20 °C up to half a year)
- Acetonitrile (Biosolve, cat. no: 012078)

! CAUTION Acetonitrile is toxic and flammable

- Alexa Fluor™ 594 C5 Maleimide or comparable maleimide-dye (Thermo Fisher Scientific, cat. no: A10256)
- Tris (Biosolve, cat. no: 200923)
- 0.5M EDTA (pH 8) (Thermo Fisher Scientific, cat. no: 15575-038)
- Sucrose (Sigma-Aldrich, cat. no: S0389)
- Imidazole (Sigma-Aldrich, cat. no: I0250)

! CAUTION Imidazole is an irritant and toxic

- NaCl (Fluka, cat. no: 71380)
- 10X PBS (Thermo Fisher Scientific, cat. no: 14200-067)

Ex vivo culture system

- Depilation cream (Veet) – for mouse skin biopsy

! CAUTION Depilation cream can cause skin damage when skin is exposed for prolonged time periods (i.e. ≥ 3-6 minutes)

- Hanks' Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, cat. no: 14175-053)

- Geltrex LDEV-Free reduced growth factor basement membrane matrix (Thermo Fisher Scientific, cat. no: A1413202)

Ex vivo culture medium

- Opti-MEM (Thermo Fisher Scientific, cat. no: 11058-021)
- Fetal calf serum (Sigma-Aldrich, cat. no: F7524)
- Penicillin–streptomycin (Sigma-Aldrich, cat. no: 11074440001)
- L-glutamine (Thermo Fisher Scientific, cat. no: 25030-024)

Conventional staining reagents

! CAUTION Please protect staining reagents from exposure to light while handling, as they are light sensitive.

▲ CRITICAL For some commercially available antibodies, such as the anti-human CD103-AF488 antibody, removal of sodium azide of the reagent is necessary in order to avoid impaired cell migration upon staining (see also 'Troubleshooting step 24' and **Fig. 2**).

- Hoechst 33342 (Thermo Fisher Scientific, cat no: H1399) ! CAUTION Hoechst 33342 is a known mutagen and should be handled with care.
- Anti-human CD1a-AF488 antibody (clone HI149, BioLegend, cat. no: 300114, https://scicrunch.org/resolver/AB_493104)
- Anti-human CD103-AF488 antibody (clone Ber-ACT8, BioLegend, cat. no: 350208, https://scicrunch.org/resolver/AB_10641844)
- Anti-human collagen type IV-AF488 antibody (clone 1042, Thermo Fisher Scientific, cat. no: 53-9871-82, https://scicrunch.org/resolver/AB_2574487)

EQUIPMENT

Production, purification and labeling of nanobodies (Box 1)

- Water bath
- Bacterial incubator with shaker (30-37 °C)
- Roller mixer
- Rotating wheel
- Centrifuge for 500 mL polypropylene bottles (e.g. Beckman Coulter, JA-10 rotor Avanti, max. speed 6,000*g*)
- Centrifuge for 50 ml and 15 ml conical tubes
- Eppendorf centrifuge for 1.5 and 2 ml tubes with a max. speed of 16,000*g*
- Orbital shaking platform
- Spectrophotometer for measuring optical density of bacterial cultures
- Biosep 3000 column (Phenomenex, cat. no: 00H-2146-K0)

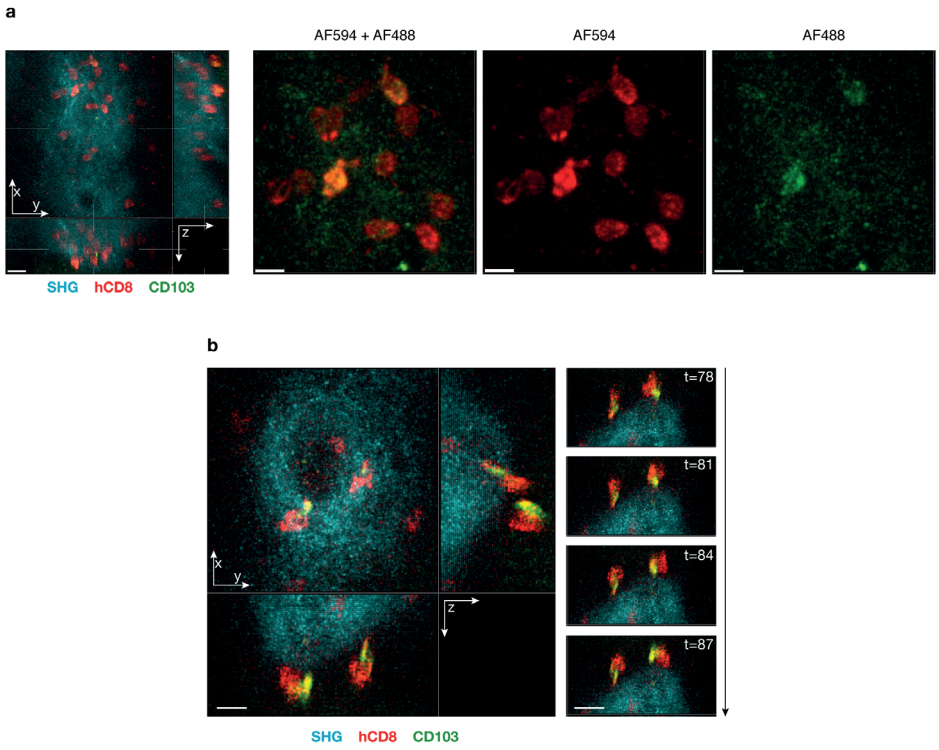

Figure 2 | Removal of sodium azide from conventional antibody reagent allows detection of migrating epidermal CD8⁺ CD103⁺ T cells. a, Left: Section view of a MP-recording of an *ex vivo* human skin biopsy stained with anti-hCD103-AF488 conventional antibody (green, 10 μg/ml final concentration, corresponding to 10% of the staining volume), also showing anti-hCD8-AF594 (red) nanobody staining and second harmonics generation signal (SHG, collagen type I, blue). Scale bar indicates 20 μm. Three right images: MP-recording top view showing single positive CD8⁺ cells (red) or double positive (red, green) cells. Note that all cells show a round morphology and are immobile (see also **Supplementary Video 2**). Scale bar indicates 10 μm. Data are representative of n=2 individuals. **b**, Left: Section view of a MP-recording of an *ex vivo* human skin biopsy stained with anti-hCD103-AF488 conventional antibody (10 μg/ml final concentration) from which the sodium azide was removed (see also 'Troubleshooting step 24'), also showing staining with anti-hCD8-AF594 nanobody (red) and SHG signal (blue). Right images: four consecutive timeframes showing CD8⁺ CD103⁺ epidermal cells migrating on top of a dermal papilla (see also **Supplementary Video 2**). Dataset in (b) is described in Fig. 3g in [22]. Scale bars indicate 15 μm. Representative images of n=3 individuals.

