Direct control of regulatory T cells by keratinocytes

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Environmental challenges to epithelial cells trigger gene expression changes that elicit context-appropriate immune responses. We found that the chromatin remodeler Mi-2β controls epidermal homeostasis by regulating the genes involved in keratinocyte and immune-cell activation to maintain an inactive state. Mi-2β depletion resulted in rapid deployment of both a pro-inflammatory and an immunosuppressive response in the skin. A key target of Mi-2β in keratinocytes is the pro-inflammatory cytokine thymic stromal lymphopoietin (TSLP). Loss of TSLP receptor (TSLPR) signaling specifically in regulatory T (Treg) cells prevented their activation and permitted rapid progression from a skin pro-inflammatory response to a lethal systemic condition. Thus, in addition to their well-characterized role in pro-inflammatory responses, keratinocytes also directly support immune-suppressive responses that are critical for re-establishing organismal homeostasis.

Skin integrity is maintained by the intimate interaction between epidermal keratinocytes and resident immune cells that supports recovery from a number of insults such as barrier disruption and bacterial or viral infection. Failure of the immune system to maintain tolerance or re-establish homeostasis after keratinocyte perturbation can cause autoimmune and chronic pro-inflammatory disorders that can give rise to skin neoplasias1–3. Despite considerable progress in keratinocyte and immune cell biology, the ways in which these distinct cell types communicate and coordinate with each other to maintain skin homeostasis remain ill-defined. Central to a productive interaction between keratinocytes and resident immune cells is an array of immune-regulatory factors that are either constitutively expressed or induced in keratinocytes or immune cells following insult.

One of the cytokines that is rapidly induced in keratinocytes under stress is TSLP. TSLP is an interleukin 7 (IL-7)-like epithelial-cell-derived cytokine that signals through a hetero-dimeric receptor comprised of the TSLPR and the alpha subunit of the IL-7 receptor (IL-7Rα) that is expressed by many lymphoid, dendritic, myeloid and neuronal cell types4,5. Ectopic expression of TSLP in mouse skin has been correlated with a T helper type 2 (Th2)-driven pro-inflammatory response in both skin and lung epithelia and an atopic dermatitis (AD)-like phenotype4. TSLP is highly expressed in both acute and chronic AD lesions in human patients, but not in non-lesional skin from the same patient4. TSLP is thought to function by inducing expression of MHC class I and II and co-stimulatory molecules on dendritic cells (DCs), which can then promote the activation and differentiation of a naïve CD4+ T cell into a pro-inflammatory Th1 cell type4. Recent reports have shown that TSLP is also highly expressed in psoriatic lesions from human patients, which suggests that TSLP is involved in the Th1 or Th17 inflammatory responses by promoting IL-23 production by DCs6,7. TSLP acts directly on CD4+ and CD8+ T cells to stimulate a pro-inflammatory response that can prevent development of skin epithelial tumors8,9.

Mechanisms that control gene expression in keratinocytes are important for the keratinocyte’s ability to respond to environmental insult and to elicit an immune response. Although signaling pathways and transcription factors are central mediators of stimulus-specific responses, chromatin regulators may also have a pivotal role in modulating transcription factor accessibility to appropriate regulatory sites following receipt of a stress signal. Mi-2β is a nucleosome remodeler and a core component of the nucleosome remodeling deacetylase (NuRD) complex that is highly expressed in hematopoietic and epithelial tissues10. In the hematopoietic system, Mi-2β associates with the Ikaros family of DNA-binding factors to control self-renewal and early lineage decisions through both positive and negative regulation of gene expression11,12. In the heart, the Mi-2β-NuRD complex is critical for maintaining cardiac muscle cell identity by repressing skeletal-muscle-specific genes13. Mi-2β also regulates cell fate decisions at different stages of epidermal differentiation14. Ectodermal precursors rely on Mi-2β for establishing their self-renewing potential. However, after establishment of self-renewal, epidermal precursors are not dependent on Mi-2β for maintenance, but rather for specification into the follicular cell fate. These findings highlight a highly dynamic role for Mi-2β and the NuRD complex in the epidermal differentiation process, possibly by engaging with stage-specific transcriptional networks.

We examined the role of Mi-2β in keratinocytes of the adult skin and found that it was critical for maintaining skin homeostasis by repressing the expression of genes normally induced in stressed keratinocytes. A key target of Mi-2β in basal keratinocytes is the gene encoding the cytokine sentinel of skin integrity, TSLP. We found that TSLPR was specifically expressed in skin-associated Treg cells and...
was required for inducing $T_{\text{reg}}$-cell-suppressive functions under pro-inflammatory conditions. In this context, TSLP's role in mounting an immunosuppressive response supersedes its role as a pro-inflammatory factor in the skin. Our findings demonstrate a previously unknown signaling mechanism that is mediated by epithelial-derived regulatory signals and has an essential role in $T_{\text{reg}}$-cell-dependent immune homeostasis in the skin.

RESULTS

Mi-2β is critical for skin homeostasis

We investigated the role of the chromatin remodeler Mi-2β, encoded by the Chd4 gene (also known as Mi-2b), in the adult skin by inducing deletion in the basal epidermis. We injected 2-month-old Mi-2βloxP/loxP Krt14-Cre-ERT2 (Mi2Δ) mice and littermate controls that lacked the Krt14-Cre-ERT2 transgene (referred to as wild type) with 4-hydroxytamoxifen (4-OHT) at days 0 and 3 and analyzed them 7–30 d later. Depletion of Mi-2β protein in keratinocytes was mosaic at day 7 and was nearly complete by day 9 (Fig. 1a,b). Potential depletion of Mi-2β in thymic epithelial cells, a subset of which expresses keratin 14 (K14), was also tested using the 4-OHT-injected Krt14-Cre-ERT2 Rosa26-YFP (yellow fluorescent protein) reporter mice. No YFP induction was seen in the thymus during the timeframe of our skin studies (data not shown).

