In Vivo Imaging of T-Cell Motility in the Elicitation Phase of Contact Hypersensitivity Using Two-Photon Microscopy

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TO THE EDITOR

The skin is not merely a physical barrier; it is also an active organ of the immune system. A variety of immune responses are constantly induced in the skin to defend against foreign pathogens. Contact hypersensitivity (CHS) is one of the most intensively investigated immune responses because it is regarded as the prototype of T cell–mediated delayed-type hypersensitivity reaction (Grabbe and Schwarz, 1998). Most of the previous studies on the pathophysiology of CHS have focused on the sensitization phase, when naive T cells are primed and differentiated into antigen-specific effector T cells. The precise mechanism of the elicitation phase of CHS, however, remains to be elucidated.

Recently, two-photon microscopy has been used to investigate the behavior of immune cells in living tissues. Real-time imaging studies have revealed that naive T cells migrate at a velocity of ~10 μm per minute in the lymph nodes (LNs). They conjugate with dendritic cells for several hours when they encounter their specific antigen (Miller *et al.*, 2002; Stoll *et al.*, 2002). Two-photon microscopy has also been used to examine the behavior of immune cells in the skin. The mechanism of neutrophil and dendritic cell recruitment against *Leishmania major* infection was analyzed in mouse ear skin (Ng *et al.*, 2008; Peters *et al.*, 2008). The motility of $CD4^+$ effector T cells was visualized in an ovalbumin-induced delayed-type hypersensitivity model in rat ear explants (Matheu *et al.*, 2008). In the present study, we induced skin inflammation by applying an irritating reagent or hapten on the skin, which is physiologically similar to irritant- and allergen-induced contact dermatitis. The motility of T cells in the skin was visualized by two-photon microscopy *in vivo*. We compared T-cell motility between the irritant dermatitis model and the allergic contact dermatitis model.

Initially, we examined the irritant (nonallergic) contact dermatitis model with phorbol-12-myristate-13-acetate (PMA) applied to the skin. In this model, naive T cells were activated and differentiated into effector T cells *in vitro* to acquire the capacity to infiltrate into inflamed peripheral tissues. $CD4^+$ and $CD8^+$ T cells were separately isolated from the LNs and the spleen of naive BALB/c mice by negative selection using automated magnetic cell sorting (auto MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). This was followed by incubation for 72 hours at 37 °C in the presence of plate-bound anti-CD3/CD28 antibodies (10 μg ml^{-1} each, eBioscience, San Diego, CA), IL-2 (20 μg ml^{-1}, R&D Systems, Minneapolis, MN), IL-12 (10 μg ml^{-1}, R&D Systems), and anti-IL-4 antibody (10 μg ml^{-1}, R&D Systems). Memory $CD4^+$ and $CD8^+$ T cells were labeled with CellTracker Red CMTPX (10 μM, Invitrogen, Carlsbad, CA) and carboxyfluorescein succinimidyl ester (10 μM, Invitrogen), respectively. A total of 1×10^7 cells of each subset were transferred intravenously into the recipient BALB/c mouse. Subsequently, 20 μl of 100 μg ml^{-1} PMA in acetone was applied to one ear; the other ear was left untreated. Twenty hours later, the mouse was anesthetized with isoflurane and the T-cell motility in the ear skin was visualized by two-photon microscopy. Without PMA application, none of the labeled T cells transmigrated to the skin (data not shown). By contrast, PMA application induced infiltration of both $CD4^+$ and $CD8^+$ T cells into the dermis but not into the epidermis (Figure 1a and b and Supplementary Movie S1 online), and they migrated smoothly in the dermis without resting. In addition, we could not find any significant difference in cell distribution or mean migration velocity between $CD4^+$ and $CD8^+$ T cells (6.3 ± 2.8 μm per minute and 6.2 ± 2.3 μm per minute, respectively; Figure 1c and d).

Next, we examined whether T-cell motility in the skin depends on T-cell receptor specificity in the CHS model. Mice were sensitized with 25 μl of 0.5% DNFB (Nacalai Tesque, Kyoto, Japan) or 100 μl of 7% trinitrochlorobenzene (TNCB; Tokyo Chemical Industry, Tokyo, Japan). Six days later, T cells were purified from each of the draining LNs and then labeled with carboxyfluorescein succinimidyl ester and CMTPX, respectively. Labeled T cells were transferred intravenously into naive mice, and the ear was subsequently challenged with 20 μl of 0.5% DNFB or 20 μl of 1% TNCB topically. Twenty hours after elicitation, both DNFB- and TNCB-sensitized T cells transmigrated into the dermis regardless of the types of haptens (Figure 2a), whereas nonactivated T cells from naive mice rarely transmigrated to the skin (data not shown). In the DNFB-applied skin, almost all the TNCB-sensitized T cells migrated smoothly in the dermis, whereas some of the DNFB-sensitized T cells remained immobile for over 1 hour (Figure 2a and b and Supplementary Movie S2 online). The mean velocity was comparable between T cells sensitized with TNCB and T cells in PMA-induced skin inflammation, whereas the mean velocity of T cells

Abbreviations: APC, antigen-presenting cell; CHS, contact hypersensitivity; LN, lymph node; PMA, phorbol-12-myristate-13-acetate; TNCB, trinitrochlorobenzene; YFP, yellow fluorescent protein

sensitized with DNFB was significantly decreased (2.9 ± 3.0 μm per minute) owing to the presence of several immobile cells (Figure 2c). In addition, reversed motile activities were observed in the TNCB-applied skin (Figure 2d).