- Reverse-phase C18 column (e.g. Atlantis)
- High-performance liquid chromatography (HPLC) instrument (e.g. Waters)
- Spectrophotometer for measuring protein concentrations (e.g. NanoDrop™ One, Thermo Fisher Scientific)

Production, purification and labeling of nanobodies: plasticware and other materials (Box 1)

- Dry ice
- 250 ml sterile Erlenmeyer flask
- 2 L baffled flask
- 50 ml conical tubes (sterile, Sarstedt, cat. no: 62.547.254)
- 15 ml conical tubes (sterile, Sarstedt, cat. no: 62.554.502)
- XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific, cat. no: EI0001)
- NuPAGE™ 4 to 12%, Bis-Tris SDS-page gel (Thermo Fisher Scientific, cat. no: NP0322BOX)
- Ultra-4 Centrifugal Filter Unit 10 kDa Amicon® (Merck Millipore, cat. no: UFC801024)
- Ultra-0.5 Centrifugal Filter Unit 100 kDa Amicon® (Merck Millipore, cat. no UFC510096)
- Ultra-0.5 Centrifugal Filter Unit 10 kDa Amicon® (Merck Millipore, cat. no: UFC501096)
- Zeba Spin Desalting Columns, 7K MWCO, 0.5 ml (Thermo Fisher Scientific, cat. no: 89882)
- Poly-Prep® Chromatography Columns (Bio-Rad, cat. no: 7311550)

Ex vivo culture system and imaging

- Biological safety cabinet
- Incubator (37 ºC, 5% CO_2)
- Water bath (37 ºC)
- Heat mat (37 ºC)

Ex vivo culture system: plasticware

- Plastic container for transport of human skin
- 50 ml conical tubes (sterile, Sarstedt, cat. no: 62.547.254)
- 100 x 20 mm culture dishes (sterile, Greiner Bio-one, cat. no: 664160)
- 145 x 20 mm culture dishes (sterile, Greiner Bio-one, cat. no: 639160)
- Pre-chilled p-1000 pipet tips (stored at -20 ºC)
- Punch biopsy instrument (sterile, 5 mm, Kai Medical, cat. no: BF-50F)
- Lumox dish 35 (sterile, for adherent cells, Sarstedt, cat. no: 946.077.331)
- Whatman Nuclepore track-etched membranes (25 mm, 8 μm, Sigma-Aldrich, cat. no: WHA110614)

Ex vivo culture system: other materials

- Ice
- Tissue paper (Kleenex)
- Scissors (sterile, e.g. Vos medical supplier, cat. no: SKU16865)

- Curved forceps (sterile, e.g. Vos medical supplier, cat. no: 00570-1)
- Gauze pads (sterile, 10 x 10 cm, non-woven, Cutisoft)
- Disposable scalpel (sterile, Swann-Morton, cat. no: 0506)
- Parafilm (Bemis Company, Inc.)

! CAUTION Work carefully when using scissors and scalpel

Multiphoton microscope (Human biopsies)

- Multiphoton laser scanning microscope DM6000 TCS SP8 (Leica), upright system
- Tuneable Spectraphysics Insight Deepsee laser (Leica)
- 25x, NA 0.95 water-immersion objective, working distance 2.5 mm (Leica Fluotar VISIR)
- External HyD-NDD detectors (Leica)
- 8,000-Hz resonant scanner (Leica)
- Band-pass filter 615/30 M 3343
- Band-pass filter ET 525/50 (M-Chroma)
- Band-pass filter D 450/65 9149
- Mercury lamp - HXP R 120 W/45 C VIS (Osram, cat. no: 4050300882772)
- FITC filter set (Leica)
- SM-8 stage (Luigs & Neumann)
- The Brick - custom humidifier - CO_2 control (Life imaging services)
- The Cube2 - custom build microscope enclosure - temperature control (37 ºC) (Life imaging services)

Confocal microscope (Mouse biopsies)

- Confocal Leica TCS SP5, inverted system (Leica)
- HCX PL APO 20x, NA 0.7 air-objective HCX (Leica)
- HyD detectors (Leica)
- Laser lines: Argon (448 nm, 65 mW) and HeNE (594 nm, 2 mW) (Leica)
- Stage inlay for 35 mm dishes (Leica)
- Mercury lamp - HXP R 120 W/45 C VIS (Osram, cat. no: 4050300882772)
- FITC filter set (Leica)
- FoilCover set for 35mm Petri dishes (HemoGenix, cat. no: 701.000)
- CultFoils for 35mm Petri dishes (HemoGenix, cat no: 701.001)
- Custom humidifier and CO_2 control
- Custom build microscope enclosure - temperature control (37 ºC)

Image acquisition and analysis software

- LAS X for acquisition (v.3.1.5., Leica)
- Imaris for analysis (v. 9.2., Bitplane)
- Computer workstation with Windows 7 64bit operating system

▲ CRITICAL Please check the Imaris website for the required specifications for computer hardware for the Imaris version in use. We used the following configuration: Intel Xeon 6C CPU (2x), 128GB DDR4-RAM, NVIDIA Quadro P5000 16GB video card, 1TB SSD & 3TB 7200 RPM hard drive. Currently, most image processing functions are multithreaded and all of the 2x6 CPU cores are used during spot detection and tracking steps. In our experience, one of the limiting time steps is the writing of the modified file to the hard drive. It is recommended to use multiple PCIe SSD-drives to speed up reading and saving of image files. Using a 4K or larger monitor at full resolution will reduce live interaction frame per seconds. We reduced the display settings to 2560 x 1440, and turned off the interpolation in display setting to speed up rendering of large files during tracking.

REAGENTS SETUP

Production, purification and labeling of nanobodies (Box 1)

All buffers can be made in advance and kept for at least a year. Sortase buffer should be stored at room temperature (RT, 20-22 ºC). TES buffer, NiNTA wash buffer and Elution buffer should be stored at 4 ºC. Buffer compositions indicate final concentrations.

TES buffer

TES buffer is 200 mM Tris (pH 8), 0.65 mM EDTA and 0.5 M sucrose.

NiNTA wash buffer

NiNTA wash buffer contains 50 mM Tris (pH 8), 150 mM NaCl and 10 mM imidazole.

Elution buffer

Elution buffer consists of 50 mM Tris (pH 8), 150 mM NaCl and 0.5 M imidazole.

Sortase buffer

Sortase buffer consists of 50 mM Tris (pH 8) and 150 mM NaCl.

Ex vivo culture medium

To prepare *ex vivo* culture medium supplement Opti-MEM with 8% (vol/vol) fetal calf serum, 0.2% (vol/vol) penicillin-streptomycin and 1% (vol/vol) L-glutamine. *Ex vivo* medium can be made in advance and kept at 4ºC. Please refer to the product datasheets for the optimal shelf life.

EQUIPMENT SETUP

Preparation of *ex vivo* culture system

Before starting with the preparation of the *ex vivo* culture system, place all the materials listed under 'Materials - *Ex vivo* culture system' in a biological safety cabinet and make the following preparations:

1. Thaw the Geltrex on ice.

▲ CRITICAL STEP Geltrex solidifies when it warms up, therefore always thaw Geltrex on ice. Thawing of a full vial takes approximately 2 hours.

2. Place a gauze pad in a 100 x 20 mm dish.
3. Place a gauze pad in a 100 x 20 mm dish and submerge in HBSS.
4. Warm the *ex vivo* culture medium in a water bath (37 ºC)

Imaging setup

Make sure the microscopy chamber containing the stage inlay for the *ex vivo* imaging dish is set at 37 ºC and the humidifier and CO_2 control are installed. Warm the *ex vivo* culture medium in a water bath (37 ºC). In addition, prepare the following materials in a biological safety cabinet:

- Heat mat (37 ºC)
- 100 x 20 cm dish
- Tissue paper
- Parafilm

PROCEDURE

▲ CRITICAL STEP This procedure describes the preparation of 4 *ex vivo* imaging dishes (e.g. 3 staining concentrations and 1 negative control) each containing 4 biopsies, and the imaging and analysis of a 4-hour recording (i.e. a standard experiment).