By 6–8 d after initiation of Mi-2β deletion in the epidermis, mice had developed a rough hair coat phenotype, indicative of a local skin inflammatory response. By days 9–11, mice exhibited flaky skin, weight loss and had become progressively less active, with a hunched posture, which are signs of progression from a local skin to a systemic response (Supplementary Fig. 1a). Nonetheless, by day 15, Mi2Δ mice showed signs of remission, and were fully recovered by day 30 (Fig. 1c). Notably, at this later time point, Mi-2β-deficient keratinocytes were no longer detectable in the epidermis, which was comprised of wild-type keratinocytes (data not shown).

Histological examination of the skin at day 9 after Mi-2β deletion revealed hyperplasia in both the basal and suprabasal epidermal layers (acanthosis), with thickening of the cornified layer (Fig. 1d). Areas of moderate-to-severe hyperplasia were also seen in the follicular infundibulum, but without hair follicle degeneration (Fig. 1d). A marked increase in the number of proliferating basal keratinocytes, normally minimal in adult skin, was observed (Fig. 1e,f). After a 2-h pulse label with bromodeoxyuridine (BrdU), 42% of Mi-2β-depleted basal keratinocytes became BrdU positive compared with 1% in wild-type skin (Fig. 1f). K6, a marker of keratinocyte hyper-proliferation, was also detected early (day 7) after Mi-2β deletion at the suprabasal layer and only in cells deficient for Mi-2β, indicating that the mutation had a cell-autonomous effect (Fig. 1a). At later time points (days 9–11), K6 expression was seen throughout the epidermis, consistent with the widespread depletion of Mi-2β (Fig. 1g). Mi-2β-deficient basal keratinocytes were able to differentiate through the epidermal layers, as indicated by an apparently normal distribution of layer-specific differentiation markers, such as K5, K1 and Loricrin, in both wild-type and Mi2Δ skin (Fig. 1g). No barrier defect was observed, as measured by an outward trans-epidermal water loss assay (data not shown). An increase in CD45+ leukocytes and CD4+ T cells in the dermis and in the cellularity of skin-draining lymph nodes (sDLNs) was seen starting on day 7 of Mi2β depletion, indicative of a rapid immune response to genetically altered keratinocytes (Fig. 1h,i and Supplementary Fig. 1b).

Taken together, our data demonstrate that loss of Mi-2β in keratinocytes induces their activation and a pro-inflammatory response that progresses to a systemic condition. Nonetheless, following displacement of the altered keratinocytes, full recovery and return to skin and organismal homeostasis is rapidly achieved.

Mi-2β represses pro-inflammatory genes in keratinocytes

The molecular basis of the epidermal phenotype that manifested following Mi-2β deletion was evaluated. Shortly after induction of Mi-2β deletion, we sorted and examined basal keratinocytes for changes in gene expression (day 7). Basal keratinocytes, expressing αv integrin (ITGA6) and lacking the CD34 and CD45 follicular stem cell and leukocyte markers, were isolated from wild-type and Mi2Δ epidermis at the telogen phase of the hair cycle (Supplementary Fig. 2a). Gene ontology analysis of upregulated genes in Mi2Δ relative to wild-type keratinocytes revealed the induction of pathways supporting cell proliferation, keratinocyte activation and mobilization, which are normally activated by skin injury (Fig. 2a and Supplementary Fig. 2b)15. In addition, expression of genes encoding immune cell regulators, such as cytokines, chemokines and stress antigens, was increased (Fig. 2a,b)3.

Among the top five most highly induced genes in all categories was the epithelial cytokine Tslp (Fig. 2b). Tgfb1, which is involved in the differentiation of $T_{\text{reg}}$ and $T_{\text{H17}}$ cells and in the migration of Langerhans cells (LCs), and Bmp7, which is required for LC differentiation, were also strongly induced (Fig. 2b)16–18. A modest increase in the expression of the cytokine genes Il17d, Il17f (IL-17fl), Il12 and Kitl and the chemokine genes Ccl22, Cxcl1, Cxcl9 and Cxcl10 was detected (Fig. 2b). Genes encoding keratinocyte stress antigens that are engaged by receptors on dendritic epidermal y8 T cells (DCTCs), including Proc, Raxel and Plxnb2, were upregulated (Fig. 2b)19–21. Cd74, which encodes a factor that is important for antigen presentation, and Icam1, which encodes a cell adhesion molecule that mediates T cell recruitment to the skin, were also upregulated (Fig. 2b)22,23. Finally, the antimicrobial and antiviral protein genes Lcn2, Oas3, Oasl1 and Oasl2 were induced (Fig. 2b)24.

