TGFβ1 Overexpression by Keratinocytes Alters Skin Dendritic Cell Homeostasis and Enhances Contact Hypersensitivity

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Overexpression of transforming growth factor beta-1 (TGFβ1) in mouse epidermis causes cutaneous inflammation and keratinocyte hyperproliferation. Here we examined acute effects of TGFβ1 overproduction by keratinocytes on skin dendritic cells (DCs). TGFβ1 induction for 2 and 4 days increased the numbers and CD86 expression of $B220^+$ plasmacytoid DCs (pDCs) and $CD207^+CD103^+$, $CD207^-CD103^-CD11b^+$, and $CD207^-CD103^-CD11b^-$ dermal DCs (dDCs) in skin-draining lymph nodes (SDLNs). The dermis of TGFβ1-overexpressing mice had significantly more pDCs, $CD207^+CD103^+$ dDCs, and $CD207^-CD11b^+$ dDCs in the absence of increased dermal proliferation. Application of dye, tetramethyl rhodamine iso-thiocyanate (TRITC), in dibutylpthalate (DBP) solution after TGFβ1 induction increased the numbers of $TRITC^+CD207^-$ dDCs in SDLNs, and augmented TRITC/DBP-induced Langerhans cell (LC) migration 72 hours post TRITC treatment. Consistent with this, LC migration was increased *in vitro* by TGFβ1 overexpression in skin explants and by exogenous TGFβ1 in culture media. Transient TGFβ1 induction during DNFB sensitization increased contact hypersensitivity responses by 1.5-fold. Thus, elevated epidermal TGFβ1 alone is sufficient to alter homeostasis of multiple cutaneous DC subsets, and enhance DC migration and immune responses to contact sensitizers. These results highlight a role for keratinocyte-derived TGFβ1 in DC trafficking and in the initiation of skin inflammation.

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INTRODUCTION

Skin contains a dense network of dendritic cells (DCs) that are initiators of a wide range of immune responses and act by bridging innate and adaptive immunity while maintaining tissue homeostasis in steady state (Steinman, 1991). Langerhans cells (LCs), which are radio-resistant, self-renewing, and characterized by the expression of langerin (CD207), are the primary DC subset in the epidermis of healthy skin (Ginhoux and Merad, 2010). The dermis contains DC subsets broadly classified as $CD207^+CD103^+$ and $CD207^-CD103^-$, with the latter subset further subdivided on the basis of CD11b and other markers (Merad *et al.*, 2008; Henri *et al.*, 2010). Skin DCs acquire and process exogenous antigens, undergo maturation, and migrate to skin-draining lymph nodes (SDLNs) where they induce the activation of naive T cells (Banchereau and Steinman, 1998).

Transforming growth factor beta-1 (TGFβ1) is one of the major regulators of DC biology in the skin. *In vitro* studies show that TGFβ1 is important for promoting LC differentiation from a $CD34^+$ promonocyte bone marrow precursor (Strobl and Knapp, 1999), whereas for both LCs and other DCs, TGFβ1 inhibits activation, maturation, and immunogenicity and promotes tolerogenic function (Geissmann *et al.*, 1999; Fainaru *et al.*, 2007; Ohtani *et al.*, 2009; Torres-Aguilar *et al.*, 2010). *In vivo* studies have demonstrated a critical requirement of both autocrine and paracrine TGFβ1 signaling for LC development and epidermal residency (Borkowski *et al.*, 1996, 1997; Kaplan *et al.*, 2007; Kel *et al.*, 2010; Zahner *et al.*, 2011). However, homeostasis and numbers of $CD207^+CD103^+$ dermal DC (dDC) appear to be unaffected in mice with a deletion of the TGFβ1 type-1 receptor in all $CD207^+$ DCs (Kel *et al.*, 2010; Zahner *et al.*, 2011) and in *Tgfb1*−/− mice (Nagao *et al.*, 2009). In contrast, for $CD207^-CD103^-$ dDCs and for DCs that infiltrate the skin under inflammatory states, there is little information on the *in vivo* role of TGFβ1.

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Abbreviations: BrdU, bromodeoxyuridine; CHS, contact hypersensitivity; DBP, dibutylpthalate; DC, dendritic cell; dDC, dermal DC; DNFB, 2,4-dinitro fluorobenzene; Dox, doxycycline; DT, double transgenic; LC, Langerhans cell; LN, lymph node; pDC, plasmacytoid DC; SDLN, skin-draining lymph node; ST, single transgenic; TGFβ1, transforming growth factor beta-1; TRITC, tetramethyl rhodamine iso-thiocyanate

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Significant increases in epidermal and skin TGFβ1 levels occur in response to inflammatory stimuli (Akhurst *et al.*, 1988), following wounding (Kane *et al.*, 1991; Levine *et al.*, 1993; Wang *et al.*, 2006), in chronic skin diseases such as psoriasis (Kane *et al.*, 1990; Flisiak *et al.*, 2002, 2003) and in premalignant keratinocytes (Glick *et al.*, 1991). Previous studies have shown that long-term overexpression of active or latent TGFβ1 in mouse epidermis causes a chronic inflammatory phenotype associated with keratinocyte hyperproliferation and T-cell infiltration (Liu *et al.*, 2001; Li *et al.*, 2004; Han *et al.*, 2010). However, recent studies have suggested that the TGFβ1-induced inflammation is not solely dependent on T cells (Michaelis *et al.*, 2010), or the interleukin 17/interleukin 23 axis (Fitch *et al.*, 2009), indicating involvement of additional pathways or immune cells.

