Cell-intrinsic regulation of murine epidermal Langerhans cells by protein S

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AXL, a member of the TYRO3, AXL, and MERTK (TAM) receptor tyrosine kinase family, has been shown to play a role in the differentiation and activation of epidermal Langerhans cells (LCs). Here, we demonstrate that growth arrest-specific 6 (GAS6) protein, the predominant ligand of AXL, has no impact on LC differentiation and homeostasis. We thus examined the role of protein S (PROS1), the other TAM ligand acting primarily via TYRO3 and MERTK, in LC function. Genetic ablation of PROS1 in keratinocytes resulted in a typical postnatal differentiation of LCs; however, a significant reduction in LC frequencies was observed in adult mice due to increased apoptosis. This was attributed to altered expression of cytokines involved in LC development and tissue homeostasis within keratinocytes. PROS1 was then excised in LysM⁺ cells to target LCs at early embryonic developmental stages, as well as in adult monocytes that also give rise to LCs. Differentiation and homeostasis of LCs derived from embryonic precursors was not affected following *Pros1* ablation. However, differentiation of LCs from bone marrow (BM) precursors in vitro was accelerated, as was their capability to reconstitute epidermal LCs in vivo. These reveal an inhibitory role for PROS1 on BM-derived LCs. Collectively, this study highlights a cell-specific regulation of LC differentiation and homeostasis by TAM signaling.

Langerhans cells | protein S | TAM signaling | epidermis | Pros1

Stratified epithelia, such as the skin epidermis and certain mucosal epithelia, contain a special type of antigen-presenting cells, named Langerhans cells (LCs). LCs represent the first line of defense against invading pathogens but are also capable of maintaining homeostasis with commensal bacteria (1–3). Epidermal LCs originate from embryonic precursors that seed the epithelium until embryonic day 18 (E18). Immediately after birth, these precursors massively proliferate while differentiating into a radioresistant and self-renewing population (4, 5). LCs in mucosal epithelia have a distinct ontogeny as they arise from bone marrow (BM) precursors—pre-DCs (predendritic cells) and monocytes. Unlike their equivalents in the epidermis, mucosal LCs are radiosensitive and continuously replaced by circulating precursors (6). Monocytes can also differentiate into epidermal LCs under certain conditions (5, 7, 8). Regardless of their origin, the differentiation of both epidermal and mucosal LCs is instructed by the epithelium. Nevertheless, intrinsic features unique to each precursor type are also likely to regulate LC development.

AXL, a member of the TYRO3, AXL, and MERTK (TAM) tyrosine kinase receptor family, is expressed on epidermal LCs and has been reported to play a role in their differentiation and activation (9). The TAM receptors and their ligands, protein S (PROS1) and growth arrest-specific 6 (GAS6), are expressed by many hematopoietic and nonhematopoietic cells and are involved in various biological, developmental, and immunological functions (10–12). GAS6 is the predominant ligand of AXL while PROS1 acts mainly via TYRO3 and MERTK (13–15). Despite the proposed role of AXL in LC development, no alteration in the epidermal LC network was found in *Axl*^{−/−} mice, which could be explained by functional redundancy known to exist among the TAM receptors (9). Nevertheless, since AXL is expressed by both LCs and kerati-

nocytes, it is hard to conclude how the absence of AXL in each cell type affects LCs. As the TAM ligands interact differentially with the various TAM receptors, exploring their function should shed light on the involvement of the TAM signaling pathways in LC ontogeny. Here, we show that, whereas GAS6 is dispensable for LC differentiation, ablation of PROS1 in specific cell types exerts distinct regulatory functions on the development of LCs.

