INTRODUCTION

As an outstanding discovery of recent years, the microbial community exhibits remarkable diversity on topographically distinct skin regions, which may be accompanied by differences in skin immune characteristics. Our aim was to compare the immune milieu of healthy sebaceous gland-rich (SGR) and sebaceous gland-poor skin areas, and to analyze its changes in an inflammatory disease of SGR skin. For this purpose, immunohistochemical, immunocytochemical, and quantitative real-time PCR analyses of thymic stromal lymphopoietin (TSLP) and other cytokines, phenotypic immune cell markers and transcription factors were carried out in samples from sebaceous gland-poor, SGR skin and from papulopustular rosacea. TSLP mRNA and protein production was also studied in cultured keratinocytes. In SGR skin, higher TSLP expression, dendritic cell appearance without prominent activation, and T cell presence with IL-17/interferon-γ cytokine milieu were detected compared with sebaceous gland-poor skin. Linoleic acid, a major sebum component, was found to induce TSLP expression dose-dependently in keratinocytes. In papulopustular rosacea, significantly decreased TSLP level and influx of inflammatory dendritic cells and T cells with IL-17/interferon-γ cytokine milieu were observed. According to our results, SGR skin is characterized by a distinct, noninflammatory immune surveillance, which may explain the preferred localization of inflammatory skin diseases, and can influence future barrier repair therapeutic concepts.


Sebaceous Gland-Rich Skin Is Characterized by TSLP Expression and Distinct Immune Surveillance Which Is Disturbed in Rosacea

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The microbial community exhibits remarkable diversity on topographically distinct skin regions, which may be accompanied by differences in skin immune characteristics. Our aim was to compare the immune milieu of healthy sebaceous gland-rich (SGR) and sebaceous gland-poor skin areas, and to analyze its changes in an inflammatory disease of SGR skin. For this purpose, immunohistochemical, immunocytochemical, and quantitative real-time PCR analyses of thymic stromal lymphopoietin (TSLP) and other cytokines, phenotypic immune cell markers and transcription factors were carried out in samples from sebaceous gland-poor, SGR skin and from papulopustular rosacea. TSLP mRNA and protein production was also studied in cultured keratinocytes. In SGR skin, higher TSLP expression, dendritic cell appearance without prominent activation, and T cell presence with IL-17/IL-10 cytokine milieu were detected compared with sebaceous gland-poor skin. Linoleic acid, a major sebum component, was found to induce TSLP expression dose-dependently in keratinocytes. In papulopustular rosacea, significantly decreased TSLP level and influx of inflammatory dendritic cells and T cells with IL-17/interferon-γ cytokine milieu were observed. According to our results, SGR skin is characterized by a distinct, noninflammatory immune surveillance, which may explain the preferred localization of inflammatory skin diseases, and can influence future barrier repair therapeutic concepts.

The tolerogenic role of TSLP is supported by recent studies where decreased TSLP level and altered microbial composition were found in Crohn’s disease (Podolsky, 2002; Round and Mazmanian, 2009). Until now, TSLP in the skin was only described under inflammatory conditions, such as atopic dermatitis (AD) and psoriasis, and its only known function in this organ so far is the promotion of Th helper (Th2) polarizing DCs (He and Geha, 2010).

In this study, we asked the question whether the above topographical differences in skin microbiota and physiology can also be accompanied by topographical differences in skin immune activity and TSLP (epiimmunome) production. The possibility that the skin immune system is characterized by distinct functional tuning on different skin regions was not challenged until now in the literature.

**RESULTS**

**TSLP protein is constitutively expressed in SGR healthy skin, but almost absent from SGP healthy skin**

To detect TSLP protein in topographically different skin regions, biopsies from sebaceous gland-poor (SGP; representing dry areas) and sebaceous gland-rich (SGR; representing seborrheic areas) healthy skin were obtained. Lesional skin of patients with severe AD was used as a positive control for TSLP staining. To confirm immunohistochemistry (IHC) results three different antibodies against TSLP were used (Figure 1a). In AD samples, strong TSLP positivity was detected in the granular and corneal but not in the basal and suprabasal layers of the epidermis. In all SGR skin biopsies, high TSLP expression was detected with all three anti-TSLP antibodies in the epidermal keratinocytes (KCs), mainly in the upper epidermal layers, and in sebocytes of sebaceous glands (Table 1). In contrast, in SGP samples, TSLP was completely or almost completely absent. Importantly, the intensity of TSLP staining (as assessed by Pannoramic Viewer software) was found to be significantly higher in SGR skin compared with SGP skin. However, TSLP expression in SGR skin was significantly lower than that in AD skin (Figure 1a and b). TSLP protein levels were also measured in the stratum corneum by immunocytochemistry and were also found to be significantly elevated in SGR skin compared with SGP skin, but did not reach the level found in AD skin (Figure 1c).