Preparation of *ex vivo* culture system | Timing: min. 2 hours

Obtain skin material | Timing: variable

1. *Human.* Obtain resected healthy human skin material directly from surgery.
   Mouse. Obtain depilated skin of mice containing $CD8^+$ T_{RM} harvested directly after sacrificing the animal.

   ■ PAUSE POINT Mounting of material should be performed on the same day that the resected skin material is obtained. Please note that the optimal timing for imaging of *ex vivo* skin material is after an overnight (12 h) recovery period and before 24 h of culture. The preparation, mounting and addition of staining reagents to the *ex vivo* culture

system takes (steps 1-15) about 2 h. Collect and store the skin material appropriately: **H**) Resected human skin can be stored in a plastic container at 4 ºC without additional hydration; **M**) Collect murine skin in a 50 ml conical tube with HBSS and store at 4 ºC. ! CAUTION Apply safety measures when working with biological materials, such as the use of gloves, protective clothes and working in a biological safety cabinet. Make sure skin material is disposed according to institutional and national guidelines. ! CAUTION All experiments should be performed in accordance with relevant institutional and national ethics regulations.

Obtain 5 mm punch biopsies | Timing: ~30 min

2. Clean the skin surface with a sterile gauze pad soaked in HBSS and collect full-thickness skin biopsies in a 50 ml conical tube containing 10 ml HBSS using a 5 mm punch biopsy instrument. Keep the samples at 4 ºC until mounting.
   ▲ CRITICAL STEP Typically, 20 biopsies are needed for a standard experiment. Please make sure to collect at least 4 biopsies more than minimally required for the intended experiment (i.e. ≥24 biopsies), to accommodate for possible issues during preparation of the biopsies for mounting.

Mount biopsies in *Ex vivo* setup | Timing: 1.5 h

3. *Human.* Pick up a human skin biopsy with a pair of forceps and place it in a 145 x 20 mm dish. Use a scalpel to cut the biopsy just below the epidermal tissue such that you are left with a ~2 mm thick epidermal plus dermal tissue piece.
   Mouse. Pick up a murine skin biopsy with a pair forceps and place it in a 145 x 20 mm dish. Use the forceps to remove any connective tissue and fat.
   ▲ CRITICAL STEP Do not squeeze the biopsy too firmly with the forceps.
4. Place the obtained biopsy sample in the 100 x 20 mm dish with the dermal side facing the HBSS-submerged gauze pad to ensure the tissue does not dehydrate before mounting.
5. Repeat steps 3-4 for all biopsies (i.e. a total of 24 biopsies).
6. Pick up a biopsy from step 5 using forceps and dry both sides of the skin by gently pushing it on the dry gauze pad in the 100 x 20 mm dish.
   ▲ CRITICAL STEP Please make sure that especially the epidermal side is dry, so that it will stick to the gas-permeable membrane of the Lumox dish in the next step.
7. Mount the biopsies of one Lumox dish (i.e. 4 in total), by placing the epidermal side facing the membrane and the dermal side pointing upwards. Flatten the tissue by spreading it on top of the membrane using forceps.
   ▲ CRITICAL STEP Open the Lumox dish by squeezing the lid. ! CAUTION Try to work relatively fast to prevent dehydration (see also 'Troubleshooting – step 7').

8. Pipet a drop of HBSS (~5 μl) on top of the dermal side of each biopsy to prevent dehydration.
   ▲ CRITICAL STEP The liquid will also aid in the mounting of the filter in step 10.
9. Repeat steps 6-8 to mount the biopsies of the remaining dishes (i.e. 4 dishes in total)
10. Place a Whatman Nuclepore track-etched filter on top of the biopsies of each dish using forceps, with the glossy surface facing the dermal side of the tissue. Use the HBSS that spreads upon placing the filter to flatten and firmly mount the biopsies in between the Whatman Nuclepore track-etched filter and the gas-permeable membrane of the Lumox dish. Press the edges of the Whatman Nuclepore track-etched filter on the membrane of the Lumox dish to enclose the biopsies.
11. Add 500 μl of Geltrex to each dish using the pre-chilled p-1000 pipet tips.
    ! CAUTION Geltrex solidifies when it warms up. Gently swirl the dish directly after pipetting to spread the liquid Geltrex evenly before it has solidified.
12. Let the Geltrex solidify completely by placing the *ex vivo* imaging dishes in a tissue culture incubator set to 37 ºC for 20 min.
13. If you do not proceed with sample staining directly after mounting, add a minimum of 750 μl *ex vivo* culture medium on top of the Geltrex to prevent the biopsies from dehydrating.

In situ **labeling of** ***ex vivo*** **skin | Timing: 12 hours**

Prepare staining | Timing: 30 min

14. Prepare staining mixes in a minimal final volume of 750 μl *ex vivo* culture medium as outlined in the table below:

Reagent	**Recommended final concentration**
Anti-mCD8-AF594 nanobody	5-10 μg/ml
Anti-hCD8-AF594 nanobody	5-10 μg/ml
Hoechst 33342	5-10 μg/ml
Anti-human CD1a-AF488 antibody	4-8 μg/ml
Anti-human CD103-AF488 antibody	5-10 μg/ml
Anti-human collagen type IV-AF488 antibody	6.25-12.5 μg/ml

▲ CRITICAL STEP Titrate Hoechst 33342 and antibodies for each donor to accommodate for variability in skin thickness and permeability. Please note that Hoechst 33342 can be toxic to cells[48,49], thus add the minimum amount of dye necessary to obtain a sufficient signal (e.g. 5 μg/ml). ▲ CRITICAL STEP Staining with anti-hCD8-AF594 nanobody can be combined with Hoechst 33342 staining and with each of the AF488-conjugated antibodies. We recommended adding label-free SHG (collagen type I) imaging to this color panel to gain spatial information on the dermal compartment and to identify autofluorescent objects.

15. Remove any pre-existing medium from the dish from step 13 and add the staining mix on top of the solidified Geltrex of each dish.

Rest and stain skin overnight | Timing: 12 h

16. Place a maximum of 3 *ex vivo* imaging dishes in a 100 x 20 cm dish in the tissue culture incubator to let the cells rest overnight (12 h).

    ▲ CRITICAL STEP The overnight recovery period is necessary for $CD8^+$ T cells to regain migratory behavior and dendritic morphology (see also **Supplementary Video 1**). ▲ CRITICAL STEP Take care to avoid environmentally induced (e.g. temperature, movement) stress responses of the T cells during relocation of the dish before imaging. To minimize the risk of such stress, we placed the dish in an incubator located in the same room as the microscope. ! CAUTION Please make sure that the humidity level of the incubator is sufficient to limit evaporation of the relatively small volume of staining solution.

Imaging of $CD8^+$ T cells in *ex vivo* skin | Timing: 4.5 h

Wash and prepare for imaging | Timing: 30 min

17. Place an *ex vivo* imaging dish in a 100 x 20 cm dish on top of a heat mat. Wash the dish twice by adding at least 1500 µl *ex vivo* culture medium per wash and swirling the dish such that the entire surface is covered.

    ▲ CRITICAL STEP Keeping the *ex vivo* dish at 37 ºC by working on a heat mat limits the chance of inducing a possible temperature-induced stress response of the T cells.

18. When using an inverted microscopy system, add 2250 µl of medium for imaging to the dish. In case the lid needs to be removed during imaging, a FoilCover ring containing a CultFoil can be placed on top of the dish in order to prevent evaporation.

    When using an upright microscopy setting, fill the dish completely with *ex vivo* culture medium. Push the lid on top of the dish and enclose the dish with parafilm to prevent leakage. Use tissue paper to remove excess liquid that is released.