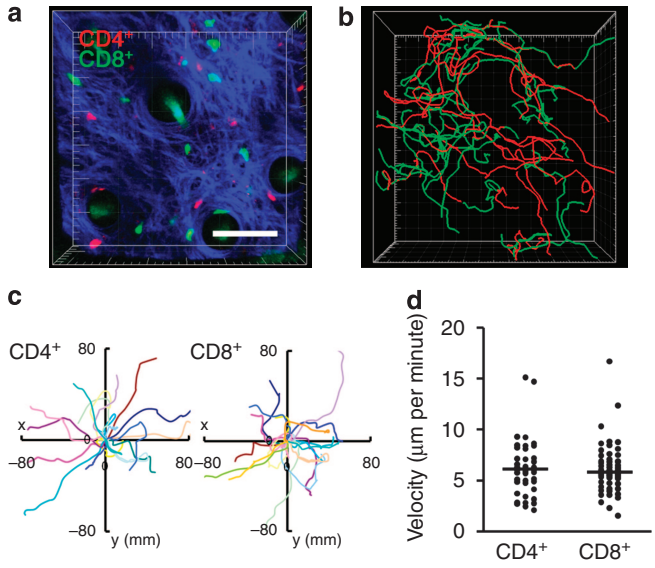

Figure 1. T-cell motility in the PMA-induced irritant dermatitis. (**a**) A snapshot of skin-infiltrated CD4$^+$ (red) and CD8$^+$ (green) T cells. Bar = 100 μm. (**b**) Sixty-minute tracks of CD4$^+$ (red) and CD8$^+$ (green) T cells from the same imaging data set. (**c**) Superimposed 10-minute tracks of 40 randomly selected CD4$^+$ (right panel) and CD8$^+$ (left panel) T cells in the *x*–*y* plane, setting the starting coordinates to the origin. (**d**) Summary of mean velocity of CD4$^+$ and CD8$^+$ T cells. Data points (n = 42 for CD4$^+$ and n = 50 for CD8$^+$ T cells) represent individual cells compiled from three independent experiments. PMA, phorbol-12-myristate-13-acetate.

These results suggest that skin-infiltrating effector T cells show low motility in the presence of T-cell receptor–specific antigen. Therefore, we examined whether immobile T cells in the skin interacted with antigen-presenting cells (APCs). We used CD11c-YFP (yellow fluorescent protein) mice as a recipient, in which CD11c$^+$ dendritic cells were labeled with YFP. CMTPX-labeled T cells from DNFB-sensitized mice colocalized with YFP$^+$ cells in the DNFB-applied skin (Figure 2e).

In this study, using the two-photon microscopic system, we have demonstrated the *in vivo* dynamic spatiotemporal T-cell mobility in irritant contact dermatitis and the elicitation phase of CHS as models of cutaneous immune responses. In irritant contact dermatitis, T cells infiltrated into the dermis and actively migrated at the mean velocity of ~6 μm per minute. T cells migrate at a velocity of ~10 μm per minute in the LNs (Miller *et al.*, 2002). Thus, T cells in the dermis migrated relatively slowly but still quite efficiently. In the elicitation phase of CHS, we observed that some DNFB-sensitized T cells remained