Interestingly, quantitative real-time PCR (RT-PCR) analysis detected nearly similar total TSLP mRNA expression in all skin types (SGR, SGP, and AD skin) (Figure 1d).

**Linoleic acid induces TSLP expression in KCs**

Sebum content, composition of commensal microbiota, and UV radiation are able to influence SGR and SGP skin differently; therefore, the effects of these factors on TSLP production in HaCaT and normal human epidermal keratinocyte cells were analyzed by using RT-PCR and ELISA. As similar TSLP protein levels were detected in hairy scalp (UV-protected) and face (UV-exposed) biopsy samples (Table 1), we did not investigate further the effect of UV.

To study the effect of chitin—a major component of *Demodex folliculorum*, which is part of the normal skin flora in SGR skin—and sebum, HaCaT KCs were treated with chitin (Figure 2a), with supernatant of cultured human SZ95 sebocytes (Zouboulis et al., 1999) (Figure 2b) and with different lipid components of sebum (Figure 2c). After chitin and sebocyte supernatant treatment, induction of TSLP mRNA could be nonsignificantly triggered. Of the used lipid components, palmitic acid, oleic acid, and linoleic acid upregulated TSLP gene expression, but only linoleic acid could elevate it significantly. Furthermore, we showed that linoleic acid induces TSLP mRNA expression in a concentration-dependent manner, reaching its maximum and significantly higher level at 150 μM (Figure 2d and e). On the other hand, the basal TSLP protein levels could not be elevated by any of the aforementioned agents (Figure 2a–c).

As sebum components influenced prominently TSLP expression in HaCaT cells, these experiments were repeated in normal human epidermal keratinocytes and similarly linoleic acid could dose-dependently elevate TSLP mRNA levels (Figure 2f and g). No TSLP protein secretion by normal human epidermal keratinocytes could be detected (not shown).

It has previously been found in AD skin that barrier damage can also lead to TSLP production by KCs (Mocsai et al., 2014); therefore, transepidermal water loss and skin pH, representing barrier functions, were measured on SGP and SGR skin regions. No differences were detected, indicating that barrier damage is most probably not the cause of distinct TSLP production in SGR and SGP skin (not shown).

**SGR skin is characterized by an elevated number of DCs without prominent activation and maturation compared with SGP skin**

The significantly higher TSLP level of SGR skin suggested that differences in other immune surveillance factors may also exist. Because DCs are the major target cells of TSLP, CD11c+ dermal myeloid DCs and CD1a+ Langerhans cells were immunolabeled and quantified in SGR and SGP skin samples. IHC revealed that CD11c+ DCs were present in significantly higher numbers (Figure 3a and d) in SGR skin compared with SGP skin and the majority of these cells were characteristically localized near sebaceous glands or the duct of the glands. In AD skin, DC count was higher compared with SGR skin and DCs were found to be diffusely infiltrated in the dermis (Figure 3a and d). In contrast, no significant differences were found between the LC counts of SGP and SGR skin samples (Figure 3g; see Supplementary Figure S1a online).