    ▲ CRITICAL STEP For the upright setting, add ~6 ml of medium (i.e. slightly more than the maximum volume of the dish) to prevent the formation of air bubbles when closing the lid and turning the dish upside down (step 19).

19. Place the dish under the microscope such that the epidermal side is facing the objective.
20. When using a water objective, add water on top of the dish.
21. Search top of epidermis through oculars by focusing on the gas-permeable membrane of the *ex vivo* dish using the mercury light and a FITC filter set.
22. *Mouse.* Set up confocal microscope using the recommended settings outlined in **Table 2**.
23. *Human.* Set up multiphoton microscope using the settings in **Table 2**.

Time-lapse imaging | Timing: 4 h

24. Time-lapse imaging: define the time interval for imaging of the cells of interest. At a minimum, the interval should be larger than the time it requires to image one z-stack. We recommend limiting exposure time as much as possible to prevent tissue damage and photo bleaching. Start recording to collect imaging data.

    Human. For MP imaging, we have used a 3-minute interval, up to 4 hours.

    Mouse. For confocal imaging of mouse tissue, we have used a 2-minute interval, up to 4 hours.

    ! CAUTION Always pause scanning when opening the microscopy chamber, as laser light is harmful to skin and eyes. ! CAUTION Please make sure skin material is disposed according to institutional and national guidelines.

Tracking analysis of CD8$^+$ T cells in *ex vivo* skin | Timing: min. 8 hours

25. Analysis of imaging data can be performed in various ways and has been reviewed in detail elsewhere[50,51]. In brief, to follow CD8$^+$ T cells over time, we advise using the automated 'spots' tracking algorithm (estimated cell size: **human**: ~10 μm; **mouse**: ~14.5 μm) in Imaris (Bitplane), with manual curation of tracks based on visual inspection (e.g. removal of autofluorescent objects and editing tracks of cells that cross paths). Alternatively, if one wants to obtain quantitative information on cell morphology (e.g. sphericity, cell ellipsoid axis, bounding box length), tracking can be performed using the 'surfaces' algorithm. Please note that after manual curation of tracks (e.g. virtually cutting the surface of two objects that cross paths), the resulting surfaces may not accurately describe cell morphology anymore.

    There are several optional steps that can be performed prior to tracking analysis to improve classification of CD8$^+$ T cells, depending on the quality of the imaging data:

- MP datasets can be processed in various ways to aid segmentation of the cells and tissue of interest (e.g. filtering, background subtraction, deconvolution, denoising). We opted for a Gaussian filter to smooth the fluorescent objects.
- If fluorescent objects deeper in the tissue are less visible than in the top, an attenuation correction can be applied, based on the measured intensities in the top and bottom of the z-stack.
- In case of minor x-, y-, or z-axis drift, migration steps can be corrected manually[22,51] or by using a 'reference frame' correction in Imaris, based on coordinates of stationary objects, such as collagen type I structures (SHG signal).

Migration statistics can be obtained from the software and analyzed further when desired, as described elsewhere[22,51]. As a means to check for an effect of the imaging procedure on cell behavior, one can for instance assess whether migration parameters stay constant throughout the recording[22] (see also 'Troubleshooting – step 24'). In order to generate visual output

Table 2 | Recommended microscopy settings

Action	Multiphoton microscopy (when using >2 colors)	Confocal microscopy
Select image size	■ 25x objective, 1.25x zoom ■ Typical max. scanned field area: 354.29 μm^2	■ 20x objective, 2x zoom ■ Typical max. scanned field area: 387.5 μm^2
Select pixel size	■ Typical x,y,z-measures: 0.462 μm^2	■ Typical x,y,z-measures: 0.8 μm^2
Select scan modus	■ Resonant scanner: 768x768 format, 8000 Hz	■ 512x512 format, 400 Hz
Select averaging	■ Line average: 3x	■ Line average: 4x
Activate scanner	■ Activate bidirectional scanning	■ NA
Choose fluorescence detectors and set gain values (10-100%)*	Band-pass emission filters: ■ 615/30 (AF594) ■ 525/50 (AF594, SHG) ■ 450/65 (Hoechst)	Collection: ■ 498-550 nm (GFP) ■ 604-700 nm (AF594)
Set pinhole	■ NA	■ 1 AU
Set shutter	■ Open laser shutter	■ NA
Tune wavelength	■ Tune laser to 800 nm (AF488, AF594, Hoechst) or 1,050 nm (SHG).	■ NA
Set scan range**	■ Z: ~130 μm ■ Step size: ~1 μm	■ Z: ~23 μm ■ Step size: ~1 μm

* Optional: collect bright field signal to include information on tissue structure.

** Optional: laser attenuation in z-direction can be used to obtain higher signal to noise ratio in deeper tissue.

of the data, recordings can be exported in a variety of ways, for instance as 2D-projections (i.e. multiple Z-planes compressed onto a single plane) or 3D time series.

In general: please consult the reference manual of the analysis software of choice for further guidance on image analysis and data visualization. In our experience, the time required for analysis of one 4-hour recording ranges from 8 hours up to a week (see also **Table 1**), and is dependent on factors such as the data quality, the processing power of the computer, and the magnitude of the $CD8^+$ T cell infiltrate.

TIMING

The optional production, purification and labeling of nanobodies (**Box 1**) takes about 5 days. The remainder of the protocol described here takes about 2.5 days and can be roughly divided into 4 phases: preparation of the *ex vivo* culture system (timing: min. 2 h), resting and *in situ* labeling of *ex vivo* skin (timing: 12 h, i.e. overnight), imaging (timing: 4.5 h) and tracking (timing: min. 8 h) of $CD8^+$ T cells and other immune cells in *ex vivo* skin. See **Table 1** for an overview of the timing of the various steps.

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
7	Biopsy does not attach to gas-permeable membrane of *ex vivo* dish during mounting	There is either too little or too much liquid on the epidermis, also edges of biopsy may curl up	If the biopsy is insufficiently moist, it will not attach to the gas-permeable membrane of the dish. Conversely, if there is liquid between the gas-permeable membrane and the biopsy, the biopsy will not stay in one place. Repeat the cycle of submerging the biopsy in HBSS and drying it on a sterile gauze pad (steps 4-6) before mounting again. Choose a different spot on the gas-permeable membrane if the original one is wet. In case the tissue curls up, use the filter and forceps as described in step 10, to enclose and flatten the biopsies on the gas-permeable membrane.
24	No fluorescent signal of staining reagent	Incorrect microscopy settings, reagent concentration is insufficient, skin tissue is too thick or impermeable, absence of cells of interest	Please check all microscopy settings (step 19-23). $CD8^+$ T cell and epidermal $CD103^+$ T cell abundance varies between individuals and skin type. In the recommended concentrations (step 14), collagen type IV, CD1a and Hoechst staining are expected to be positive in all healthy skin samples, however fluorescence intensity may vary between biopsies. If there is no fluorescent signal detected in any of the recommended 4 biopsies per staining concentration, repeat the experiment with skin material from another individual. To investigate whether cells of interest are present in the skin, analyze material by flow cytometry[22], immunohistochemistry or immunofluorescence following standard protocols, using either a separate biopsy or biopsy material post imaging.
	Fluorescent signal decreases over time	Photo-bleaching	Decrease laser exposure, for instance by lowering the laser power, increasing Z-step size, and/or increasing the time interval between stacks.
	x,y,z-axis drift	Movement of biopsy	If shift in z-axis is minimal, choose new coordinates for a recording of the same imaging area. If local shift is more profound, search new imaging area of the same biopsy. If shift is too profound, image a different biopsy. Optional: x,y,z-axis drift can be corrected virtually, see also step 25.
	Images are dim or out of focus	Water on objective has dried out	In case of long imaging times, the water solution may evaporate. If possible, apply new water solution during the interval time, or pause the recording to apply more water.
	Failure of HyD-detector during scanning	Scanning of highly fluorescent object (e.g. hair, pigment)	HyD-dectors are highly light sensitive and when a highly fluorescent object exceeds the detection threshold, HyD-detectors go into a fail-safe mode to prevent damage. Restart the recording to proceed with imaging.