Results

Expression of TAM Receptors and Ligands in the Murine Ear Skin. To study the impact of TAM ligands on epidermal LCs, we first characterized the expression of TAM receptors in epidermal cells. LCs (CD45⁺ MHCII⁺ CD11c⁺ EpCAM⁺ langerin/CD207⁺) were found to express AXL, but not MERTK or TYRO3 (Fig. 1A). A large fraction of CD45-negative epidermal cells, representing keratinocytes, expressed AXL whereas MERTK expression was not detected (Fig. 1B). Expression of TYRO3 was observed in part of the keratinocytes using both flow cytometry and Western blot analyses (Fig. 1B and C). We next examined the expression of the TAM ligands. PROS1 and GAS6 were detected via Western blot in epidermal sheet lysate (Fig. 1C). Immunofluorescence staining further visualized both ligands in keratinocytes while PROS1 appeared to be located in the suprabasal epidermal layers (Fig. 1D–F). Western blot analysis of sorted keratinocytes, LCs, and γ BT cells revealed that PROS1 is expressed mainly by keratinocytes (Fig. 1C). PROS1 was undetectable in LCs also using flow cytometry (SI Appendix, Fig. S1). Similar staining for GAS6 using different

Significance

Langerhans cells (LCs) are the exclusive antigen-presenting cells of the epidermis, capable of mounting immunity and tolerance. LCs maintain themselves locally by self-renewing throughout life, a process that is regulated by both LCs and keratinocytes. Nevertheless, the mechanisms underlying this process are not clearly understood. Using targeted genetic ablation, we demonstrate that lack of protein S (PROS1) in keratinocytes, but not in LCs, results in reduced numbers of terminally developed LCs. This is due to increased apoptosis of LCs and associated with altered expression of cytokines involved in tissue homeostasis. Furthermore, PROS1 also down-regulates LC differentiation from bone marrow precursors. Together, these identify PROS1 as a regulator of LC development and homeostasis.

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Fig. 1. Expression of TAM receptors and ligands in the murine skin epidermis. Epidermal cells were prepared from 8-wk-old mice, and representative flow cytometry plots present the expression of AXL, MERTK, and TYRO3 receptors on (A) LCs and (B) keratinocytes. Data of one of three independent experiments are provided, and each experiment included at least five separately analyzed mice. (C) Western blot analysis showing the presence of MERTK, TYRO3, PROS1, GAS6, and GAPDH as a control in lysates of total epidermal cells from adult WT *Gas6*^{+/+} and *Gas6*^{-/-} mice or sorted keratinocytes, LCs, and dendritic epidermal T cells (DETC) from 8-wk-old WT mice. +, positive control. Representative results of one of three independent experiments are shown. (D–F) Ear cross-sections (D, Upper image presents the actual image while the Lower image provides a magnification of a selected area) or whole epidermal sheets (E) were stained for PROS1 (green), langerin (red), and DAPI (blue) for nuclear visualization. The white dotted line demarcates the basal membrane and the arrowhead indicates the epidermis. (F) Whole epidermal sheets were stained with GAS6 (red) and langerin (green). Immunofluorescence microscopy images are shown representing one of three independent experiments. (G) Relative expression levels of *Pros1* and *Gas6* in whole epidermal tissues and sorted cells isolated from 8-wk-old mice were analyzed by RT-qPCR. Bar graphs present the fold change in mRNA levels among the various groups, which was normalized to *Gas6*^{+/+} or *Cre*⁻ mice, respectively, $\pm$ SD ($n = 5$). D, dermis; E, epidermis. * $P < 0.05$. Results of one of two independent experiments are presented.

antibodies did not provide technically reliable results. To overcome this technical hurdle, we fractionated epidermal cell populations and quantified *Gas6* and *Pros1* mRNA using RT-qPCR (reverse transcription quantitative polymerase chain reaction). As depicted in Fig. 1G, significant *Gas6* and *Pros1* mRNA levels were detected in keratinocytes and, to a lesser extent, in LCs, but not in cells derived from *Gas6*^{-/-} and keratin 5 (*K5*)/*Pros1*^{fl/fl}, which served as negative controls, respectively. In *K5/Pros1*^{fl/fl} mice, the *Pros1* alleles are homozygously deleted in keratinocytes. Taken together, among the various TAM receptors, AXL is strongly expressed by most keratinocytes and LCs whereas TYRO3 is expressed at lower levels or only by a small fraction of keratinocytes. Such an expression pattern fits well with previous observations demonstrating that expression of TAM receptors and ligands is restricted to certain cellular layer/s within the stratified epithelium (9, 16). Regarding TAM ligands, both PROS1 and GAS6 are expressed by keratinocytes while LCs might express either no protein or only a limited amount of the ligands.