To further analyze the characteristics of DCs, their classical maturation and/or activation markers CD80, CD83, CD86, and DC-lysosomal associated membrane protein were investigated on the mRNA level. As the classical proinflammatory effect of TSLP is to boost Th2 polarizing DCs in allergic diseases, thymus- and activation-regulated chemokine (TARC) (also known as chemokine [C-C motif] ligand 17 [CCL17]), an atopic eczema-specific, DC-secreted chemokine, and CD83 were also assessed by IHC. Although the number of CD83 positive cells (Figure 3c and f) and mRNA levels of CD80 (Figure 3h), CD83 (Figure 3i), CD86 (Figure 3k), and lysosomal associated membrane protein 3 (CD208) (Figure 3j) could be found in somewhat higher amounts in SGR skin compared with SGP, none of the investigated markers’ expression differed significantly, whereas significantly higher numbers of CD83+ cells were detectable in AD samples (Figure 3c and f). TARC was completely absent from both types of healthy skin, but was present in AD samples (Figure 3b and e).
Figure 1. TSLP is absent from SGP skin, but constitutively expressed in SGR skin and attenuated in PPR skin. (a) Representative images for immunostaining of TSLP with three different TSLP antibodies (TSLP Ab 1: rabbit polyclonal antihuman TSLP Ab; TSLP Ab 2: sheep polyclonal antihuman TSLP Ab; TSLP Ab 3: mouse monoclonal antihuman TSLP Ab) in SGP, SGR, AD, and PPR skin sections. Size bars = 100 μm. Ig or isotype controls are presented in the bottom-right corner of SGR and AD samples. Quantification of (b) epidermal TSLP protein levels, (c) stratum corneum TSLP protein levels, and (d) TSLP mRNA levels. The graphs show the mean ± standard error of the means of measured protein and mRNA levels (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by the Newman-Keuls test). Ab, antibody; AD, atopic dermatitis; ANOVA, analysis of variance; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich; TSLP, thymic stromal lymphopoietin.
Elevated T cell number and noninflammatory IL17/IL-10 cytokine milieu feature SGR skin

Next, CD3+ and CD4+ cells were stained in SGR and SGP skin samples. CD3+ (Figure 4a and d) and CD4+ (Figure 4b and e) T cells were present in significantly higher numbers in SGR skin compared with SGP skin. The localization of T cells was similar to that of DCs, and the clear majority of T cells were Th cells.

As a next step, representative cytokines of Th subsets (IL-10: regulatory T cell [Treg]; IL-13: Th2; IL-17: Th17, and IFN-γ: Th1) were immunostained. IHC revealed that no IL-13+ and IFN-γ+ cells could be detected in either of the healthy skin types. IL-10+ and IL-17+ cells showed similar patterns; they were detected at very low levels or absent from SGP skin, but were found at significantly higher levels in SGR skin (Figure 5a–d; see Supplementary Figure S1b–e). RT-PCR analyses of the aforementioned cytokines were also performed and showed a similar pattern to that found at the protein levels, although the differences were not significant (Figure 5e–h). In SGP skin, the cytokine content was very low, in contrast to the characteristic IL-17/IL-10 cytokine milieu of SGR skin.

Then, the mRNA levels of transcription factor characteristic of different Th cell subsets were investigated. Expression of T-bet (TBX21 gene), mediating inflammatory Th17 [Th17(23)] and Th1 cell responses (Figure 5i), and GATA3, mediating Th2 responses (Figure 5j), were detected at similar levels in SGP and SGR skin. On the other hand, RORγt (RORC gene), mediating noninflammatory Th17 [Th17(23)] and Th17(23) development (Figure 5k) and FOXP3, characteristic of Tregs (Figure 5l), showed notably higher expression levels in SGR compared with SGP skin. CCR4 (Figure 5m) and CCR8

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**Table 1. Characteristics and TSLP protein expression in the skin of the studied individuals**