Table 3 | Troubleshooting table (continued)

Step	Problem	Possible reason	Solution
	$CD8^+$ T cells change behavior over time (unrelated to any treatment)	Photo-toxicity	Decrease the laser exposure, for instance by lowering the laser power, increasing Z-step size and/or increasing the time interval between stacks. The consistency of migration parameters over time (e.g. speed) can be analyzed to check whether the imaging process affects cell behavior. Analysis of $CD8^+$ T cells in murine *ex vivo* skin can serve as a positive control[22].
	Staining with a high volume of conventional antibody results in round and immobile $CD8^+$ T cells	Sodium azide	Commercially available antibodies contain sodium azide in order to prevent bacterial growth. In our experience, when high volumes of antibody (≥10% of final volume) are necessary to obtain fluorescent signal in *ex vivo* human skin, $CD8^+$ T cells display a round morphology and immobility (see also **Fig. 2a** and **Supplementary Video 2** for co-staining with anti-hCD103-AF488). To overcome this issue (**Fig. 2b**), sodium azide can be removed by use of an Ultra-0.5 Centrifugal Filter Unit 100 kDa Amicon® (Merck Millipore, cat. no: UFC510096), according to the manufacturer's protocol, and the obtained antibody can subsequently be diluted in PBS. Note that the shelf life of the resulting antibody solution is reduced due to an increased chance of bacterial contamination after the removal of sodium azide.

ANTICIPATED RESULTS

This protocol allows for the labeling and longitudinal (4 h) tracking of $CD8^+$ T cells in *ex vivo* murine and human skin[22]. Please note that all *ex vivo* human samples display an autofluorescent layer at the top of the epidermal side of the biopsy (**Fig. 3**). **Figure 4** shows anti-hCD8-$AF594^+$ nanobody-labeled T cells and **Supplementary Video 3** gives examples of $CD8^+$ T cell tracking in *ex vivo* human skin. Note that the number of skin-resident $CD8^+$ T cells can vary between individuals and biopsies, ranging from 0 up to approximately 30 in both the epidermis and papillary dermis per imaging field (as defined in[22], n=4). Individual $CD8^+$ T cells show variable migration speeds (median 0.5 μm/min, up to 6 μm/min), which is in part correlated with the local collagen type I density for dermal $CD8^+$ T cells[22]. In addition to labeling of $CD8^+$ cells, labeling with Hoechst (**Fig. 5a**) and antibodies against proteins such as CD1a (i.e. Langerhans cells, **Fig. 5b**), CD103 (**Fig. 2b**) and collagen type IV (**Fig. 5c**), allows for the real-time study of other immune cell compartments within their anatomical context[22]. Furthermore, label-free imaging of collagen type I can be used to provide additional anatomical context. $CD8^+$ T cells in the epidermis are expected to migrate in the basal layer below sessile Langerhans cell bodies, along the lining of the dermal collagen type I structures. CD103 signal is primarily located at the trailing end of migrating epidermal $CD8^+$ T cells. In the papillary dermis, $CD8^+$ T cells are expected to migrate in collagen type I poor and dense areas, with some cells moving along collagen type IV positive dermal vessels[22].

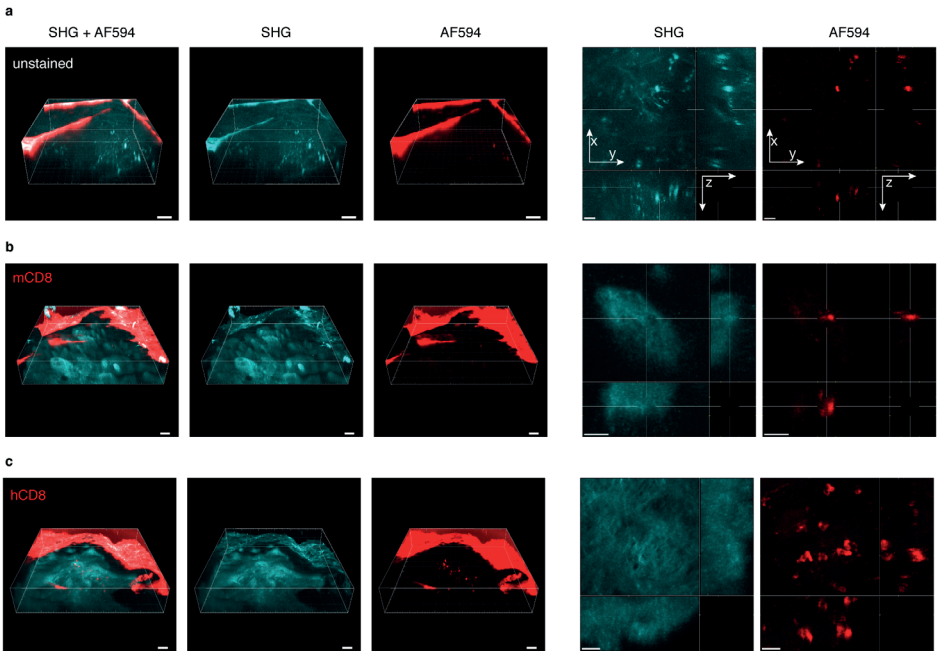

Figure 3 | Second harmonics generation (SHG) and AF594⁺ signal in *ex vivo* human biopsies stained with control and anti-human CD8 nanobodies. Left panels: Perspective view of MP-recordings of an ex vivo human skin sample that was left untreated (**a**), stained with control anti-murine CD8-AF594 nanobody (**b**), or stained with anti-human CD8-AF594 nanobody (**c**). Red signal indicates the AF594 channel and the blue signal represents second harmonic generation signal (SHG). Note that there is AF594⁺ SHG⁺ fluorescent signal at the epidermal side in all conditions (a-c). Right panels: MP-recordings showing a section view of areas where AF594⁺ signal was observed. Note that there is autofluorescent signal (i.e. AF594⁺SHG⁺ objects) (a), minimal background signal (b) and specific signal (c) in these biopsies. Data are representative of n=3 (a, b) or n=4 (c) individuals. Dataset in (b) and (c) are described in Fig. 3a in [22]. Scale bar indicates 50 μm (left panels) and 10 μm (right panels).