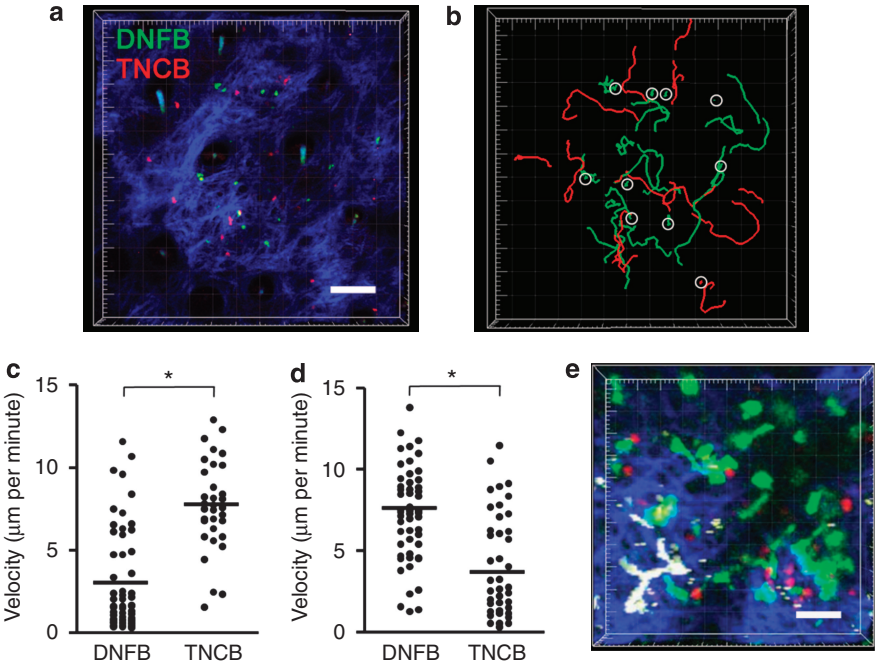

Figure 2. T-cell motility in the elicitation phase of CHS. (**a**) An image of skin-infiltrated DNFB-sensitized (green) and TNCB-sensitized (red) T cells. Bar = 100 μm. (**b**) Thirty-minute tracks of DNFB-sensitized (green) and TNCB-sensitized (red) T cells from the same imaging data set. Circles in panel **b** represent unmoved cells. (**c**, **d**) Summary of mean velocity of DNFB- and TNCB-sensitized T cells in DNFB-elicited skin (panel **c**) and in TNCB-elicited skin (panel **d**). Data points represent individual cells compiled from three independent experiments. *P<0.001 between the indicated groups. (**e**) An image of skin-infiltrated DNFB-sensitized T cells (red) and CD11c$^+$ cells (green). Bar = 40 μm. CHS, contact hypersensitivity; DNFB, dinitrofluorobenzene; TNCB, trinitrochlorobenzene.

still whereas others actively migrated in the DNFB-applied skin. We assume that this distinct migratory activity may be due to the fact that not all T cells in the LNs after DNFB sensitization were DNFB specific. In contrast, almost all TNCB-sensitized T cells actively migrated when DNFB was applied. In addition, we showed reverse motile activities in the TNCB-painted skin. Moreover, skin-infiltrating T cells colocalized with APCs. These findings suggest that skin-infiltrating T cells may actively scan for antigens, and when they meet APCs carrying their specific antigens, they stop migrating and stably interact with APCs. Our results also indicate that in the elicitation phase of CHS, hapten seems to be presented mainly by APCs in the dermis. Additional detailed studies are needed to clarify which subset of dermal APCs is essential for hapten presentation and whether epidermal Langerhans cells contribute.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Human Hair Follicle and Epidermal Melanocytes Exhibit Striking Differences in Their Aging Profile which Involves Catalase

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TO THE EDITOR

Canities or senile hair graying, a universally recognized sign of aging, remains unresolved in terms of physiological causes, although a strong genetic contribution is understood (Gunn *et al.*, 2009). As the hair fiber continues to grow long after melanin production ceases, we suggest that melanocytes in the hair follicle may be more sensitive to the impact of chronological aging than are keratinocytes. Moreover, follicular melanocytes also age more markedly than those in the overlying epidermis. The hair follicle provides a unique opportunity to decouple the impact of age on two hair follicular tissue functions: hair formation and hair pigmentation. Previous studies have pointed to a link between cellular aging mechanisms, including oxidative stress and hair graying (Arck *et al.*, 2006; Wood *et al.*, 2009). Commo *et al.* (2004) and others (Nishimura *et al.*, 2005) have suggested that repopulation of the early anagen hair bulb with precursors of active melanocytes is also increasingly likely to fail with aging. However, most studies differentiate follicles on the basis of the level of pigmentation, not donor age. We also know that hair graying may be partially reversed in some skin disorders or after certain drug therapies (Reynolds *et al.*, 1998; Shaffrali *et al.*, 2002). Thus, graying may not necessarily indicate a complete deletion of the melanocyte stem cell population. We wanted to explore the impact of chronological age on melanocyte behavior to further understand the graying process and to identify associated molecular changes.

This study provides analysis of race, age, and anatomically matched cultures of adult human epidermal and hair follicle melanocytes (HFMs) (Supplementary Table S1 online), and to our knowledge, this is previously unreported. Cultured HFMs showed at least three distinct sub-populations, including highly pigmented/dendritic bulbar melanocytes, less-differentiated tripolar cells, and an undifferentiated amelanotic bipolar sub-population (Supplementary Figure S1 online). By contrast, epidermal melanocytes (EMs) largely consisted of a homogeneous population of highly dendritic and uniformly weakly pigmented cells (Supplementary Figure S1 online). Unlike EMs, the most active melanocytes of the bulb do not survive when explanted *ex vivo*.

Abbreviations: EM, epidermal melanocyte; HFM, hair follicle melanocyte; SOD, superoxide dismutase