We previously showed that induction of TGFβ1 in papillomas caused a rapid increase in tumor-infiltrating macrophages and DCs, and an increase in the numbers of $CD11c^+$ and $CD11b^+$ cells in SDLNs (Mohammed *et al.*, 2010), suggesting that tissue inflammation and migration of DCs may be a primary response to elevated TGFβ1 levels. Here we show that induction of active TGFβ1 in the basal layer of mouse epidermis causes significant and rapid changes in cutaneous DC migration and influx, and enhances contact hypersensitivity (CHS) responses. These results provide insight into the role of keratinocyte-derived TGFβ1 in skin DC homeostasis and in the initiation of skin inflammation.

RESULTS

Elevated keratinocyte TGFβ1 increases DC numbers in SDLNs

To determine the effect of elevated epidermal TGFβ1 levels on cutaneous DC populations, we placed 7-week-old double transgenic (DT) (K14rTAxtetOTGFβ1) mice on doxycycline (Dox) chow to induce active TGFβ1 in keratinocytes and then analyzed migratory DC subset accumulation in the SDLNs. We gated on $MHCII^{hi}$ lymph node (LN) cells to identify DCs migrating from the skin and excluded any LN-resident $CD8^+CD103^+CD207^+$ DCs that are $MHCII^{int}$ (Dakic *et al.*, 2004; Kissenpfennig *et al.*, 2005). We identified five skin-derived DC subsets in the SDLNs of FVB/n mice (Figure 1a) similar to those in C57BL/6 mice (Henri *et al.*, 2010): $B220^+$ plasmacytoid DCs (pDCs), $CD207^+CD103^-$ (LC), $CD207^+CD103^+$dDCs, and $CD207^-CD103^-$dDCs, which are either $CD11b^+$ or $CD11b^-$ (referred to as $CD207^-CD11b^+$ dDCs and $CD207^-CD11b^-$ dDCs, respectively). At steady state, $CD207^+$ dDCs constituted the highest percentage of DCs in the LN (36%). Two days after induction of TGFβ1 expression in keratinocytes, there was a significant expansion of the $CD207^-CD11b^-$ subset relative to the other DCs, and this persisted through 4 days (Figure 1b). The percentage of pDCs also increased after

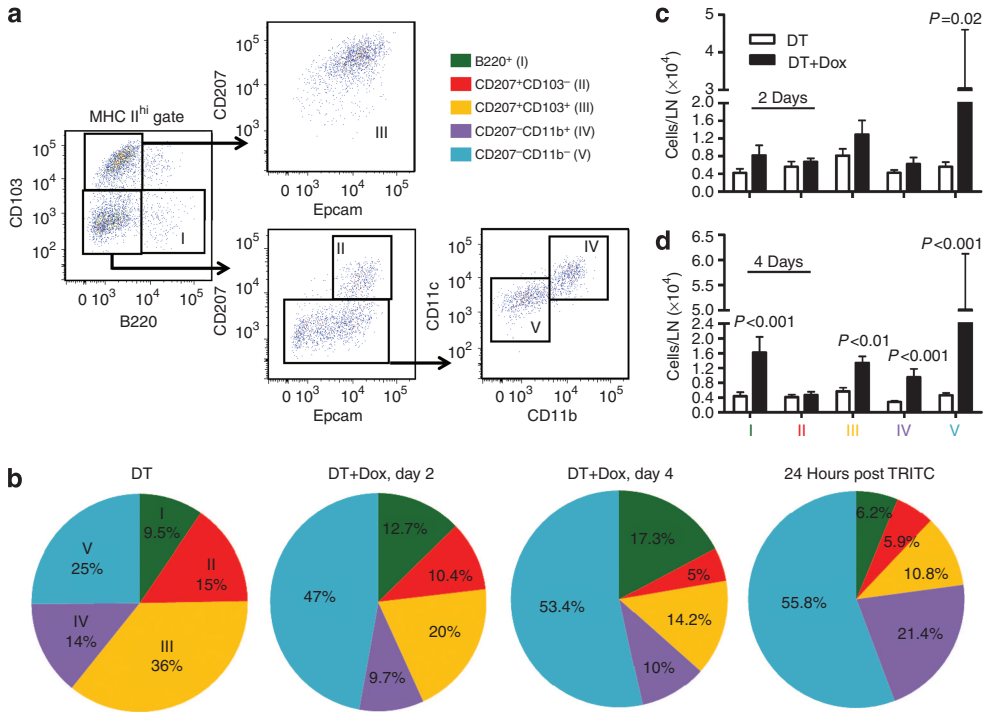

Figure 1. Elevated keratinocyte transforming growth factor beta-1 alters migratory dendritic cell (DC) percentages and numbers. Double transgenic (DT) mice were given regular or doxycycline (Dox) chow (1 g kg^{-1}) for 2 or 4 days and skin-draining lymph nodes (LNs) were harvested for immunophenotypic analysis of $MHCII^{hi}$ cells. (**a**) Gating strategy to identify $B220^+$ plasmacytoid DCs, Langerhans cell, $CD207^+$, and $CD207^-$ dermal DC subsets. (**b**) Analysis of five DC subsets identified in **a** for their percentages following 2 and 4 days of Dox treatment. In addition, tetramethyl rhodamine iso-thiocyanate $(TRITC)^+$ DC percentages were analyzed 24 hours following TRITC painting in a 1:1 solution of acetone and dibutylpthalate. (**c**) Analysis of five DC subsets identified in **a** for their numbers following 2 and 4 days of Dox treatment ($N = 4$–8, repeated at least twice with similar results). Error bars = ± SEM.