<table>
<thead>
<tr>
<th>Healthy individuals (HI)/patients (P)</th>
<th>Sex</th>
<th>Age</th>
<th>Localization</th>
<th>Count of sebaceous glands</th>
<th>Intensity of TSLP staining (visual scoring)</th>
<th>Intensity of TSLP staining (Pannoramic Viewer software)</th>
</tr>
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<tbody>
<tr>
<td>SGP skin (n = 8)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HI 1</td>
<td>M</td>
<td>77</td>
<td>Shin</td>
<td>−</td>
<td>+</td>
<td>1.50E⁻03</td>
</tr>
<tr>
<td>HI 2</td>
<td>M</td>
<td>85</td>
<td>Shin</td>
<td>−</td>
<td>−</td>
<td>7.01E⁻04</td>
</tr>
<tr>
<td>HI 3</td>
<td>F</td>
<td>72</td>
<td>Lower arm</td>
<td>−</td>
<td>−</td>
<td>6.43E⁻04</td>
</tr>
<tr>
<td>HI 4</td>
<td>F</td>
<td>81</td>
<td>Lower arm</td>
<td>−</td>
<td>−</td>
<td>5.70E⁻04</td>
</tr>
<tr>
<td>HI 5</td>
<td>M</td>
<td>40</td>
<td>Lower arm</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>HI 6</td>
<td>F</td>
<td>72</td>
<td>Lower arm</td>
<td>−</td>
<td>+</td>
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<tr>
<td>HI 7</td>
<td>F</td>
<td>86</td>
<td>Hand</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>HI 8</td>
<td>F</td>
<td>56</td>
<td>Shin</td>
<td>−</td>
<td>−</td>
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<td>Mean age ± SD</td>
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<td></td>
</tr>
<tr>
<td>HI 9</td>
<td>F</td>
<td>77</td>
<td>Heary scalp</td>
<td>+</td>
<td>++</td>
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<tr>
<td>HI 10</td>
<td>M</td>
<td>62</td>
<td>Mandibula</td>
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<tr>
<td>HI 11</td>
<td>F</td>
<td>57</td>
<td>Nose</td>
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<td>+++</td>
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</tr>
<tr>
<td>HI 12</td>
<td>F</td>
<td>61</td>
<td>Nose</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>HI 13</td>
<td>F</td>
<td>42</td>
<td>Scapula</td>
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<td>++</td>
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<tr>
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<td>Chin</td>
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<td>M</td>
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<td>Shoulder</td>
<td>+++</td>
<td>+</td>
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<td>Heary scalp</td>
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<td>+++</td>
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<tr>
<td>HI 17</td>
<td>F</td>
<td>19</td>
<td>Face (central part)</td>
<td>+++</td>
<td>+++</td>
<td>8.01E⁻03</td>
</tr>
<tr>
<td>HI 18</td>
<td>M</td>
<td>66</td>
<td>Face (lateral part)</td>
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<td>+++</td>
<td>7.07E⁻03</td>
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<td>Mean age ± SD</td>
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<tr>
<td>P 1</td>
<td>F</td>
<td>65</td>
<td>Face</td>
<td>+++</td>
<td>++</td>
<td>6.78E⁻03</td>
</tr>
<tr>
<td>P 2</td>
<td>F</td>
<td>71</td>
<td>Face</td>
<td>+++</td>
<td>−</td>
<td>9.18E⁻04</td>
</tr>
<tr>
<td>P 3</td>
<td>M</td>
<td>70</td>
<td>Nose</td>
<td>+++</td>
<td>−</td>
<td>1.03E⁻04</td>
</tr>
<tr>
<td>P 4</td>
<td>F</td>
<td>68</td>
<td>Face</td>
<td>+++</td>
<td>−</td>
<td>1.01E⁻03</td>
</tr>
<tr>
<td>P 5</td>
<td>F</td>
<td>57</td>
<td>Nose</td>
<td>+++</td>
<td>+</td>
<td>3.01E⁻03</td>
</tr>
<tr>
<td>P 6</td>
<td>M</td>
<td>69</td>
<td>Nose</td>
<td>+++</td>
<td>+</td>
<td>3.83E⁻03</td>
</tr>
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<td>P 7</td>
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<td>66</td>
<td>Face</td>
<td>++</td>
<td>+</td>
<td>4.25E⁻03</td>
</tr>
<tr>
<td>P 8</td>
<td>M</td>
<td>62</td>
<td>Eyebrow</td>
<td>+++</td>
<td>+++</td>
<td>6.41E⁻03</td>
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<tr>
<td>P 9</td>
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<td>65</td>
<td>Forehead</td>
<td>++</td>
<td>+</td>
<td>3.79E⁻03</td>
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<tr>
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<td>Eyebrow</td>
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<td>+</td>
<td>4.56E⁻03</td>
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<tr>
<td>Mean age ± SD</td>
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Scoring of sebaceous gland count was performed according to the number and size of sebaceous glands in the field of view on x10 magnification: samples containing n ≤ 1 sebaceous gland were defined as negative (−), those containing n ≥ 3 sebaceous glands were defined as positive and scored in accordance with the area of sebaceous glands in percentage of dermal surface: (+) 5–15%; (+++) 15–30%, and (+++++) more than 30%. Visual scoring of TSLP staining was performed according to the percentage of the epidermal surface positively stained for TSLP: (−) 0–5%; (+) 5–15%; (+++) 15–30%, and (+++++) more than 30%.