TSLP expression in keratinocytes is normally induced in response to a variety of perturbations in skin structure or function25–30. Several lines of evidence suggest that induction of TSLP is a direct consequence of Mi-2β deletion. First, during the initial phase of Mi-2β deletion, immunohistochemistry confirmed early and cell-autonomous induction of TSLP only in Mi2Δ keratinocytes in mosaically deleted skin (Fig. 2c). Second, TSLP was induced in cultured keratinocytes after deletion of Mi-2β in vitro, eliminating the possibility of an indirect effect by an undetected barrier perturbation. 48 h after 4-OHT addition, induction of Tslp mRNA and secretion of TSLP protein were readily observed (Fig. 2d,e). Given that cultured keratinocytes were proliferating before induction of Mi-2β deletion, induction of TSLP mRNA following Mi-2β deletion was not secondary to a keratinocyte proliferative response. Finally, the direct regulation of Tslp by Mi-2β was confirmed by Mi-2β chromatin immunoprecipitation combined with quantitative PCR. We detected Mi-2β enrichment at previously characterized Tslp enhancer elements that are controlled by the transcription factor NF-κB (−3.9 kb) and the retinoid X receptor/ vitamin D receptor complex (−4.2 kb), respectively (Fig. 2f)26,31. In contrast with these enhancer sites, no enrichment was detected at the Tslp promoter (−0.3 kb) or at intron 3 (Fig. 2f).

Thus, the chromatin remodeler Mi-2β appears to be a key regulator of skin homeostasis by repressing the expression of genes that support both activation and mobilization of keratinocytes and their immune cell neighbors. These genes are normally induced by transcriptional mechanisms that respond to environmental inputs, but are actively repressed by the Mi-2β-NuRD complex under homeostatic conditions (Supplementary Fig. 2c).
We observed an extensive expansion of the epidermal layers in both Mi2Δ mice (Supplementary Fig. 3a). A skin pro-inflammatory phenotype demarcated by expansion of the epidermal layers was also seen in RMKO mice by day 9, but was milder than that observed in Mi2Δ mice (Supplementary Fig. 3a). We observed an extensive expansion in a variety of myeloid cells, including LCs and DCS, in RMKO compared with other mice (Supplementary Fig. 4). However, the systemic response and rapid demise was only seen in TMKO mice, indicating that this phenotype is lymphocyte dependent (Fig. 3a).

We further evaluated the type of pro-inflammatory response induced by Mi-2β depletion and the effect of TSLPR signaling in this process. Expression of genes encoding pro-inflammatory cytokines was determined in skin biopsies. An increase in the expression of genes encoding Il2 and the T11- and T117-related cytokines Il1α, Il1β, Il6, Il18, Il23a, Tnf and Ifng was detected in both Mi2Δ and TMKO skin, whereas the T12 cytokine encoding gene Il4 was only upregulated in Mi2Δ mice. This result is consistent with a previously reported role of TSLP in supporting a T12 response. Among the commonly upregulated cytokine genes, Il2, Il1α, Il18 and Ifng were further induced in TMKO compared with Mi2Δ mice (Fig. 3b).

We also examined phenotypic changes in epidermal keratinocytes and resident immune cells. Induction of keratinocyte activation and pro-inflammatory factors, such as K6, the Ki67 antigen and TSLP, was observed in Mi2Δ mice (red nuclear staining) compared with wild type (WT, yellow nuclei) epidermis. Scale bars represent 50 μm. The epidermis and was likely associated with the rapid development of a demarcated by expansion of the epidermal layers was also seen in Mi2Δ mice, becoming lethargic and died around day 10. In contrast, mice with Mi-2β skin deletion and lacking all lymphocytes showed a similar disease onset and recovery as Mi2Δ mice (Rag1Δ/Δ Mi-2βloxP/loxP Krt14-Cre-ERT2, referred to as RMKO; Fig. 3a). A skin pro-inflammatory phenotype demarcated by expansion of the epidermal layers was also seen in RMKO mice by day 9, but was milder than that observed in Mi2Δ mice (Supplementary Fig. 3a). We observed an extensive expansion in a variety of myeloid cells, including LCs and DCS, in RMKO compared with other mice (Supplementary Fig. 4). However, the systemic response and rapid demise was only seen in TMKO mice, indicating that this phenotype is lymphocyte dependent (Fig. 3a).

Figure 1 Loss of Mi-2β in the epidermis causes rapid keratinocyte activation. (a,b) Depletion of Mi-2β protein was evaluated by immunofluorescence at days 7 (a) or 9 (b) after 4-OHT treatment. DAPI stained nuclei were shown in blue at day 7 and in red at day 9. At day 7, K6 expression (red) was confined to keratinocytes that lacked Mi-2β protein (green). At day 9, extensive depletion of Mi-2β was seen in Mi2Δ mice (red nuclear staining) compared with wild type (WT, yellow nuclei) epidermis. Scale bars represent 50 μm. (c) Time course of disease development and staging in Mi2Δ mice. No clinical expression of disease; 1, rough hair coat on the ventral side; 1.5, rough hair coat on both sides; 2, loss of weight; 2.5, less movement; 3, hunched; 3.5, no movement, very thin; 4, dead. (d) Hematoxylin- and eosin-stained sections of WT and Mi2Δ skin. Scale bar represents 50 μm. (e,f) Proliferation of basal keratinocytes was measured by BrdU pulse labeling. Skin sections with BrdU positive keratinocytes (red) are shown in blue at day 7 and in red at day 9. At day 7, K6 expression (red) was confined to keratinocytes that lacked Mi-2β protein (green). At day 9, extensive depletion of Mi-2β was seen in Mi2Δ mice (red nuclear staining). Scale bars represent 50 μm. (g) Distribution of keratinocytes was assessed by BrdU pulse labeling. Skin sections with BrdU positive cells (red) are shown in blue at day 7 and in red at day 9. At day 7, K6 expression (red) was confined to keratinocytes that lacked Mi-2β protein (green). At day 9, extensive depletion of Mi-2β was seen in Mi2Δ mice (red nuclear staining). Scale bars represent 50 μm.
**Figure 2** Mi-2β actively represses genes involved in keratinocyte activation and immune cell regulation. (a) Pathway analysis of genes that were significantly upregulated in Mi2Δ keratinocytes relative to wild-type keratinocytes (upregulated genes: 1,201, P < 0.05, more than twofold difference). Selected biological process GO terms and their associated P values (–log10) for pathway discovery are shown. (b) Expression of genes relevant to immune cell regulation shown as normalized exon mapping reads. (c) Early induction of TSLP expression (red) correlated with Mi-2β protein depletion in keratinocytes and was confined to cells that lacked Mi-2β (green). Scale bar represents 50 μm. (d,e) Mi-2β depletion in primary cultured keratinocytes resulted in induction of Tslp mRNA and protein secretion. (d) Induction of Tslp mRNA at 48 h after in vitro induction of Mi-2β deletion. Sorted keratinocytes from in vivo wild-type and Mi-2β-depleted epidermis were used as a control. D, DMSO; T, 4-OHT. (e) Secretion of TSLP protein in the culture medium at 72 h after induction of Mi-2β deletion. (f) Mi-2β ChIP-qPCR analysis of the TSLP locus in primary keratinocytes. VDR, Vitamin D3 response site; NF, NF-κB response site; Pr, Promoter; IN3, Intron 3. Data were generated from two independent experimental groups with pooled samples from Mi2Δ (n = 7) mice in (e), three independent experiments in (f) (mean ± s.e.m., two independent PCR reactions in each experiment).