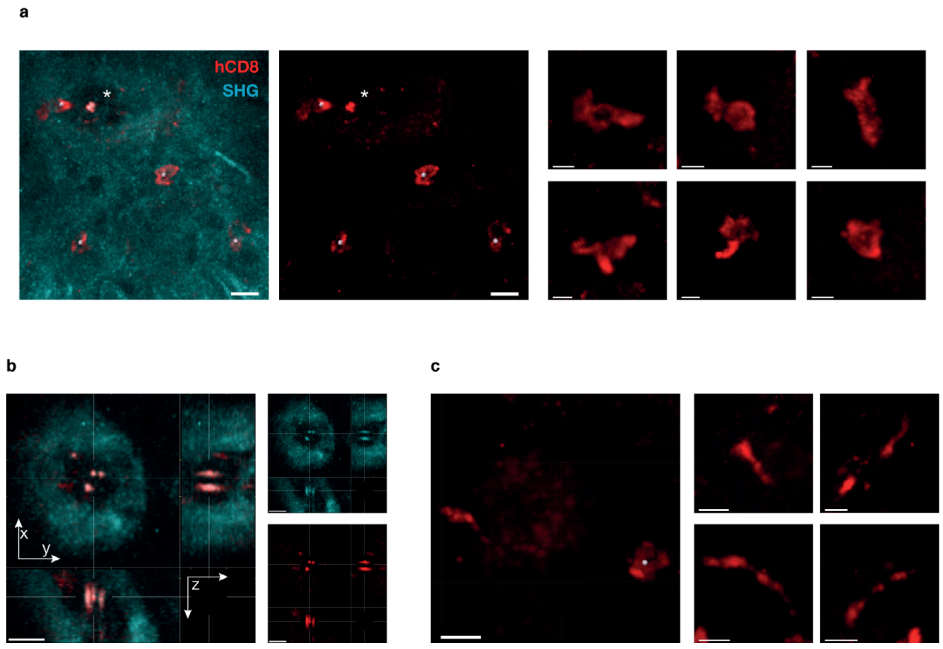

Figure 4 | Labeling and tracking of CD8$^+$ T cells in *ex vivo* human skin. MP-recordings showing an *ex vivo* human skin biopsy stained with anti-hCD8-AF594 nanobody (red) also depicting second harmonics generation signal (SHG, collagen type I, blue). **a**, Top view of stained biopsies showing AF594$^+$ objects classified as CD8$^+$ T cells (i.e. ~10 μm, intact). Asterisk indicates autofluorescent object (AF594$^+$SHG$^+$). While dots indicate CD8$^+$ T cells. Scale bar indicates 15 μm (left) or 10 μm (right, zoomed views). Note that CD8$^+$ T cells display various shapes. **b**, Section view of stained biopsy depicting examples of irrelevant autofluorescent objects (AF594$^+$SHG$^+$). Scale bar indicates 15 μm. **c**, Top view of stained biopsies depicting examples of AF594$^+$ objects that are occasionally observed in biopsies and are not classified as CD8$^+$ T cells (i.e. stretched, fragmented). White dot indicates CD8$^+$ T cell. Scale bars indicate 10 μm. Right images show zoomed views. See also **Supplementary Video 3** for examples of a-c. Dataset in (a-c) is described in Fig. 4a in [22]. Representative of n=4 individuals.

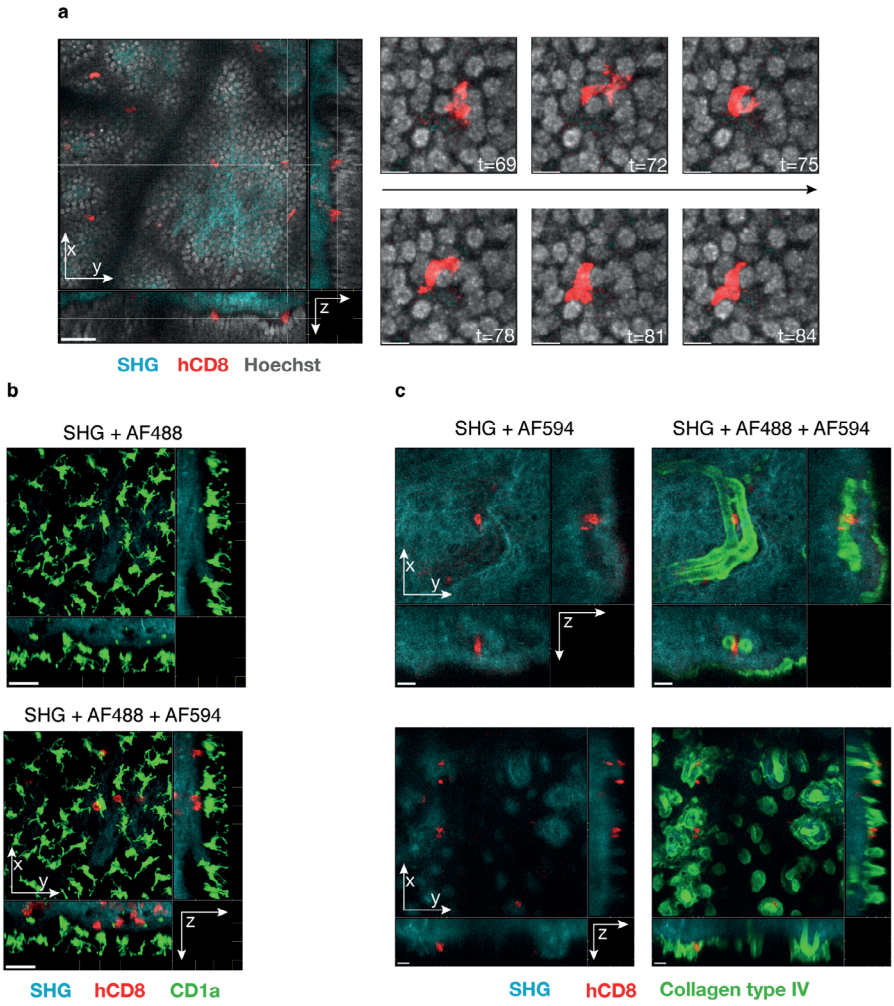

Figure 5 | Visualization of immune cells and structural components in *ex vivo* human skin using conventional staining reagents. a, Left: Section view of MP-recording of *ex vivo* human skin showing collagen type I signal (SHG, blue), and staining with anti-hCD8-AF594 nanobody (red) and Hoechst 33342 (grey). Scale bar indicates 50 μm. Right: Top view of $CD8^+$ T cell migrating in between $Hoechst^+$ epidermal skin cells. Scale bar indicates 10 μm. Representative of n=4 individuals. Dataset is described in Supplementary Video 9-II in [22]. **b**, Sectioning of MP-recording of *ex vivo* human skin tissue showing collagen type I signal (SHG, blue), and staining with anti-CD1a-AF488 conventional antibody (Langerhans cells, green) and anti-hCD8-AF594 nanobody ($CD8^+$ T cells, red). Note that the Langerhans cells point their dendrites upwards and are located above the $CD8^+$ T cells that are present in the dermis (blue) and epidermis (black). Representative of n=4 individuals. Scale bar indicates 50 μm. Dataset is described in Fig. 3h in [22]. **c**, Section view showing two MP-recordings (top and bottom) depicting collagen type I signal (SHG, blue), and staining with anti-hCD8-AF594 nanobody (red) and anti-collagen type IV-AF488 conventional antibody (green). Note that collagen type IV staining demarks the basement membrane and dermal vessels. Dataset in c (bottom) is described in Fig. 4c (bottom) in and Supplementary Video 14-III[22]. Data representative of n=3 individuals. Scale bars indicate 20 μm. See also **Supplementary Video 4** for examples (a-c).

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Author contributions

F.E.D. and D.W.V. conceived the idea of the murine *ex vivo* imaging setup. F.E.D. designed and performed experiments with murine and human skin, and analyzed imaging data. M.T. generated nanobodies and nanobody conjugates. M.H. performed multiphoton imaging experiments. F.E.D. and M.M. designed imaging analysis. T.R.M. and M.B.M.T. organized human skin material. F.E.D., M.T., M.H., T.R.M., M.B.M.T., D.W.V., R.M.L. and T.N.S. contributed to protocol design. F.E.D. and T.N.S. wrote the manuscript with input from all co-authors.