Abbreviations: PPR, papulopustular rosacea; SD, standard deviation; SGP, sebaceous gland poor; SGR, sebaceous gland rich; TSLP, thymic stromal lymphopoietin.
Treatment of HaCaTs with chitin (RT-PCR)

Treatment of HaCaTs with chitin (ELISA)

Treatment of HaCaTs with chitin (ELISA)

Treatment of postconfluent NHEKs with FFAs (RT-PCR, 6h)

Treatment of preconfluent NHEKs with FFAs (RT-PCR, 6h)

Treatment of preconfluent NHEKs with linoleic acid (RT-PCR, 6h)

Treatment of HaCaTs with linoleic acid (RT-PCR, 24h)

Treatment of HaCaTs with SZ95 sebocytes' supernatant (RT-PCR)

Figure 2. Linoleic acid upregulates TSLP gene expression in HaCaT keratinocytes. HaCaT KCs were incubated with (a) different chitin concentrations (0.2, 0.5, and 2 mg/ml), with (b) SZ95 sebocyte culture medium and SZ95 sebocyte supernatant (40% and 80%) and with (c) different sebum components, for 24 hours. Concentration-dependent effect of linoleic acid (37.5, 75, 112.5, and 150 μM) and Poly (I:C) after (d) 6-hour and (e) 24-hour treatment. TSLP mRNA levels were detected after treating pre- and postconfluent NHEK cells with different sebum components (f) and with different concentrations of linoleic acid (g) for 6 hours. No TSLP secretion of NHEKs could be detected by ELISA (not shown). Higher concentrations of linoleic acid than 150 μM highly decreased the viability of both cell types, and incubation of NHEKs with linoleic acid for 24 hours had a toxic effect. TSLP mRNA and protein levels were quantified by RT-PCR and ELISA. The graphs show the mean ± standard error of the means of measured protein and mRNA levels (*P < 0.05, as determined by one-way ANOVA followed by the Newman-Keuls test). ANOVA, analysis of variance; FFA, free fatty acid; KC, keratinocyte; NHEK, normal human epidermal keratinocyte; RT-PCR, quantitative real-time PCR; TSLP, thymic stromal lymphopoietin.
Figure 3. Elevated DC count with low activation state and without TARC positivity is detected in SGR skin. A robust influx of CD83+, TARC-negative DCs is characteristic to PPR. Representative images for immunostaining of (a) CD11c, (b) TARC, and (c) CD83 in SGP, SGR, AD, and PPR skin sections. Size bars = 100 μm. Cell counts of (d) CD11c+ DCs, (e) TARC+ DCs, (f) CD83+ DCs, and (g) Langerhans cells were blindly analyzed by the Pannoramic Viewer software. Quantification of (h) CD80, (i) CD83, (j) LAMP3, and (k) CD86 mRNA levels by RT-PCR. (l) Strong, but not significant inverse correlation was found in PPR skin between the TSLP level and DC count by the Pearson r test (P = 0.0526; Pearson r = −0.7219). The graphs show the mean ± standard error of the means of measured protein and mRNA levels (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by the Newman-Keuls test). AD, atopic dermatitis; ANOVA, analysis of variance; DC, dendritic cell; LAMP3, lysosome-associated membrane glycoprotein 3; PPR, papulopustular rosacea; RT-PCR, quantitative real-time PCR; SGP, sebaceous gland poor; SGR, sebaceous gland rich; TARC, thymus- and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin.
Figure 4. SGR skin sites are characterized by the remarkable T cell presence and similar macrophage count compared with SGP skin. In PPR skin, robust influx of both cell types was observed. Representative images for immunostaining of (a) CD3, (b) CD4, and (c) CD163 in SGP, SGR, and PPR skin sections. Cell counts of (d) CD3+, (e) CD4+ T cells, and (f) CD163+ macrophages were blindly analyzed by the Pannoramic Viewer software. Size bars = 100 μm. (f) Comparison of the presence of eosinophils, neutrophils, and mast cells by a professional pathologist after May-Grunwald-Giemsa staining. Scoring system: (−) no cell observed; (+) low cell count; (++) moderate cell count; (+++) high cell count. The graphs show the mean ± standard error of the means of measured protein levels (*P < 0.05; **P < 0.01; ***P < 0.001, as determined by one-way ANOVA followed by the Newman-Keuls test). ANOVA, analysis of variance; PPR, papulopustular rosacea; SGP, sebaceous gland poor; SGR, sebaceous gland rich.
Supplementary Figure S1 f). Neither neutrophils nor eosinophils were present in healthy skin (Figure 5 i) and FOXP3 (Figure 5 l) were significantly higher in mRNA levels of cytokines corresponded to their protein absent (Figure 5 a). Compared with SGR skin samples, an especially robust IFN-γ detectable in PPR skin (Figure 3 b and e). Moreover, strong, in-crease in the number of IL-10 formed in PPR skin samples. Parallel to the prominent in-
ences in its mRNA levels were found (Figure 1 d). Infiltrating CD11c+ DCs (Figure 3 a and d), CD3+, and CD4+ T cells (Figure 4 a, b, d, and e) were detected in significantly higher numbers in PPR compared with SGR skin and were present diffusely through the dermis. CD80, CD83, DC-lyosomal associated membrane protein, and CD86 activation and/or maturation markers of DCs (Figure 3 h–k) were all significantly upregulated on mRNA levels compared with SGR skin. Although CD83+ DCs were present in significantly elevated numbers (Figure 3 c and f), TARC positivity was almost undetectable in PPR skin (Figure 3 b and e). Moreover, strong, but nonsignificant correlation was detected between the increase of DC count and the decrease of TSLP level in PPR samples (Figure 3 l). No difference was found between SGR and PPR skin regarding the number of Langerhans cells (Figure 3 g; see Supplementary Figure S1 a). Significantly higher numbers of macrophages, mast cells, and neutrophils could be detected in PPR skin compared with SGR, whereas eosinophils were absent from both SGR and PPR samples (Figure 4 c, f; g; see Supplementary Figure S1 f).