4 days of TGFβ1 induction. The alteration in skin-derived DC percentages in SDLNs at 2 and 4 days after induction was similar to tetramethyl rhodamine iso-thiocyanate (TRITC)$^+$ DC subsets 24 hours post TRITC/dibutylpthalate (DBP) application (Figure 1b), confirming their skin origin and suggesting that the effects of TGFβ1 overexpression and the irritant DBP on skin CD11b$^-$ dDCs were similar. There was also a 4–5-fold increase in absolute numbers of CD207$^-$CD11b$^-$ DCs at 2 days, which increased to 11-fold relative to steady state at 4 days, a 2-fold increase in the number of CD207$^+$ CD103$^+$ dDCs, a 3-fold increase in CD207$^-$CD11b$^+$ DCs, and a 4-fold increase in B220$^+$ pDCs in the SDLNs at 4 days post TGFβ1 induction (Figure 1c and d). Thus, TGFβ1 appears to mobilize primarily CD207$^-$CD11b$^-$ dDCs but not LCs, although other DC subsets are significantly affected.

Keratinocyte TGFβ1 causes selective influx and proliferation of DCs in the dermis

To further examine TGFβ1-induced changes in cutaneous DC homeostasis, we analyzed the percentage of MHCII$^+$ cells in the dermis. Two days post TGFβ1 induction, there was a significant increase in the frequency of MHCII$^+$ cells in the dermis, which remained high through 4 days (Figure 2a). The increase in MHCII$^+$ cells was primarily due to an increase in B220$^+$ pDCs (Figure 2b and c). In addition, 4 days after TGFβ1 induction, there was a 2.5-fold higher percentage of CD207$^+$103$^+$ and CD207$^-$CD11b$^+$ dDCs (Figure 2c).

To test whether TGFβ1 induction altered proliferation of resident skin DC subsets, we injected mice with 1.5 mg bromodeoxyuridine (BrdU) 3 hours before necropsy following 2 days of TGFβ1 induction and determined the percentage of BrdU-positive cells within individual dDC subsets by flow cytometry. Although significantly more MHCII$^+$ and B220$^+$ pDCs were noted in the dermis, no changes in BrdU incorporation in any of the DC subsets was detected (Figure 3a). Alternatively, we dosed mice continuously with BrdU while inducing TGFβ1 at the same time for 4 days. There was a two- and three-fold increase in BrdU labeling in CD207$^+$CD103$^+$ and CD207$^-$CD11b$^-$ dDCs, respectively, but no difference in other DC subsets including pDCs (Figure 3b). Thus, TGFβ1 causes an increase of pDCs in the dermis without affecting proliferation and selectively promotes proliferation of other dDC subsets.

CD86 expression of skin-derived DC subsets is increased in SDLNs but not in the dermis

Consistent with increases in the numbers of skin-derived DC subsets in SDLNs, MHCII$^+$ cells in dermal sheets were detected in numerous distinct dermal cords, a characteristic feature of DCs aligning in the dermal lymphatics before migration, following 4 days of TGFβ1 induction (Figure 4a). DCs upregulate CD86 levels upon maturation by antigen encounter and/or inflammatory cytokines, which is linked to their migration to SDLNs (Banchereau and Steinman, 1998). We evaluated dDC subsets and their counterparts in SDLN for CD86 expression following TGFβ1 induction. In steady-state dermis, CD207$^+$CD103$^+$ dDCs had the highest and CD207$^-$CD11b$^-$ dDCs had the lowest CD86 levels. TGFβ1

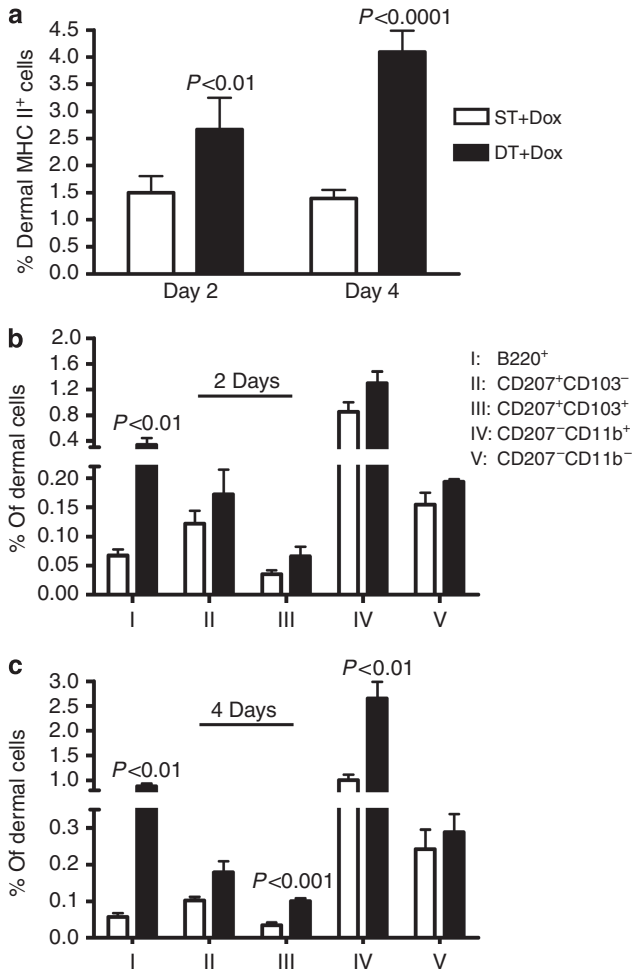

Figure 2. Increase in plasmacytoid dendritic cells (DCs) and dermal DCs (dDCs) in the dermis following transforming growth factor beta-1 (TGFβ1) induction. Single transgenic (ST) and double transgenic (DT) mice were given doxycycline (Dox) chow for 2 and 4 days, and dermal cells prepared from mouse ears following separation of the epidermis and dermis. Percentage of MHCII^{hi} cells (**a**) and different dDC subsets of the total dermal cells were analyzed by flow cytometry at day 2 (**b**) and day 4 (**c**) of TGFβ1 induction. Error bars = ± SEM.

caused a significant increase in CD86 expression only in the CD207$^-$CD11b$^-$ dDC subset at day 2 and no change in CD86 could be detected in any subset at day 4 (Figure 4b and c). However, there was an increase in CD86 expression in the SDLNs of all of the skin-derived DC subsets (Figure 4d), with the greatest increase occurring in the CD207$^-$CD11b$^+$ and CD207$^-$CD11b$^-$ dDC subsets (2.8- and 4.8-fold, respectively), and this was sustained through day 4 post TGFβ1 induction (data not shown). Supplementary Figure S1 online shows representative histograms for CD86 expression on dDC subsets in the dermis and SDLNs.