Competing interests

The authors declare no competing interests.

Data availability

Examples of the primary datasets underlying all data Figures and Supplementary Videos presented in this article can be found on 10.5281/zenodo.3843094. **Supplementary Video 1-4** and **Supplementary File 1** are available upon request from the first author.

Related links

Key reference using this protocol:

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Box 1 | Expression, purification and labeling of nanobodies. Timing: ~5 days

This procedure describes the expression, purification and labeling of nanobodies. Nanobodies are approximately 10-fold smaller in size than conventional antibodies (i.e. 15 kDa versus 150 kDa[43]), which may improve penetration into tissues such as the skin. The production of nanobodies in *Escherichia coli* is relatively straightforward and cost-effective. In addition, nanobodies can be easily modified via genetic engineering, and coupled to a fluorescent label of choice via a sortase reaction[43,47].

Note that steps 22-24 (labeling of GGGC peptide to maleimide dye) can be performed prior or in parallel to steps 1-21 (nanobody expression and purification).

Nanobody expression and purification. Timing: 4 days

1. Transform the pHEN6c plasmid containing the LPETGG-6xHis sequence and the desired nanobody into *E. coli* WK6 by heat shocking for 45 sec at 42 °C. The pelB leader sequence present in the pHEN6c vector ensures that the protein is expressed in a soluble form in the periplasm of the WK6 *E. coli*. Please see **Supplementary data 1** for the empty pHEN6c-[NcoI-BstEII]-LPETGG-6xHis plasmid sequence. Note that restriction sites NcoI and BstEII can be used to clone the desired nanobody sequence into the expression vector.
2. Add 1 ml of Luria-Bertani (LB) medium to the bacteria and inoculate 50 ml of LB supplemented with 50 μg/ml carbenicillin in a 250 ml sterile Erlenmeyer flask.
3. Grow the preculture overnight at 37 °C shaking at 200 r.p.m.
4. Inoculate 500 ml of Terrific Broth (TB) (supplemented with 0.4% (vol/vol) glycerol and 50 μg/ml carbenicillin) in a 2 L baffled flask with 10 ml of the preculture.
   ▲ CRITICAL STEP The culture volume can be easily scaled up to 3 L in order to obtain more protein.
5. Shake the mixture at 37 °C and 200 r.p.m. until it reaches an OD_{600} of 0.6-0.8. This will take about 2-3 h; measure OD_{600} regularly after 2 h.
   ▲ CRITICAL STEP Aim for an OD_{600} of 0.6, in order to obtain the highest nanobody yield after induction with IPTG in the next step.
6. Induce nanobody expression with 1 mM IPTG (final) and grow overnight at 200 r.p.m. at 30 °C.
7. Collect the bacteria by centrifugation for 20 min at 8,000*g* at room temperature (RT, 20-22 °C).
8. Carefully resuspend the cell pellet of 500 ml of culture in 15 ml of ice-cold **TES buffer** (200 mM Tris (pH 8), 0.65 mM EDTA, 0.5 M sucrose), and incubate for at least 1 h at 4 °C on an orbital shaking platform at 70 r.p.m.

9. To selectively release the periplasmic fraction into the supernatant via an osmotic shock, add 30 ml of **0.25 x TES buffer** (diluted in H_20) to resuspend the pellet and continue rotating at 4 °C overnight.
10. Centrifuge the suspension for 15 min at 8,000*g* at 4 °C and recover the supernatant (i.e. the periplasmic extract).
11. Repeat step 10 with the supernatant.
12. In order to subsequently purify the His-tagged nanobodies from the periplasmic extract, prepare for nickel-based gravity-flow chromatography while step 11 is ongoing. Prepare 2 ml of NiNTA beads (i.e. 4 ml of a 50% (vol/vol) beads suspension) by adding them to a 50 ml conical tube and washing them with 25 ml **NiNTA wash buffer** (50 mM Tris (pH 8), 150 mM NaCl, 10 mM imidazole).
13. Pellet beads at 300*g* for 2 minutes. Remove wash.
14. The supernatant can now be transferred to the 50 ml conical tube with NiNTA beads. Incubate for 1 h at 4 °C on a roller mixer, and centrifuge for 3 min at 300*g* at 4 °C.
15. Remove and discard the supernatant.
16. Wash beads by filling the 50 ml conical tube with 25 ml **NiNTA wash buffer**, and spin down the beads for 3 min at 300*g* at 4 °C. Repeat once, so that the beads have been washed with approx. 50 ml of wash buffer.
17. Resuspend beads in 5 ml wash buffer and transfer from the 50 ml conical tube to a disposable Poly-Prep® chromatography column. After the column has settled, rinse the 50 ml conical tube with another 5 ml of wash buffer.
18. Elute with 6 ml **elution buffer** (50 mM Tris (pH 8), 150 mM NaCl, 0.5 M imidazole) into a 15 ml tube.
19. Measure the protein concentration on a spectrophotometer (e.g. NanoDrop). Optional: check purity on a 4-12% gradient SDS PAGE gel.
20. Purify the obtained nanobody by gel filtration chromatography (e.g. using a Biosep 3000 Phenomenex column), for instance on an HPLC system. Collect the appropriate fraction(s). Optional: check purity on a 4-12% gradient SDS PAGE gel.
21. Measure protein concentration on a spectrophotometer (e.g. NanoDrop). The expected yield is 10-15 mg per 500 ml of culture (e.g. of the anti-mCD8 or anti-hCD8 nanobody).

Labeling of GGGC peptide to maleimide dye. Timing: ~1 day and freeze dry overnight

▲ CRITICAL STEP These steps can be performed prior or in parallel to steps 1-21.

22. To label the GGGC peptide with the fluorescent dye, add 1 mg of maleimide dye (e.g. Alexa Fluor™ 594 C5 maleimide) in 50 µl DMSO, 10 mM $NaHCO_3$ and 200 µM GGGC peptide and incubate for 2 h at RT.

23. Purify on a C18 column on an HPLC system and elute with a gradient of acetonitrile.
24. Collect the fraction, freeze dry and dissolve in DMSO at a concentration of 4 mM.
    ■ PAUSE POINT The GGGC dye can be stored for at least a year at -20 °C.

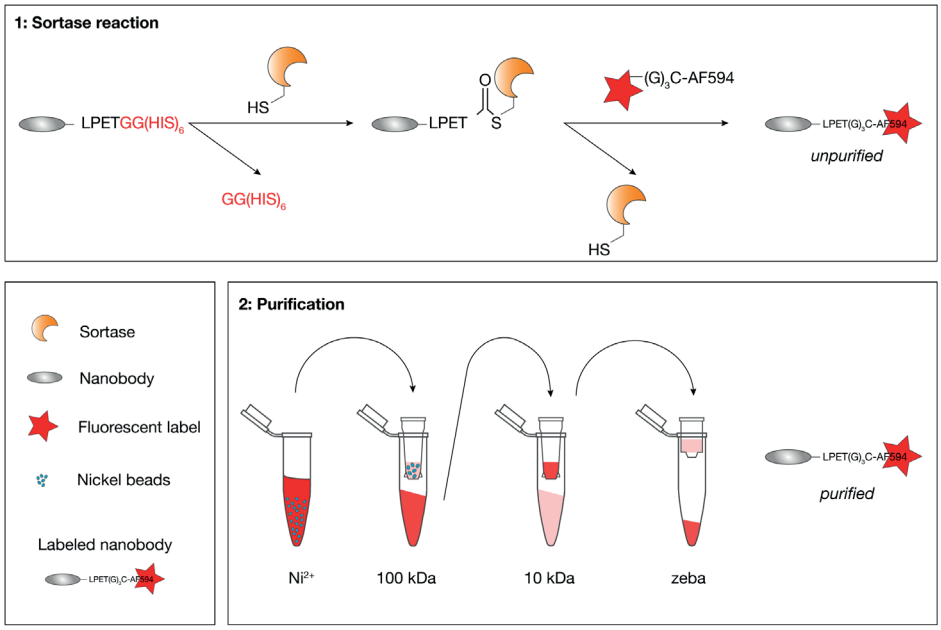
Labeling of nanobody to GGGC dye via sortase reaction and purification. Timing: ~ 3 h