The characterization of cytokine milieu was also performed in PPR skin samples. Parallel to the prominent in-
crease in the number of IL-10+ and IL-17+ cells in PPR compared with SGR skin samples, an especially robust IFN-γ+ cell presence was detected, whereas IL-13+ cells were absent (Figure 5 a–d; see Supplementary Figure S1 b–e). The mRNA levels of cytokines corresponded to their protein levels (Figure 5 e–h). Gene expression levels of TBX21 (Figure 5 i) and FOXP3 (Figure 5 l) were significantly higher in PPR compared with SGR skin, whereas RORC (Figure 5 k) and GATA3 (Figure 5 j) gene expression levels were lower than in healthy SGR skin. Expressions of both Treg homing receptors (CCR4 and CCD8) were significantly higher in PPR samples than in SGR skin (Figure 5 m and n).

DISCUSSION

Skin microbial community exhibits remarkable differences on seborrheic, dry, and moist regions probably connected to the different physiology of these sites (Grice et al., 2009; Grice and Segre, 2011). Because skin microbiota has mutu-alistic connection with the skin immune system (Belkaid and Segre, 2014; Belkaid and Tamoutounour, 2016; Grice and Segre, 2011; Naik et al., 2015), possible immunological distinctions between topographically different healthy skin sites can be postulated but have not been revealed.

Previous investigations on the immune activity of intestinal mucosa indicated a distinct presence of TSLP in particular gut sections; thus, in our study, TSLP also an important cytokine of KCs, was studied first (Rimoldi et al., 2005; Swamy et al., 2010). A constitutive TSLP protein expression was detected by two methods (IHC and immunocytochemistry) in healthy SGR skin areas; in contrast, in healthy SGP areas, TSLP was practically absent. Although TSLP mRNA expression has already been detected in healthy skin, its protein expression (Bjerkan et al., 2015; Fornasa et al., 2015) was found only in inflamed epidermis (AD, psoriasis) until now (Soumelis et al., 2002; Ziegler, 2012). The conflicting data between the previous publications and our current study might be explained by that the other investigators most probably used healthy SGP skin and no SGR samples as controls of AD or psoriasis.

Although the protein expression of TSLP showed remark-able differences between healthy SGP and SGR skin, mRNA levels were similar in all samples. The discrepancy between the protein and mRNA expressions of TSLP can be explained by important, but presently unknown posttranscriptional modifications during KC differentiation. Bogiatzi et al. (2007) detected a basal TSLP mRNA expression without the presence of the protein in cultured KCs, and this mRNA content was not upregulated in the presence of proallergic cytokines. On the other hand, when whole skin explant, a model that preserves the differentiation of KCs, was used, TSLP protein could be measured after cytokine incubation (Bogiatzi et al., 2007). The importance of the posttranscriptional modifications can also explain our observations that, although linoleic acid could significantly and dose-dependently elevate TSLP mRNA levels, protein production was not increased. Until now, no study investigated the effect of sebum components and S295 sebocyte supernatant on TSLP mRNA and protein expression of cultured or HaCaT KCs, and only one research group examined the outcome of chitin treatment on primary and HaCaT KCs’ TSLP protein production (Koller et al., 2011). Although mRNA levels were not investigated, the authors detected significantly elevated TSLP protein levels in a concentration-dependent manner, which could not be confirmed by us. Nevertheless, according to our results, sebum lipid content may have a role in the initiation of TSLP production in SGR skin.