Keratinocyte TGFβ1 enhances DC migration in response to sensitizer application in skin

Although TGFβ1 alone altered homeostasis of dDC subsets, it can also suppress the action of other inflammatory signals.

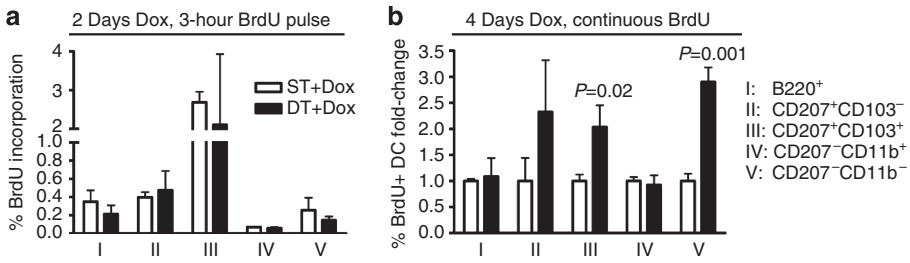

Figure 3. Transforming growth factor beta-1 induction does not alter local proliferation of plasmacytoid dendritic cells (DCs) and dermal DCs in the dermis. (**a**) Dermal cells were prepared from mouse ears following 2 days of doxycycline (Dox) treatment and 3 hours after bromodeoxyuridine (BrdU; 1.5 mg per mouse) injection, $N = 4$–6. (**b**) Dermal cells were prepared following 4 days of Dox treatment and continuous BrdU treatment either in drinking water (0.8 mg ml^{-1}) or daily intraperitoneal injections (1 mg per mouse), $N = 4$–5. Flow cytometric analysis was performed on dermal cells to detect the percentage of BrdU$^+$ DC subsets following the manufacturer's recommended protocols. DT, double transgenic; ST, single transgenic. Error bars = ± SEM.

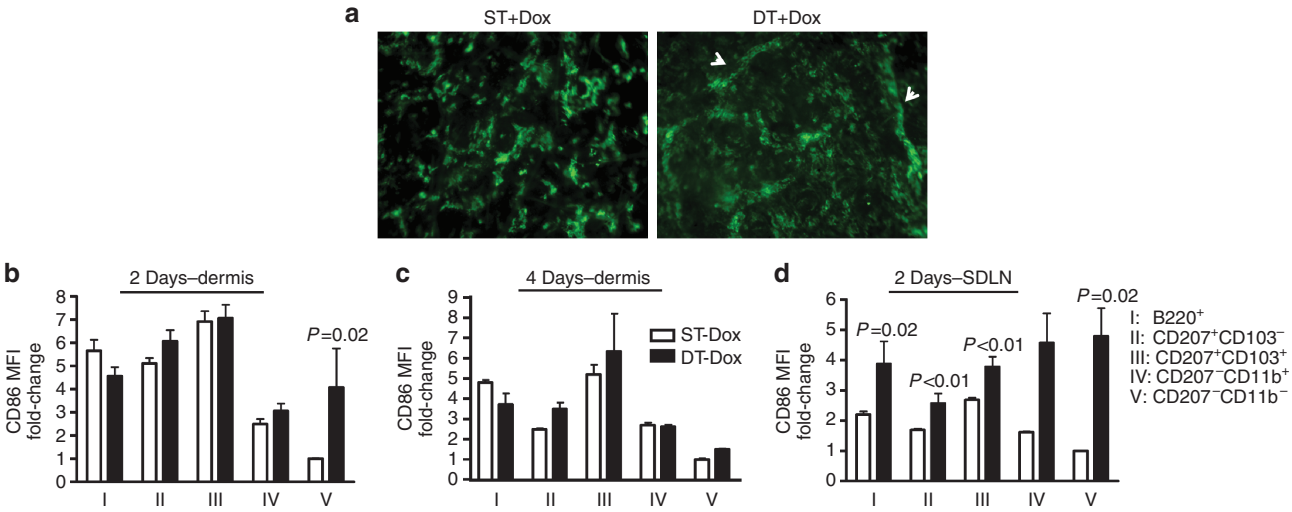

Figure 4. Keratinocyte transforming growth factor beta-1 promotes migration and increases CD86 expression of migratory dendritic cell (DC) subsets in skin-draining lymph nodes (SDLNs). (**a**) Dermal sheets were prepared from mouse ears after 4 days of doxycycline (Dox) treatment and analyzed for MHCII + cells. White arrows represent dermal cords ($N = 4$–6). Dermal cells prepared from mouse ears following 2 (**b**) and 4 days (**c**) of Dox treatment were analyzed by flow cytometry for CD86 mean fluorescent intensities (MFI) on various DC subsets. The average raw MFI values from controls for DC subset V were normalized to 1. Data are represented as fold change between the groups ($N = 5$–6). (**d**) SDLNs harvested from single transgenic (ST) and double transgenic (DT) mice following 2 days of Dox treatment and migratory DCs identified in Figure 1a were analyzed for CD86 expression. Data are represented as fold change as in **b** ($N = 5$–7). Error bars = ± SEM.