**Activation of skin T{sub}reg cells is dependent on TSLP**

We further evaluated the role of TSLPR signaling in skin-associated T cells under homeostatic and pro-inflammatory conditions (Mi2Δ). An increase in sDLN cellularity and in the absolute number of CD4+ and CD8+ T cells was seen in both Mi2Δ and TMKO relative to wild-type mice (Fig. 4a and Supplementary Fig. 5a). These cell numbers were modestly reduced in TMKO compared with Mi2Δ mice, supporting TSLP’s contribution to the skin inflammatory response in Mi2Δ mice, but also highlighting the fact that TSLP is not the only contributing factor in this mouse pro-inflammatory model.
We observed a marked increase in immunosuppressive CD4+Foxp3+ Treg cells in sDLNs from early time points of Mi2Δ deletion (Fig. 4a,b). Treg cells from Mi2Δ sDLNs displayed an activated effector cell phenotype with higher expression of CD25, CD44, CTLA4, CD103, KLRG1, TNFR2 and the Ki67 antigen (Fig. 4b and Supplementary Fig. 5b). Furthermore, Treg cells associated with Mi2Δ pro-inflammatory skin had a strong in vitro immunosuppressive activity compared with normal skin-associated Treg cells (Fig. 4c and Supplementary Fig. 5c). However, in the absence of TSLPR signaling, Treg cell numbers were very modestly increased, especially compared with wild-type Treg cells from Mi2Δ skin (approximately threefold reduction in TMKO compared with Mi2Δ; Fig. 4a,d). In addition, expression of activation receptors, co-inhibitory molecules, and memory and effector markers, were lower in TSLPR∆ Treg cells compared with wild-type Treg cells from Mi2Δ sDLNs (Fig. 4b and Supplementary Fig. 5b).

In contrast with the significant reduction in Treg cells, CD4+Foxp3+ effector T cells (Teff cells) were less affected by the loss in TSLPR, and we observed only a small reduction in cell numbers in TMKO compared with Mi2Δ (~1.2-fold reduction; Fig. 4a,d). Teff cells from both mutants displayed an activated effector cell phenotype with increased expression of the activation markers CD44 and CD69 (Supplementary Fig. 5b), and expressed higher amounts of TNF, IL-2 and IFN-γ following in vitro re-stimulation compared with their wild-type sDLN counterparts (Supplementary Fig. 5d). Protein expression of IL-4, IL-13 and IL-17A were not readily detected in the CD4+ T cell re-stimulation assay with cells from either TMKO or Mi2Δ mice (data not shown).

In summary, our data indicate that, although the role of TSLP as a skin pro-inflammatory factor can be redundant, its apparent role as an immune-suppressive factor is not. Notably, TSLPR signaling correlates with the rapid activation of skin Treg cells and potent immunosuppressive function.

Direct regulation of skin Treg cells by TSLP

We then evaluated the potential direct role of TSLPR in the functional activation of skin-associated Treg cells. Treg cells isolated from the skin and sDLNs expressed more TSLP than other CD4+ T cells present at these sites (Fig. 5a). Nonetheless, the increase in TSLPR expression in Treg cells compared with other CD4+ T cells was diminished in the spleen (Fig. 5a). These data suggest that skin-associated Treg cells are specifically conditioned to respond to TSLP, normally induced by keratinocyte perturbation, by higher expression of the TSLP signaling receptor.

The direct role of TSLPR signaling in Treg cell function was tested in adoptive transfer studies. Wild-type and Mi2Δ mice, bearing the Foxp3-IREs-EGFP knock-in alleles, were lethally irradiated and used as recipients for bone marrow from mice with conditional deletion of TSLPR in Treg cells (Foxp3-IREs-Cre CrIf2lox/lox; referred to as TSLPR∆ Treg cells). 8–12 weeks after transplantation, we treated both wild-type and Mi2Δ chimeras with 4-OHT and harvested them 8–11 days later. Foxp3+ Treg cells were subdivided into TSLPR∆ donor and TSLPR wild-type recipients on the basis of EGFP expression (Supplementary Fig. 6a,b). Under homeostatic conditions, the ratio of TSLPR∆ donor to wild-type recipient cells was ~2:1 in wild-type skin and sDLN (Supplementary Fig. 6c). However, following skin depletion of Mi-2β, the ratio was reduced to 0.71 (Supplementary Fig. 6c), reflecting a preferential expansion of TSLPR wild-type recipients relative to the TSLPR∆ donor Treg cells (Supplementary Fig. 6b,c). The Mi2Δ bone marrow chimeras with TSLPR∆ Treg cells developed disease scores similar to those seen in Mi2Δ mice, possibly as a result of expansion of the endogenous radio-resistant TSLPR wild-type Treg cells that provided immunosuppression and protection against development of a lethal systemic response.