25. To covalently link the purified nanobodies to the GGGC-dye, perform a sortase-mediated ligation. Perform the reaction in 500 µl **sortase buffer** (50 mM Tris (pH 8), 150 mM NaCl), according to the following table using either 5M or 7M sortase*:

Calcium-dependent sortase (5M)	Calcium-independent sortase (7M)
2.5 µM purified nanobody-LPETGG-6xHis	2.5 µM purified nanobody-LPETGG-6xHis
40 µM GGGC-dye	40 µM GGGC-dye
0.4 µM penta-mutant (5M) sortase*	0.4 µM hepta-mutant (7M) sortase*
10 mM $CaCl_2$	-

* Sortase is produced in-house according to a previously described protocol using sonication instead of French press (timing: 4-5 days)[47].

26. Incubate the reaction for 2h at 4 °C.
27. Remove the unbound nanobody fraction and free label using 25 µl of Ni-NTA beads (i.e. 50 µl of a 50% (vol/vol) beads suspension).
28. Rotate the suspension gently on a rotating wheel for 30 min at 4 ºC.
29. Remove the NiNTA beads by running the suspension over a 100 kDa-cutoff filter and centrifuging according to the manufacturer's protocol. Collect the eluate which contains the labeled nanobody.
30. Add flow through from the previous step to a 10 kDa-cutoff filter and centrifuge according to the manufacturer's protocol. To exchange the buffer with PBS, repeat the centrifugation step 3 times by adding PBS on top of the filter. Concentrate to ~130 µl.
31. Purify the material further using a zeba spin column by loading the concentrated protein from step 30 on the pre-washed (in PBS) zeba column according to the manufacturer's protocol, and collect the flow through. This contains the purified labeled nanobody. The nanobody reagent can be stored in aliquots at -20 °C up to a year.
    ▲ CRITICAL STEP To adjust for variability in labeling efficiency between nanobody batches, titrate each batch on relevant (e.g. CD8 positive) and irrelevant cells (e.g. CD8 negative), and check the performance of each batch on an *ex vivo* skin sample.



Box 1 | Sortase-mediated labeling and purification of nanobodies. 1: Sortase reaction Nanobodies containing an $LPETGG(HIS)_6$ tag are covalently linked to the GGGC-dye via sortase-mediated ligation. **2: Purification** In order to purify the nanobody solution, four subsequent purification steps are performed. First, to remove the non-modified nanobody fraction (i.e. still containing the His-tag), the nanobody solution is incubated with Ni-NTA (i.e. nickel, $Ni2^+$) beads. Second, the solution is loaded onto a 100 kDa cutoff filter to remove the $Ni2^+$ beads. Third, in order to remove free fluorescent label, the flow through is loaded onto a 10 kDa cutoff filter and exchanged in PBS. Fourth, the concentrated nanobody solution recovered from the 10 kDa filter is loaded onto a zeba spin column to remove any remaining free fluorescent label.

SUPPLEMENTARY INFORMATION

Supplementary Files

Supplementary File 1 | pHEN6c-[NcoI-BstEII]-LPETGG-6xHis plasmid sequence. DNA sequence of the empty pHEN6c expression vector containing the LPETGG (i.e. Sortag) and 6xHis (i.e. Histag) sequence and the NcoI and BstEII cloning sites, which can be used to insert the desired nanobody sequence.

Supplementary Videos

Supplementary Video 1 | CD8$^+$ T cells in murine *ex vivo* skin regain motility and dendricity after overnight culture. Maximum confocal microscopy projection of OT-I GFP$^+$ CD8$^+$ T cells (green) in mouse skin imaged directly after mounting in *ex vivo* imaging setup. Cells start off round and immobile and regain mobility and dendricity over time (time in minutes). N=2 recordings and 2 independent observations. Scale bar indicates 50 μm.

Supplementary Video 2 | Removal of sodium azide from conventional antibody reagent allows detection of migrating epidermal CD8$^+$ CD103$^+$ T cells in *ex vivo* human skin. First segment: Section view of MP recording showing *ex vivo* human skin biopsy stained with anti-hCD103-AF488 conventional antibody (green) without removal of sodium azide, also showing anti-hCD8-AF594 nanobody staining and second harmonics generation signal (SHG, blue). Note that CD8$^+$ T cells display a round morphology and are immobile. Representative of n=2 individuals. Scale bar indicates 20 μm. Second segment: Section view of MP recording showing *ex vivo* human skin biopsy stained with anti-hCD103-AF488 conventional antibody (green) after removal of sodium azide, also showing anti-hCD8-AF594 nanobody staining and second harmonics generation signal (SHG, blue). Note that CD8$^+$ CD103$^+$ T cells display a variable morphology and migrate. Data in second segment adapted from Supplementary Video 11 in [22]. Representative of n=3 individuals. Scale bar indicates 15 μm.

Supplementary Video 3 | Labeling and tracking of CD8$^+$ T cells in *ex vivo* human skin. First segment: Top view of MP-recordings showing examples of tracked anti-hCD8-AF594 nanobody labeled T cells (red) that migrate in *ex vivo* human skin. Second segment: Examples of autofluorescent and fragmented AF594$^+$ objects in *ex vivo* human skin biopsies stained with anti-hCD8-AF594 nanobody (red), also showing second harmonics generation signal (SHG, collagen type I, blue). Both segments: lines indicate tracked cells. Datasets are described in Fig. 4a in [22]. Data representative of n=4 individuals. Scale bars indicate 10 μm.

Supplementary Video 4 | Visualization of immune cells and structural components in *ex vivo* human skin using conventional staining reagents. First segment: Top view of MP-recording showing anti-hCD8-AF594 nanobody-labeled CD8$^+$ T cell (red) migrating in between Hoechst$^+$ nuclei (grey) in the epidermis. Dataset described in Supplementary Video 9-II in [22]. Representative of n=4 individuals. Scale bar indicates 10 μm. Second segment: Section view showing anti-hCD8-AF594 nanobody-labeled CD8$^+$ T cell (red) migrating below anti-hCD1a-AF488 antibody labeled Langerhans cells (green), also showing second harmonics generation signal (collagen type I, blue). Dataset described in 3h and Supplementary Video 12-I in [22]. Representative of n=4 individuals. Scale bar indicates 50 μm. Third segment: Section view of MP-recording showing anti-hCD8-AF594 nanobody labeled CD8$^+$ T cells (red) migrating alongside anti-human collagen type IV positive structures (i.e. basement membrane and dermal vessels, green), also showing second harmonics generation signal (collagen type I, blue). Dataset is described in 4c (bottom) and Supplementary Video 14-III in [22]. Representative of n=3 individuals. Scale bar indicates 20 μm.