To determine whether elevated epidermal TGFβ1 altered DBP-induced DC migration, mice were given Dox chow and 1% TRITC in a 1:1 mixture of acetone, and DBP was applied to the shaved abdominal skin 18–24 hours later. At both 24 and 72 hours post TRITC/DBP treatment, TGFβ1 overexpression significantly enhanced the total number of TRITC$^+$ MHCII^{hi} cells in SDLNs (Figure 5a). At 24 hours, migration of TRITC$^+$ CD207$^-$CD11b$^-$ dDC, which constituted the majority of TRITC$^+$ DCs in SDLNs, was enhanced by TGFβ1. At 72 hours, LCs that migrate slower than other DC subsets (Kissenpfennig *et al.*, 2005) were the largest subpopulation of TRITC$^+$ cells in both groups, and overexpression of TGFβ1 enhanced DBP-induced LC migration by 1.77-fold. In addition, TGFβ1 expression also caused a two-fold increase in CD207$^-$CD11b$^+$ dDC and a three-fold increase in CD207$^-$CD11b$^-$ dDC numbers at the 72-hour time point (Figure 5a). A small number of TRITC$^+$B220$^+$ pDCs were detected at 24 and 72 hours post TRITC, although their numbers did not change significantly following TGFβ1 induction. In addition, there was no effect at any time point on TRITC$^+$CD207$^+$CD103$^+$ dDC migration to SDLNs. These results show that, under inflammatory conditions induced by DBP application, TGFβ1 differentially increases numbers of CD207$^-$CD103$^-$ dDCs and LCs in SDLNs.

Elevated endogenous and exogenous TGFβ1 increases migration of LCs *in vitro*

To further evaluate the effects of elevated keratinocyte TGFβ1 on LC migration, we used skin explant cultures, an inflammatory setting that causes DC migration (Stoitzner *et al.*, 1999). Explants from single transgenic (ST) and DT mice placed on Dox chow for 24 hours were cultured, and migrated skin DCs were analyzed after 48 hours of culture. Under these conditions, 60–70% of DCs that migrated out of the skin were LCs, and TGFβ1 induction *in vivo* caused a two-fold increase in their migration *in vitro* (Figure 5b).