To further evaluate the functionality of Treg cells expanded in the skin by cell autonomous TSLPR signaling, we generated a second adoptive transfer model. Rag or RMKO mice were sublethally irradiated and reconstituted with bone marrow from mice with TSLPR∆ Treg cells. Bone marrow from mice with intact TSLPR signaling in Treg cells (Foxp3-IREs-Cre CrIf2lox/lox; referred to as TSLPR+ Treg cells) was used as a control. 8–12 weeks after transplantation, we treated both Rag and RMKO chimeras with 4-OHT. By day 7 after Mi-2β deletion, RMKO mice reconstituted with TSLPR∆ Treg cells, but with an otherwise TSLPR-competent immune system, developed a severe disease phenotype (RMKO:TSLPR∆ Treg; Fig. 5b). This result was in
contrast with RMKO mice that received TSLPR\(^+\) T\(_{reg}\) cells (RMKO: TSLPR\(^-\) T\(_{reg}\) Fig. 5b). Both the severity and time frame of the disease in RMKO:TSLPR\(^-\) T\(_{reg}\) chimeras and the milder disease phenotype seen in RMKO:TSLPR\(^+\) T\(_{reg}\) chimeras were similar to those previously seen in TMKO or in Mi\(2\Delta\) mice. Thus, under skin pro-inflammatory conditions, TSLPR signaling in T\(_{reg}\) cells prevents progression to a severe systemic response. Consistent with an impairment in T\(_{reg}\) cell function, the sDLN cellularity was greater in RMKO:TSLPR\(^-\) T\(_{reg}\) chimeras than in RMKO: TSLPR\(^+\) T\(_{reg}\) chimeras (Fig. 5c). A greater expansion of T\(_{eff}\) cells was also seen in both the skin and sDLNs of RMKO:TSLPR\(^-\) T\(_{reg}\) chimeras (Fig. 5d–g and Supplementary Fig. 7a). Although the number of skin-associated T\(_{reg}\) cells with or without TSLPR signaling was similar in the two types of RMKO chimeras, possibly as a result of the short time of allowed analysis after Mi\(2\beta\) deletion (Fig. 5d–g and Supplementary Fig. 7a), induction of activation markers, such as CTLA4, CD103 and CD25, was consistently reduced in the absence of TSLPR signaling in these cells, similar to the TMKO mice (Fig. 5h and Supplementary Fig. 7b). Thus, under skin pro-inflammatory conditions, TSLPR signaling in skin T\(_{reg}\) cells was required for their activation and ability to repress expansion of local effector T cells. We also tested the role of IL-2R signaling in Mi\(2\Delta\) skin T\(_{reg}\) cells. Treatment of mice with an antibody to IL-2R\(\alpha\) (anti-CD25) has been shown to cause a transient reduction in T\(_{reg}\) cells\(^38\). After anti-CD25 treatment, a severe reduction in T\(_{reg}\) cells was observed in normal skin under homeostatic conditions compared with untreated controls (Supplementary Fig. 8). However, after Mi\(2\beta\) deletion in the skin (days 8–14), the number of skin T\(_{reg}\) cells was increased whether the mice were previously treated with anti-CD25 or not (Supplementary Fig. 8). At both early and later time points of Mi\(2\beta\) deletion, an expansion of skin T\(_{reg}\) cells was seen in spite of the anti-CD25 treatment. Thus, although IL-2 is critical for T\(_{reg}\) cell maintenance under skin homeostatic conditions, TSLP secreted by activated keratinocytes can drive T\(_{reg}\) cell expansion under skin pro-inflammatory conditions. In summary, skin T\(_{reg}\) cells are conditioned to respond to changes in their local environment by expressing TSLPR. Early induction
of TSLPR signaling in skin Treg cells is critical for balancing local immune cell responses and preventing their uncontrolled dissemination to the periphery.

**TSLPR signaling augments T<sub>reg</sub> effector transcription**

We next examined the global effect of TSLPR signaling on the expression of genes responsible for T<sub>reg</sub> cell effector function. Wild-type or TSLPR<sup>Treg</sup> cells were isolated from sDLNs associated with Mi-2β-depleted pro-inflammatory skin (WT<sup>Treg</sup>Mi2Δ and TSLPR∆<sup>Treg</sup>Mi2Δ) and used for RNAseq studies. Wild-type T<sub>reg</sub> cells from sDLNs associated with homeostatic skin were used as controls (WT<sup>Treg</sup>Hom). Differential gene expression analysis of wild-type T<sub>reg</sub> cells from pro-inflammatory compared with homeostatic skin revealed that 1,321 genes were upregulated and 925 genes were downregulated (Fig. 6a and Supplementary Tables 1 and 2). A similar trend of up- and downregulated genes was seen with TSLPR∆<sup>Treg</sup> cells from pro-inflammatory skin (TSLPR∆<sup>Treg</sup>Mi2Δ versus WT<sup>Treg</sup>Mi2Δ; Fig. 6a). However, the changes in gene expression detected in TSLPR∆<sup>Treg</sup> cells never reached the level detected in wild-type T<sub>reg</sub> cells under similar pro-inflammatory conditions (Fig. 6a–c).