Ablation of GAS6 Minimally Affects the Development of Epidermal LCs. Since AXL has been reported to play a role in LC development and homeostasis (9), we examined whether GAS6, the high affinity ligand of AXL, is also involved in this process. Similar frequencies of LCs, as well as $\gamma\delta$ T cells, the other major leukocyte population in the epidermis, were found in the ear epidermis of adult *Gas6*^{-/-} mice and littermate controls (Fig. 2A). A whole-mount immunofluorescence

staining of epidermal sheets with MHCII and langerin further demonstrated the presence of a typical LC network in the epidermis of *Gas6*^{-/-} mice (Fig. 2B). Interestingly, the staining intensity of langerin in *Gas6*^{-/-} mice appeared to be brighter in comparison with the control, a phenomenon that was further verified using flow cytometry (Fig. 2C). Nevertheless, expression of the maturation markers MHCII and CD86 was not altered in *Gas6*^{-/-} mice. Concurring with the above findings, expression of *Tgfb1*, a cytokine controlling LC development and maturation, was similar in the epidermis of both groups (Fig. 2D). We also quantified *Il-34* and *Bmp7* expression, other cytokines involved in LC development (17, 18), and, whereas *Il-34* remained unchanged, expression of *Bmp7* was up-regulated in GAS6-depleted epidermis. We then checked the expression of other TAM receptors and ligands in *Gas6*^{-/-} mice by RT-qPCR. Elevated *Axl* and *Mertk* mRNA levels, but not *Pros1* or *Tyro3*, were found in the epidermis of *Gas6*^{-/-} mice compared with their littermate controls (Fig. 2E). In line with our overall results, postnatal differentiation of LCs was normal in 10-d-old *Gas6*^{-/-} mice, based on the frequencies of epidermal MHCII⁺ cells, langerin expression, and expression of AXL on LC precursors and keratinocytes (SI Appendix, Fig. S2). Taken together, although AXL was proposed to regulate epidermal LC development, the lack of its predominant ligand GAS6 hardly affected murine LCs.

Excision of PROS1 in Keratinocytes Reduces LC Frequencies. We next investigated the impact of PROS1 on LCs. Since total knockout of PROS1 yields nonviable mice (19), we crossed *K5-cre* mice

Fig. 2. LC differentiation is not impaired due to the lack of GAS6. (A) Epidermal cells were purified from adult *Gas6*^{-/-} mice and littermate controls (WT). Representative flow cytometry plots present the frequencies of LCs (langerin⁺) and $\gamma\delta$ T cells ($\gamma\delta$ TCR⁺) among total CD45⁺ leukocytes. Data are representative of three independent experiments, and each experiment included at least four separately analyzed mice. (B) Whole-mount immunofluorescence staining on epidermal layers prepared from adult *Gas6*^{-/-} mice and littermate controls, stained for MHCII (red) and langerin (green). Representative confocal microscopy images are presented, representing one of four independent experiments (*n* = 3 mice in each experiment). (C) Representative flow cytometry plots demonstrating the expression levels of MHCII, CD86, and langerin on epidermal LC cells of adult *Gas6*^{-/-} mice and littermate controls. Data of one of three independent experiments are provided (*n* = 5 in each experiment). (D and E) Relative expression of various cytokines or TAM receptors and ligands in epidermal layers of adult *Gas6*^{-/-} mice and littermate controls were analyzed by RT-qPCR (*n* = 5 mice). RNA expression data of one of two independent experiments are presented as the mean values $\pm$ SD. **P* < 0.05.

with *Pros1*^{fl/fl} mice to generate *K5/Pros1*^{fl/fl} mice in which the *Pros1* alleles were homozygously deleted in keratinocytes, as demonstrated in *SI Appendix, Fig. S3*. Analyzing epidermal tissues of *K5/Pros1*^{fl/fl} adult mice by immunofluorescent staining and RT-qPCR showed a considerable reduction in PROS1 expression, thus confirming the effectiveness of the system (Fig. 3A and B). The LC network was then examined, and a significant reduction in the frequencies and absolute numbers of LCs was found in *K5/Pros1*^{fl/fl} mice compared with *Cre*⁻ littermate controls (Fig. 3C). In fact, a gradual decrease in LC frequencies was observed during the second (~30% reduction) and fifth (~50% reduction) months after birth while no further reduction was found 10 mo after birth. A whole-mount immunofluorescence staining of epidermal tissues with MHCII and langerin antibodies further revealed empty patches in the LC network only in *K5/Pros1*^{fl/fl} mice (Fig. 3D). Moreover, in epidermal areas of *K5/Pros1*^{fl/fl} mice that contain LCs, expression of langerin and MHCII appeared to be more distributed in the cell body and dendrites compared with littermate controls (Fig. 3E). These data suggest that expression of PROS1 by keratinocytes is important for the development or maintenance of an intact LC network in the epidermis.