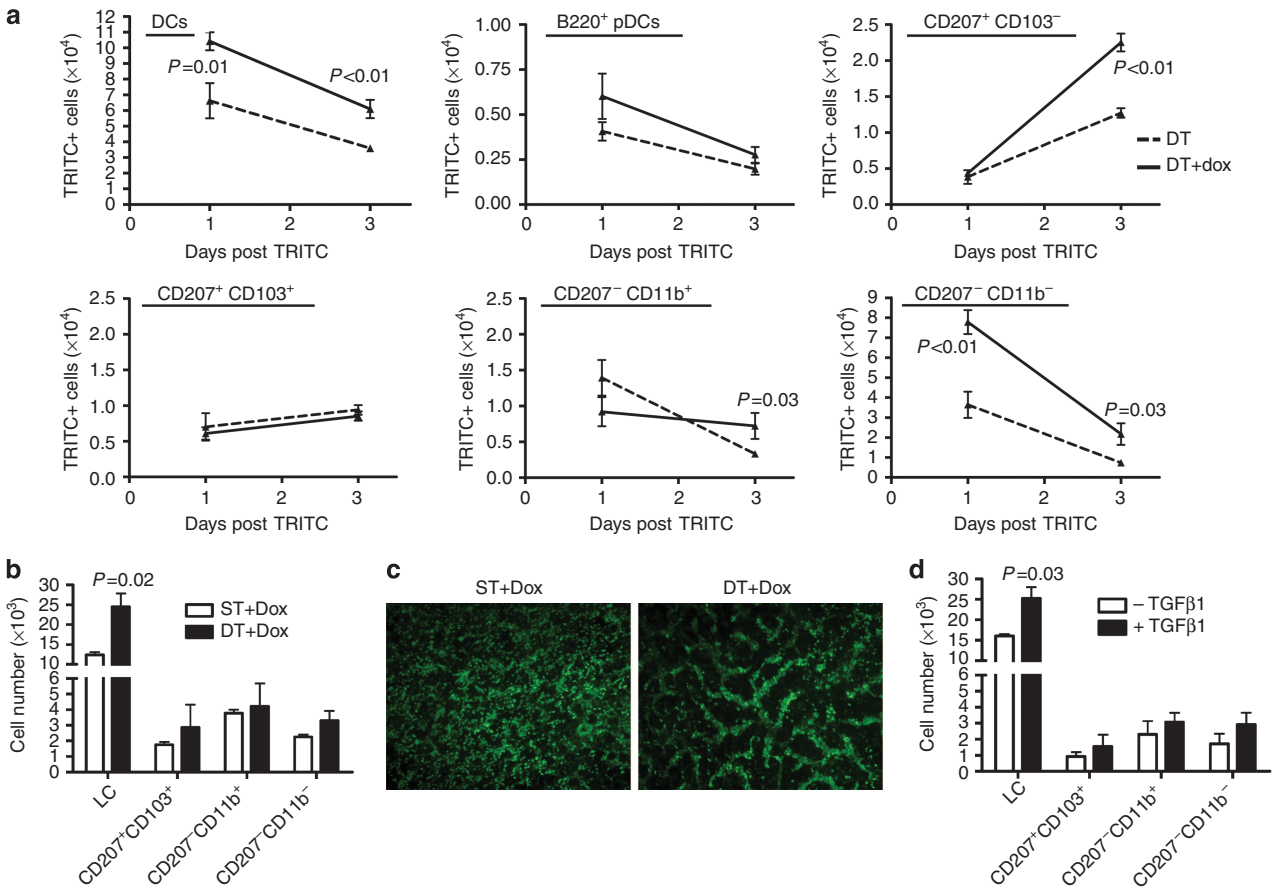

Figure 5. Elevated transforming growth factor beta-1 (TGFβ1) enhances Langerhans cell migration following tetramethyl rhodamine iso-thiocyanate (TRITC)/dibutylpthalate (DBP) treatment *in vivo* and in skin explant cultures *in vitro*. Double transgenic (DT) mice were given doxycycline (Dox) chow 18–24 hours before painting of shaved belly with 1% TRITC in 1:1 acetone and DBP. Skin-draining lymph nodes were harvested 24 and 72 hours post TRITC painting and analyzed for TRITC$^+$ dendritic cell (DC) subsets. (**a**) Kinetics of the number of individual TRITC$^+$ DC subsets at 24 and 72 hours post TRITC ($N = 4$–6). Error bars = ± SEM. (**b**) Single transgenic (ST) and DT mice were given Dox chow for 24 hours and ear skin explant cultures initiated. Cells accumulating in the media were harvested after 48 hours, counted, stained for flow cytometric analysis, and represented as the number of DCs migrating into the media per ear. (**c**) Dermal sheets were prepared from ST and DT ear skin explants following 48 hours of culture and MHCII immunofluorescence performed. (**d**) FVB/n mouse ears were split and floated in complete RPMI media containing TGFβ1 and cultured for 48 hours. Cells in the media were analyzed as in (**b**). For **b** and **c**, the results are the average of three independent experiments. LC, Langerhans cell; MHCII, major histocompatibility complex II; pDC, plasmacytoid DC. Error bars = ± SEM.

In addition, TGFβ1 caused a 1.5-fold increase in migration of the CD207$^-$CD11b$^-$ dDC subset that represented 10% of the migrated DC population. The increase in migration correlated with the formation of numerous well-organized dermal cords containing DCs in TGFβ1-overexpressing samples as opposed to scattered DCs and fewer dermal cords in ST dermis following 48 hours of skin culture (Figure 5c). Similarly, treatment of ear explant cultures from FVB/n mice with exogenous TGFβ1 stimulated LC migration (Figure 5d) to nearly the same extent as the DC chemokine CCL21 (Kissenpfennig *et al.*, 2005) (Supplementary Figure S2 online).

Elevated keratinocyte TGFβ1 promotes skin CHS

As the overexpression of TGFβ1 in the epidermis enhanced migration of DCs in response to topical application of the contact sensitizer, we hypothesized that TGFβ1 might affect CHS responses. We induced TGFβ1 expression using a one-time intraperitoneal injection of Dox 18–24 hours before sensitization with 0.5% DNFB. TGFβ1 expression caused a significant increase in the number of CD4$^+$CD44$^+$ CD62L$^+$ central memory T cells and CD4$^+$IFNγ$^+$ cells in SDLNs compared with ST mice (Figure 6a and b) following 5 days of sensitization. When mice sensitized in the presence of TGFβ1 were challenged with 0.3% DNFB, there was a 1.4-fold increase in ear thickness and weight (Figure 6c and d). These results show that the alteration of skin DC homeostasis by elevated keratinocyte TGFβ1 results in enhanced CHS responses.

DISCUSSION

DCs constantly migrate from peripheral tissues to draining LNs in steady state at low frequencies and generally promote tolerance and maintain tissue homeostasis (Steinman *et al.*,

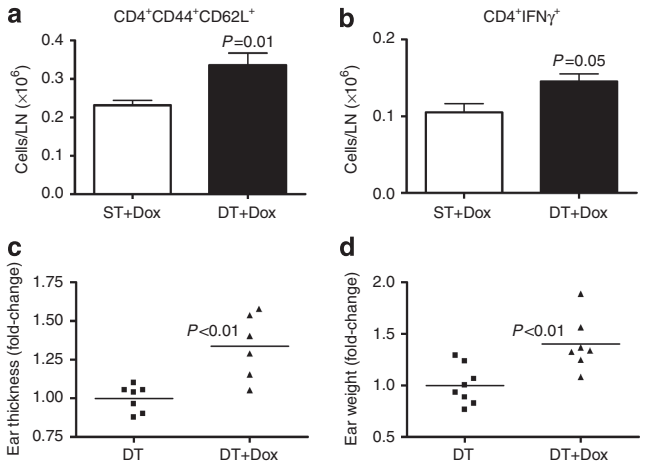

Figure 6. Transforming growth factor beta-1 induction during sensitization enhances contact hypersensitivity response to DNFB (2,4-dinitro fluorobenzene). Single transgenic (ST) and double transgenic (DT) mice were injected with 500 ng doxycycline (Dox) per mouse 18–24 hours before sensitization with 0.5% DNFB. Skin-draining lymph nodes (LNs) were harvested 5 days post sensitization and analyzed for CD4 central memory cells (**a**) and $CD4^+IFN\gamma^+$ cells (**b**) by flow cytometry ($N = 2$–3). Error bars = ± SEM. Alternatively, mice were challenged on both sides of the ear at day 5 using 0.3% DNFB and ears thickness (**c**) and ear weight (**d**) analyzed 24 hours post challenge.

2000; Hawiger *et al.*, 2001; Waithman *et al.*, 2007). Peripheral inflammation from infection, injury, and autoimmunity can increase the rate of DC migration and numbers in draining LNs and sustain T-cell activation (Ohl *et al.*, 2004; Coquerelle and Moser, 2010). Compared with steady state, TGFβ1 overexpression in keratinocytes caused profound changes in composition and increased the numbers of skin-derived dDC subsets and $B220^+$ pDCs in the SDLNs. The striking resemblance of these TGFβ1-induced changes to those occurring 24 hours following application of skin irritant DBP suggests that increased TGFβ1 and DBP alter the skin microenvironment in a similar way. However, direct effects of TGFβ1 on DCs promoting their migration cannot be ruled out.