Genes encoding transcription factors required for the transition of naive T<sub>reg</sub> cells to an activated effector state, such as Irf4 and Prdm1 (Blimp1), and those important in T<sub>reg</sub>H17 cells, such as Ahr, Rora and Ikzf3, were upregulated in wild-type T<sub>reg</sub> cells under skin pro-inflammatory conditions compared with homeostatic conditions (Fig. 6d)39–41. Genes involved in migration and epithelial localization of T<sub>reg</sub> cells, such as Ccr4, Ccr8, Ccr10 and Fat17, were also strongly induced, whereas Ccr7 was reduced in activated compared with resting wild-type T<sub>reg</sub> cells (Fig. 6d)35,36,42,43. Genes involved in Treg-cell-mediated immunosuppression, such as the co-stimulatory molecule Icos, the co-inhibitory molecules Cita14, Igals1 (galectin1), Tigit and Havcr2 (Tim3), the cytotoxic molecule Gzmb (granzyme B), and the effector molecules Fgl2 (fibrinogen-like protein 2) and Ebi3 (component of IL-35), were also strongly upregulated (Fig. 6d)44,45. The immunosuppressive cytokine Il10 was not markedly expressed in either activated or resting T<sub>reg</sub> cells (Fig. 6d). Notably, induction of these genes involved in the immunosuppressive response was greatly compromised in TSLPR∆<sup>Treg</sup> cells (Fig. 6d).

In summary, TSLPR signaling is a major contributor to the expression of genes that support a T<sub>reg</sub> cell's immunosuppressive function in the context of a pro-inflammatory skin response (Supplementary Fig. 9). Many of these genes were induced in the absence of TSLPR signaling; however, their expression overall was insufficient to support T<sub>reg</sub> cell suppressor function.

**DISCUSSION**

In its function as the barrier between the body and the outside world, the skin must respond to pathogenic insult by rapidly initiating a repair response and by deploying a pro-inflammatory reaction. We found that the chromatin remodeler Mi-2β in the NuRD complex holds these two components of a skin response poised for rapid deployment following injury. The first component includes growth factors and structural proteins that comprise an autocrine complex holds these two components of a skin response poised for rapid deployment following injury. The second component includes a panoply of stress-induced antigens, anti-microbial proteins, cytokines and chemokines that selectively target activation and mobilization of skin-resident immune cells in response to environmental insults. Loss of Mi-2β in basal keratinocytes was demarcated by hyperplasia in both the basal and suprabasal
Of the pro-inflammatory cytokines induced in Mi-2β-deficient keratinocytes, TSLP was the most prevalent. The Tslp gene was directly repressed by Mi-2β binding to an enhancer region that was also regulated by the vitamin D receptor and NF-κB. TSLP is a known sentinel of epithelial cell barrier integrity that can promote T<sub>12</sub>-cytokine-mediated skin inflammation. Consistent with previous studies, in the absence of TSLPR signaling, the T<sub>12</sub> response was diminished in the Mi-2β-deficient skin. However, T<sub>11</sub> and T<sub>11</sub> responses were largely unaffected and possibly increased. Activation of skin resident immune populations, such as DETCs and DCs, was also observed regardless of TSLPR signaling. These findings indicate that, although TSLPR signaling contributes to a skin pro-inflammatory response initiated by the loss of Mi-2β, this pro-inflammatory activity is redundant with other signals.

In contrast, we observed an unexpected and non-redundant role of TSLPR signaling in resolving an inflammatory response in these experiments. A rapid increase in T<sub>reg</sub> cells with an effector phenotype and strong in vitro immunosuppressive properties was seen in Mi-2βΔ skin and sDLNs. TCR ligation is not normally sufficient to activate T<sub>reg</sub> cells, and an additional signal provided by the environment in the form of a cytokine such as IL-2 is required for full T<sub>reg</sub> cells suppressive activity in situ. Unexpectedly, TSLP, a pro-inflammatory cytokine that is secreted by Mi-2β-deficient keratinocytes, was critical for the activation of skin T<sub>reg</sub> cells. In principle, TSLPR signaling could activate skin T<sub>reg</sub> cells either directly or indirectly by acting through another immune cell such as a DC. Using bone marrow chimeras generated by reconstituting lymphocyte-deficient mice with an immune system in which TSLPR signaling was defective only in T<sub>reg</sub> cells, we found that TSLPR signaling was directly required in skin T<sub>reg</sub> cells to promote their activation, to control expansion of local T<sub>eff</sub> cells and to protect against development of a lethal systemic response. T<sub>reg</sub> cells from Mi-2β-depleted skin showed a highly activated effector phenotype that was underscored by an increase in expression of transcription factors such as Irf4 and Prdm1, the co-inhibitory and effector molecules Clda4, Tigit, Lgals1, Fgl2 and Gzmb, and chemokine receptors such as Ccr4, Ccr8 and Ccr10. Induction of these factors, which are critical for T<sub>reg</sub> cell function, was impaired by the loss of TSLPR signaling. It is noteworthy that TSLP also induces expression of the CCR4 ligands CCL17 and CCL22 in DCs, thereby affecting the migration of activated skin T<sub>reg</sub> cells by influencing two independent cell types. The transcriptional profile of Mi2Δ-skin-associated T<sub>reg</sub> cells complemented the transcriptional profile of Mi-2β-depleted keratinocytes that express many T<sub>11</sub>- and T<sub>11</sub>17-promoting cytokines and the T<sub>12</sub> phenotype of CD4 T cells associated with Mi-2β-depleted sDLNs. Both T cell and DC inhibitory factors were strongly upregulated in these skin effector T<sub>reg</sub> cells, raising the possibility that a lack of proper induction following the loss of TSLPR signaling may allow aberrant activation of DCs and effector T cells that is rapidly disseminated in the periphery.