Because DCs are the major target cells of TSLP action, the significantly higher TSLP levels of SGR skin proposed that...
differences in other immune surveillance factors may also exist between SGR and SGP areas. Although no difference was found in the LC count, the number of CD11c+ DCs was significantly elevated in SGR skin compared with SGP areas. Despite their high number, DCs did not exhibit noticeable activity. Moreover, the complete absence of TARC+ cells indicates that TSLP expressed by SGR skin does not act as it is described in AD skin, where TSLP induces TARC+ DCs promoting Th2 responses (Soumelis et al., 2002). The fact that TSLP has not only an inflammatory but also a tolerogenic function (especially in lower amounts) on DCs is known from immunological studies of the gut (Zeuthen et al., 2008; Ziegler and Artis, 2010). A plethora of evidence supports the crucial role of TSLP in the maintenance of intestinal immune homeostasis and tolerance to commensal flora (Rimoldi et al., 2005; Zaph et al., 2007). We propose that TSLP found in SGR skin might have a similar role, because (i) SGR skin samples were clinically healthy without any signs of inflammation; (ii) the amount of TSLP was lower than found in AD samples; and (iii) DCs were TARC negative without noticeable activation.

We also examined and found differences between SGR and SGP skin sites regarding the number of T cells and levels of cytokines, as well as transcription factors characteristic to different Th subsets. Significantly more T cells were present in SGR skin, which were dominantly Tregs and probably also Th17(β) cells. The presence of Tregs was proven by the higher expression of FOXP3 and CCR4 as well as CCR8 Treg homing receptors accompanied by significantly higher IL-10+ cell counts. According to recent data, approximately 20% of CD4+ cells in healthy human skin express FOXP3 and these Tregs are effector memory cells being associated with hair follicles (Nomura et al., 2014; Sanchez Rodriguez et al., 2014); however, these authors did not compare different topographical skin sites. The higher Treg content detected in our study was also supported by a recent clinical investigation; that is, skin metastases from solid-organ tumors were found most commonly on head and neck areas, and the authors also hold the higher number of Tregs responsible for the higher probability of metastases on these sites (Schulman et al., 2016).

Recently Th17 cells were divided into nonpathogenic Th17(β) and pathogenic Th17(23) cells. Th17(β) cells are characterized by IL-17 and IL-10 production and the expression of RORγt, whereas Th17(23) cells play an important role in the development of inflammatory and autoimmune diseases, produce IL-17, INF-γ, and GM-CSF, and express T-bet and RORγt transcription factors (Geginat et al., 2014; Nomura et al., 2014). Although significantly more IL-17+ and IL-10+ cells were present in SGR skin, IFN-γ+ cells were completely absent, and besides the higher RORγt expression, T-bet mRNA levels did not differ compared with SGP skin. Therefore, we suppose that nonpathogenic Th17(β) cells were detected in SGR skin (Geginat et al., 2014; Nomura et al., 2014). Innate lymphoid cells and γδT cells must also be taken into consideration as they are known to produce IL-17 (Sutton et al., 2012). The noninflammatory T-cell milieu of SGR skin was also supported by the similarly low appearance of innate immune cells in both healthy skin areas.

In conclusion, our results suggest that similar to skin microbiota and chemical milieu, a fine topographical difference does exist in the activity of the human skin immune system regarding an epidermal factor (TSLP), DCs, and T cells, although in this study moist skin regions were not investigated (the immune characteristics of healthy, moist skin areas are under investigation by our workgroup; manuscript under preparation). Hence these results may provide explanation of the characteristic localization of certain immune-mediated skin diseases in special topographical skin areas (i.e., AD on SGP and PPR on SGR skin sites). Moreover, our data highlight the importance of correctly used topologically identical control skin samples in scientific studies. Furthermore, our study may influence future barrier repair therapeutic approaches.

After detecting this special immune surveillance in healthy SGR skin, we wondered how this can be changed in an immune-mediated skin disease like PPR, which is exclusively localized to SGR skin regions. In PPR, special activation of both the innate and adaptive immune mechanisms was previously described despite the absence of an obvious infectious or dangerous trigger, and literature suggests that decreased tolerance could be responsible for this increased skin sensitivity.