TGFβ1 induction also increased DBP-driven migration of $CD207^-CD11b^-$ and $CD207^-CD11b^+$ dDC subsets. Although we did not observe TGFβ1-induced mobilization of LCs, TGFβ1 expression significantly enhanced DBP-induced LC numbers in SDLNs. Consistent with this, ear explant cultures, which mimic an inflammatory environment, from TGFβ1-induced mice had a two-fold increase in LC migration, and LC numbers were comparable between skin explant cultures treated with either exogenous TGFβ1 or the CCR7 ligand, CCL21. Thus, within the context of an inflammatory microenvironment such as that established by DBP or explant culture, elevated TGFβ1 stimulates LC migration rather than inhibiting it. It is not clear whether this is mediated by effects of TGFβ1 on LCs within the context of other proinflammatory cytokines, or by indirect activation mediated by TGFβ1-induced upregulation of proinflammatory cytokines along with other changes in the tissue microenvironment. The lack of inhibition of LC homeostatic trafficking to the LNs by elevated keratinocyte TGFβ1 contrasts with studies showing that TGFβ1 type-I receptor ablation in LCs provokes LC maturation and migration (Kel *et al.*, 2010; Zahner *et al.*, 2011). These differences suggest that elevated keratinocyte-derived TGFβ1 and LC TGFβ1 signaling may have distinct effects on LC maturation and migration.

Although activated DCs express elevated levels of the costimulatory molecule CD86 before LN migration (Banchereau and Steinman, 1998), we observed no significant change in CD86 expression in dDCs except for the $CD207^-CD11b^-$ dDC subset. It is possible that coexistence of migrating DCs with higher CD86 and influx of precursors with less CD86 expression results in no apparent change in the mean CD86 levels. However, the CD86 expression was higher in all DC subsets in SDLNs as early as 2 days post TGFβ1 induction and remained high through 4 days. Whether this results from the influence of $CD207^-CD11b^-$ dDCs on CD86 levels in other migratory DC population or simply that other dDC subsets increase CD86 levels *en route* to SDLNs remains to be determined. Further analysis of other markers of migration and maturation such as CCR7 and E-cadherin (Kel *et al.*, 2010; Zahner *et al.*, 2011) will also clarify the differential migration of dDCs versus LCs in response to TGFβ1.

The pDCs participate in antiviral immune responses and may have an important role in the pathogenesis of autoimmune diseases such as psoriasis and lupus (Gilliet *et al.*, 2008). The pDCs infiltrate the skin after wounding, in tumors, in psoriasis, and following imiquimod application (Palamara *et al.*, 2004; Gilliet *et al.*, 2008; Nestle *et al.*, 2009; Gregorio *et al.*, 2010). Here we report for the first time that TGFβ1 overexpression causes a rapid increase in pDCs in the dermis. The lack of proliferation in these cells suggests that the increase is due to direct infiltration rather than expansion of a skin-resident population. Although pDCs infiltrating skin during psoriasis or lupus may be activated by complexes of the antimicrobial peptide, LL37, produced by neutrophils, and self-DNA from apoptotic cells in a Toll-like receptor 9–dependent manner (Nestle *et al.*, 2009; Guiducci *et al.*, 2010), it remains to be determined whether this pathway of pDC activation occurs in this mouse model. We have previously reported induction of apoptosis in primary keratinocytes cultured in excess TGFβ1, as well as in telogen follicles of DT mice upon TGFβ1 induction (Liu *et al.*, 2001), suggesting that nucleic acids from the apoptotic cells could trigger Toll-like receptor 7/9–mediated pDC activation. As pDCs did not migrate in significant numbers following hapten application in TGFβ1-induced mice, it appears that increased pDCs in the skin of TGFβ1-overexpressing mice might modulate or support local activation and maturation of myeloid DCs as in psoriasis (Gilliet *et al.*, 2008). Similar to pDCs, we also noticed increases in percentages of $CD207^+$ dDCs and $CD207^-CD11b^+$ dDCs but not the $CD207^-CD11b^-$ dDCs following 4 days of induction. As the predominant dDC subset migrating in response to TGFβ1 induction was $CD207^-CD11b^-$ (11-fold at 4 days),

it appears that a balance is maintained between infiltrating/proliferating and migrating $CD207^{-}CD11b^{-}$ dDC subset.

LCs constantly renew in the epidermis throughout life (Merad *et al.*, 2002), and dDCs are maintained by local proliferation with some infiltration of blood-derived precursors in steady state (Bogunovic *et al.*, 2006). Because of increased migration of dDC subsets to SDLNs at 4 days of TGFβ1 induction, we predicted an increase in self-renewal potential to maintain their normal homeostatic percentages in the dermis. Despite increases in dermal percentages of most DC subsets, proliferation only occurred in $CD207^{+}$ and $CD207^{-}CD11b^{-}$ dDC subsets. Hence, it appears that TGFβ1 alters dDC homeostasis by promoting influx of blood-derived pDCs and non-self-renewing DC precursors in the dermis while also influencing proliferation of $CD207^{+}CD103^{+}$ dDCs and $CD207^{-}CD11b^{-}$ dDCs. The absence of any increases in dermal percentage of $CD207^{-}CD11b^{-}$ cells may reflect the large and rapid migration of this subset to the SDLNs.