STAT5 was recently reported to be critical for the suppressive function of T<sub>reg</sub> cells. STAT5 is activated in response to IL-2R signaling in T<sub>reg</sub> cells and its activation is sufficient to rescue their function in the absence of IL-2R. In this context, it is noteworthy that TSLPR signaling activates STAT5 in other cell types and also contributes to STAT5 activation in T<sub>reg</sub> cells. We also found that anti-CD25 treatments that are sufficient to deplete T<sub>reg</sub> cells in normal skin did not prevent activation of T<sub>reg</sub> cells following Mi-2β deletion in keratinocytes in vivo. We therefore propose that the TSLP-TSLPR-STAT5 cascade...
Thymic stromal lymphopoietin links keratinocytes and dendritic cells

Identification of bone morphogenetic protein 7 (BMP7) as an anti-inflammatory cytokine in keratinocytes

Regulatory T cells in skin diseases, in which the prevalent paradigms are immunosuppressive

The role of the chromatin remodeler Mi-2beta in hematopoietic stem cell self-renewal and multilineage differentiation. Genes Dev. 22, 1174–1189 (2008).

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Preparation of single cell suspension from ear skin and dorsal skin. Ear or dorsal skins were cut in pieces, and incubated for 20–30 min at 37 °C in 0.1% collagenase D (Roche) and 100 units/ml DNase I. Cells were then washed three times in ice-cold RPMI containing 10% FCS and 2 mM EDTA.

Flow cytometry of immune cell populations. Phenotypic analysis of cells from skin or sDLNs was performed by flow cytometry using a FACSCanto (BD Biosciences). Data was acquired with the FlowJo software (Tristar). The antibodies used were: CD45 (55M11, 1:300), CD3ε (2C11, 1:200), CD4 (GK1.5, 1:100), CD8 (53-6.7, 1:200), CD45 (183-1, 1:200), CD3-25 (PC61, 1:200), CD44 (IM7, 1:200), CD69 (H1.2F3, 1:200), IL-2 (JES5-128, 1:100), TNF (MP6-XT22, 1:100), IFN-γ (XM16.2, 1:200), Ki67 (6F5, 1:100), CCL2 (4G9, 1:200), CCL5 (G04-17, 1:200), CD49a (2B11, 1:200), CD133 (2E7, 1:200), CD44 (G04-17, 1:200), and TRAP (Non-1, 1:200).

Isolation of basal keratinocytes from dorsal epidermis. Dorsal epidermis was separated from the dermis after overnight digestion at 4 °C in 0.25% trypsin. The epidermis was washed three times in PBS and stirred for 10 min in PBS containing 10% CaCl2-depleted FCS (FCS). Cells were then stained with anti-CD45 (30-F11, 1:200), anti-CD34 (RAM34), and anti-ITGAV6 (eBioGoH3) and basal keratinocytes were sorted as ITGA6+CD34+CD45- using a MoFlo sorter (Cytomation). Two independent sorts of basal keratinocytes from wild-type N = 3 and Mi2A N = 2 mice were used for two RNA-seq experiments.

RNA-sequencing, gene-expression and pathway analysis. Total RNA was isolated with RNeasy kit (Qiagen). NEXArray RNAseq kit (BIOO scientific) or a TrueSeq stranded mRNA sample prep kit (Illumina) were used for construction of cDNA libraries for RNA-Sequencing. The cDNA libraries were ligated with indexed primers, followed by 15 cycles of PCR amplification. The amplified libraries were multiplexed and sequenced by Illumina HiSeq2000 at the Baurer Center of Harvard University. Raw sequencing files were subjected to quality control using FastQC. Read alignment was performed on the mm9 or mm10 assembly of the mouse genome using either the Burrows-Wheeler or the STAR genome alignment algorithms.55,56 Read normalization and differential gene expression was performed with the Deseq2 algorithm through HOMER implementation of the R platform.57,58 Heat maps of normalized exon read counts for differentially expressed genes were generated with Cluster 3.0 (open source software developed by Michael Eisen) and visualized with Java TreeView (open source software developed by Alok J. Saldanha). Differentially expressed genes shown in the heatmap of Figure 6a were filtered for having a value of 64 normalized raw reads in at least one sample and a difference of 1 between the maximum and minimum log2 transformed normalized raw reads. DAVID Bioinformatics Resources 6.7 was used for Gene Ontology analysis.59 Changes in gene expression as determined by cumulative distribution function plot was also analyzed by the Kolmogorov-Smirnov test (GraphPad Prism, P < 0.05 was considered significant. The RNA-seq data sets were deposited to the GEO series database (GSE90573).

Induction of Mi-2β deletion in primary cultured keratinocytes. Cultures were prepared from newborn Mi-2βloxF/loxF Krt14-CreERT2 mice and grown to confluence in low-calcium keratinocyte growth medium ( Gibco) at 37 °C, 5% CO2. After reaching 80% confluence, cells were treated with 300 nM 4-OHT in DMEM for 16 h at 37 °C, 5% CO2. Cells were then washed three times with PBS, fresh media were added and cells were incubated for 32 h before RNA preparation or for 56 h before collection of conditioned media.