According to our findings, in PPR skin, TSLP was lost, DCs became activated, T cells turned to inflammatory type [Th1 and Th17(23)], and their numbers were highly elevated, similarly to a recent study (Buhl et al., 2015), resulting in the disruption of the noninflammatory immune milieu of SGR skin. Although the Treg presence was also higher, this is not a contradiction, as their accumulation was usually detected in inflammatory skin diseases (Nomura et al., 2014). These changes were accompanied by the significant influx of macrophages, neutrophils, and mast cells. We were also able to show that linoleic acid, an important component of sebum, exerted an effect on TSLP expression; moreover, literature data described altered sebum composition in patients with rosacea (Ni Raghallaigh et al., 2012; Two et al., 2015). On the basis of these findings, we hypothesize that this altered sebum production in rosacea and the consequently occurring loss of tolerogenic TSLP may be one of the main events during PPR development (Figure 6). This change in TSLP level may influence DC and T-cell activation (Kinoshita et al., 2009; Spadoni et al., 2012). At the same time, because DCs can potentially...
be exposed directly to changes of sebum, chemical milieu, and microbiota of the skin, the disruption of the noninflam-
matory milieu can also be initiated by DCs or T cells (Mittal et al., 2013; Naik et al., 2015).

MATERIALS AND METHODS

Skin biopsies
Skin punch biopsies (0.5–1 cm²) were taken from lesional skin of 8 patients with AD, from 10 patients with PPR, and from normal skin of 18 healthy individuals (8 from SGP and 10 from SGR skin sites; Table 1) after obtaining written, informed consent, according to the Declaration of Helsinki principles. The study was approved by the local ethics committee of University of Debrecen, Hungary. All bi-
opsies were cut into two pieces. For IHC, samples were paraffin-
embedded, whereas for RT-PCR, samples were stored in RNAlater (Qiagen, Hilden, Germany) at −70 °C until RNA isolation. After
hematoxylin and eosin staining, samples were sorted according to
the number of sebaceous glands and were defined as SGP skin when containing n ≤ 1 sebaceous glands and as SGR skin when contain-
ing n ≥ 3 sebaceous glands in the field of view on ×10 magnification in the microscope (Table 1).

Cell culture
HaCaT and normal human epidermal keratinocyte KCs and SZ95 sebocytes (Zouboulis et al., 1999) were cultured and seeded at
50,000 cells/well in 12-well plates for further RT-PCR and cytokine
ELISA measurements. For a detailed description, see Supplementary Materials and Methods online.

RNA isolation and quantitative real-time PCR
RNA was extracted from the skin tissue specimens and HaCaT KCs
with Tri Reagent (Sigma-Aldrich, Dorset, UK) and converted to cDNA
by using the High Capacity cDNA Archive Kit (Invitrogen, Life
Technologies, San Francisco, CA). Levels of TSLP, CD80, CD83,
CD86, lysosomal associated membrane protein 3, IL-10, IL-13, IL-
17A, IFN-γ, TBX21, GATA3, RORC, FOXP3, CCR4, and CCR8 tran-
scripts were also examined by using predesigned Minor Groove
Binding assays ordered from Applied Biosystems (Life Technologies).
For a detailed description, see Supplementary Materials and Methods.

IHC, routine staining, and whole-slide imaging
Paraffin-embedded sections were stained with antibodies against hu-
man TSLP (3 different antibodies), CD3, CD4, CD1a, CD11c, CD163,
CD83, TARC, IL-10, IL-13, IL-17A, IFN-γ, TBX21, GATA3, RORC,
FOXP3, CCR4, and CCR8 transcripts were also examined by using
predesigned Minor Groove Binding assays ordered from Applied Biosystems (Life Technologies). For a detailed description, see Supplementary Materials and Methods.

Stratum corneum TSLP measurement
The tape-stripping method and immunostaining were carried out
according to the method described in a previous report (Morita et al., 2010). For a detailed description, see Supplementary Materials and Methods.

ELISA
The concentration of TSLP in the supernatant was quantified in trip-
licates by using antihuman TSLP Quantikine ELISA (R&D Systems,
Minneapolis, MN).
Measurement of transepidermal water loss and skin pH
For a detailed description, see Supplementary Materials and Methods.

Statistical analysis
To determine the statistical significance between the groups, the one-way analysis of variance test and Newman-Keuls posttest were used. Differences between the groups were demonstrated using mean ± standard error of the mean. P-values <0.05 were considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001). Analysis of correlations was performed by the Pearson r test. Two-tailed P-values <0.05 were considered statistically significant (*P < 0.05, **P < 0.01).

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 www.jidonline.org. and at http://dx.doi.org/10.1016/j.jid.2016.12.025.

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