Our results highlight the potential proinflammatory role of TGFβ1 in skin immunity and show that TGFβ1 overexpression by keratinocytes alters DC homeostasis and enhances adaptive immunity in the context of CHS, although the DC subset that mediates this response has not been determined. Nevertheless, the effects of TGFβ1 on dDC migration, pDC influx, and enhanced CHS provide insight into the onset of psoriasis-like skin inflammation following chronic TGFβ1 induction in keratinocytes (Li *et al.*, 2004), and suggest that elevated TGFβ1 in human psoriasis and other conditions of elevated cutaneous TGFβ1 may directly impact DC homeostasis, activation, and T-cell immunity.

MATERIALS AND METHODS

Mice

ST K14rTA or tetoTGFβ1 and DT K14rTA-tetoTGFβ1 mice in FVB/n background that were sex- and age-matched (7–10 week) were used for all the experiments. To induce keratinocyte TGFβ1, DT mice were given 1 g kg^{-1} Dox chow (Bio-serve, Frenchtown, NJ). Animals were treated according to approved Institutional Animal Care and Use protocols.

Antibodies

The following antibodies were purchased from Ebioscience, San Diego, CA: anti-CD16/32 (93), APC eFluor 750-anti-CD45 (30-F11), FITC- and eFluor 450-anti-MHCII (M5/114.15.2), Alexa 700-anti-CD11c (N418), FITC-anti-CD4 (GK1.5), PECy5-anti-CD8α (53-6.7), PE-anti-CD103 (2E7), PECy7-anti-B220 (RA3-6B2), PE-anti-PDCA-1 (eBio-927), PercpCy5.5-anti-CD11b (M1/70), eFluor 450-anti-F4/80 (BM8), PE-anti-CD62L (MEL-14), and PECy5-anti-CD44 (IM7). The following antibodies were purchased from BD Pharmingen, San Diego, CA: PE-anti-CD45 (30-F11), Alexa 700-anti-CD86 (GL1), and PECy7-anti-IFNγ (XMG1.2). FITC-anti-BrdU (ABFM18) was purchased from Phoenix Flow Systems, San Diego, CA. Alexa 568-anti-Epcam (G8.8) and Alexa 647-anti-CD207 (L31) antibody conjugates were generated as previously described (Gaiser *et al.*, 2012).

Isolation and flow cytometric analysis of DC

Inguinal lymph nodes were gently disrupted using forceps and incubated in Hank's balanced salt solution containing 0.1% collagenase D (Roche, Nutley, NJ) and 0.05% DNase I (Sigma, St Louis, MO) for 30 minutes at 37 °C. Epidermal and dermal cell suspensions were prepared as described previously, with slight modification (Nagao *et al.*, 2009). Dermal components were cut into small pieces and incubated for 45 minutes in 0.1% collagenase D and 0.05% DNase I for 45 minutes at 37 °C, filtered using a 70-μm cell strainer, and suspended in flow cytometry staining buffer. Single cells were incubated with panels of monoclonal antibodies following CD16/32 preincubation. For anti-CD207 staining, cells were fixed and permeabilized using Foxp3 fixation/permeabilization buffers (Ebioscience) and incubated with anti-langerin antibody in 0.2% saponin buffer. Cells were acquired on BD Fortessa LSRII flow cytometer and analyzed using the FlowJo software (Tree Star, Ashland, OR).

Analysis of cell proliferation

Mice were initially injected with 1 mg BrdU in sterile phosphate-buffered saline and then continuously given 0.8 mg ml^{-1} BrdU in drinking water, which was changed daily. Alternatively, 1 mg BrdU was injected intraperitoneally daily. Single cells were prepared from inguinal LNs, epidermis, and dermis as described above, and were first stained for surface markers and CD207. BrdU staining was performed using the BrdU labeling flow kit (BD Pharmingen) following the manufacturer's protocol.

Skin explant cultures

Ears of ST and DT mice previously treated with Dox chow for 18–24 hours were excised, rinsed in 70% ethanol, and then in a solution containing 200 IU ml^{-1} penicillin and 200 μg ml^{-1} streptomycin for 5 minutes. Ear skin was split into dorsal and ventral halves, and at least four split ears were cultured in complete RPMI media, 10% fetal calf serum for 48 hours at 37 °C. FVB/n mouse ears were cultured as described above and treated with either 100 ng ml^{-1} CCL21 (R&D systems, Minneapolis, MN) or 500 pg ml^{-1} TGFβ1 (R&D systems). The migratory cells from the explant were harvested, enumerated, and analyzed by flow cytometry.

Epidermal and dermal sheet immunofluorescence

Epidermal and dermal sheets were prepared as described previously (Nagao *et al.*, 2009). For staining, dermal sheets were rehydrated in phosphate-buffered saline and incubated overnight with FITC-anti-MHCII antibody at 4 °C. Slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed on an Olympus BX61 microscope (Olympus America, Center Valley, PA).

TRITC painting

ST and DT mice were kept on 1 g kg^{-1} Dox chow 18–24 hours before treatment with 1% TRITC (Invitrogen, Eugene, OR) in 1:1 solution of acetone and DBP on shaved abdominal skin. After 24 and 72 hours post TRITC application, inguinal LNs were harvested and single cells prepared as described above. Total viable cells per LN were counted and stained for specific markers and analyzed by flow cytometry.

Contact hypersensitivity

ST and DT mice were injected intraperitoneally with 500 ng Dox 18–24 hours before sensitization with 25 μl of 0.5% DNFB (Sigma) in 4:1 solution of acetone/olive oil on shaved abdominal skin. Mice were challenged 5 days later with 20 μl of 0.3% DNFB on both sides of the right ear. The left ear was treated with vehicle and served as a

control for baseline thickness. Measurements of ear thickness (minus baseline thickness), weight, and histological analysis were taken 24 hours following challenge, in a blinded manner.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA). A two-tailed Student's *t*-test was performed to compare the groups. Under certain circumstances where the variances were significant, a Mann–Whitney test was performed.

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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