RT-qPCR. Total RNA was extracted with Trizol (Invitrogen) and purified with RNeasy kit (Qiagen), followed by reverse transcription with the Superscript III RT-PCR system (Invitrogen). qPCR analyses were performed on 7000 real-time PCR system (Applied Biosystems) using gene-specific primer pairs. Data were normalized by the abundance of the Actb transcript. The sequences of primers are listed in Supplementary Table 3.

Antibody array. Secretion of TSLP in conditioned media was measured by the Mouse Cytokine Antibody Array 4 (RayBio) according to the manufacturer’s protocol.

ChIP-qPCR. Mi-2β ChIP was performed as described previously. Briefly, chromatin was prepared from primary cultured keratinocytes from newborn wild-type mice, followed by ChIP with anti-Mi-2β (16G4, 2G8, 17H11 and 16F5; produced in house). ChIPed DNA was subjected to analysis by real-time qPCR. Fold enrichment was calculated as target/ negative control (intergenic region of Chr5) for anti-Mi-2β ChIP/ [target/ negative control] for the input. The sequences of primers are listed in Supplementary Table 3.

Preparation of epidermal sheets and Immunofluorescence. The epidermal sheet of ear skin was separated from the dermal layer by digestion at 37 °C in 0.25% trypsin for 10 min. The epidermis was washed three times in PBS, fixed for 15 min at −20 °C in ice-cold acetone, washed two times in PBS, and rehydrated for 30 min at 25 °C in PBS. Nonspecific binding on the epidermis was then blocked with two incubations in 1% BSA and 0.1% Tween-20 in PBS, followed by overnight incubation at 4 °C with FITC-conjugated anti-CD3ε (2C11) or anti-CD207 (4C7), followed by TRITC-conjugated secondary antibodies.

Purification of CD4+ T cell subsets. CD4+Foxp3- GFP+ Treg cells, CD4+CD62L+Foxp3-GFP- naive T cells and CD4+Foxp3-GFP+ Treg cells were
Mice were injected i.p. with 400 IL-2R6. Cells were fixed overnight at 4 °C in 70% ethanol. Fixed 2–∆ε bone marrow from reg- A two-tailed unpaired t-test was used to calculate differences between two groups (GraphPad Prism). P < 0.05 was considered significant. The F test was applied to determine variation within groups and Welch’s correction to the t-test was applied to groups with unequal variances. Statistical tests were not used to pre-determine group size, samples were not randomized and investigators were not blinded to experimental group allocations.

Data availability statement. Supporting data are available from M.K. and K.G. Gene Expression Omnibus: RNA-seq data sets have been deposited under the accession code GSE90573. The source data for Figures 1–6 are available online.

In vivo Treg cell suppression assay. Sorted CD4+Foxp3-GFP+ Treg cells were co-cultured with sorted CD4+CD62L+Foxp3-GFP naive T cells that were stimulated with anti-CD3e (1 µg/ml) in the presence of irradiated antigen-presenting cells (APCs) for 3 days at 37 °C, 5% CO2. APCs, isolated from wild-type spleens after T cell depletion using anti-CD4 and anti-CD8 magnetic beads (Miltenyi Biotec), or Rag1−/− spleens were irradiated before use.

In vitro re-stimulation of CD4+ effector T cells. Sorted CD4+Foxp3-GFP+ cells were co-cultured for 16 h in RPMI containing 10% FCS in the presence of brefeldin A. Cells were then re-stimulated with PMA plus ionomycin for 4 h in the presence of anti-CD3 µg/ml propidium iodide (PI), and DNA content was analyzed by FACSCanto and FlowJo software.

Cell-cycle analysis. Cells were fixed overnight at 4 °C in 70% ethanol. Fixed cells were stained for 30 min at 37 °C with propidium iodide staining buffer (250 µg/ml RNase A and 50 µg/ml propidium iodide (PI)), and DNA content was analyzed by FACSCanto and FlowJo software.

Generation of bone marrow chimeras. Bone marrow from Foxp3-IRESCre Crlf2loxP/loxF mice was used in adoptive transfers as a source of TSLRΔ Treg cells. Bone marrow from Foxp3-IRESCre Crlf2loxP/loxF mice with intact TSLR signaling in Treg cells was used as a control. 4 × 10⁶ bone marrow cells were transferred into lethally irradiated (500 rads twice, 16 h apart) wild-type or MipΔ recipients or sublethally irradiated (300 rads twice, 16 h apart) RagΔ or RMKO recipients. 8–12 weeks after reconstitution Mi-2β deletion was induced with 4-OHT delivered twice within a 3-d interval. WT and MipΔ chimeras were analyzed 8–11 d after 4-OHT administration. Rag and RMKO chimeras were analyzed at 7 d later.

In vivo IL-2Rα blockade. Mice were injected i.p. with 400 µg/mouse anti-CD25 (PC61) 5 days before inducing Mi-2β deletion with 4-OHT™. A PBS-injected group was used as a control.

Statistical analysis. A two-tailed unpaired t-test was used to calculate differences between two groups (GraphPad Prism). P < 0.05 was considered significant. The F test was applied to determine variation within groups and Welch’s correction to the t-test was applied to groups with unequal variances. Statistical tests were not used to pre-determine group size, samples were not randomized and investigators were not blinded to experimental group allocations.

Data availability statement. Supporting data are available from M.K. and K.G. Gene Expression Omnibus: RNA-seq data sets have been deposited under the accession code GSE90573. The source data for Figures 1–6 are available online.

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