Maturation and Activation of LCs Is Not Altered in *K5/Pros1*^{fl/fl} Mice. We next sought to check whether the reduction in LCs was due to an accelerated maturation/activation of these cells. First, we examined the impact of PROS1 deletion on the expression of other TAM molecules in epidermal cells of adult mice. Using flow cytometry, we found an up-regulation of AXL on both LCs and keratinocytes of *K5/Pros1*^{fl/fl} mice whereas TYRO3 expression was not altered (Fig. 4A). Epidermal expression of *Gas6* and *Mertk* in these mice was normal based on RT-qPCR quantification (Fig. 4B). We also quantified the expression of *Il-34*, *Tgfb1*, and *Bmp7* and found reduced expression of *Tgfb1* and *Bmp7* but not *Il-34* (Fig. 4B). It has been shown that AXL is up-regulated in antigen-

presenting cells (APCs) and epithelial cells upon activation, acting as a negative regulator on the positive feedback loop of IFN-I signaling that facilitates inflammatory cytokines production (16, 20, 21). Therefore, we examined, by RT-qPCR, the levels of certain cytokines involved in this regulatory process. An up-regulation in the expression of IFN- α and IFN- β was detected in the epidermis of *K5/Pros1*^{fl/fl} mice (Fig. 4C). However, expression of the proinflammatory cytokines TNF- α and IL-1 β was either unchanged or significantly reduced, respectively. In line with the latter finding, MHCII expression on keratinocytes was not induced due to the intrinsic absence of PROS1 (Fig. 4C). These results suggest that, although the IFN-I/AXL regulatory pathway was modified in the epidermis of *K5/Pros1*^{fl/fl} mice, based on the inflammatory indicators assayed, the epidermis of these mice is not more inflamed in comparison with littermate controls.

We then examined LC maturation using flow cytometry and found normal expression of MHCII and CD86 while langerin was slightly reduced in *K5/Pros1*^{fl/fl} mice (Fig. 4D). Using RT-qPCR, we detected reduced *Cd86* expression in *K5/Pros1*^{fl/fl} mice whereas expression of *Ccr7* and *Cdhl* (E-cadherin), known to be involved in LC migration to the lymph nodes (LNs) upon maturation, was not altered (Fig. 4E). Concomitantly, no increase in migratory DCs was found in the draining LNs of *K5/Pros1*^{fl/fl} mice; in fact, this population was reduced compared with controls due to the reduction of LCs (*SI Appendix, Fig. S4*). These results suggest that LC reduction in *K5/Pros1*^{fl/fl} mice is not due to an accelerated activation of the LCs or keratinocytes. Finally, we examined the development of contact-hypersensitivity (CHS) response in *K5/Pros1*^{fl/fl} mice since elevated CHS responses were previously reported in TAM KO mice 2 wk after challenge (9). Nevertheless, such a phenomenon was not observed in *K5/Pros1*^{fl/fl} mice (Fig. 4F), suggesting that regulation of keratinocyte activation is intact in our system.

Fig. 3. Ablation of PROS1 in keratinocytes reduces LC frequencies. (A) Whole-mount immunofluorescence staining on epidermal layers prepared from adult *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice, stained for PROS1 (green) and DAPI (blue). Representative fluorescent images are shown, representing one of two independent experiments ($n = 3$ mice in each experiment). Negative control, primary antibody was omitted. (B) Quantification of mRNA levels of PROS1 in epidermal layers of adult *K5/Pros1^{fl/fl}* and *Cre⁻* mice using RT-qPCR ($n = 5$ mice). Data of one of two independent experiments are presented as the mean values $\pm$ SD. (C) Frequencies and absolute numbers (per a half ear) of LCs ($CD45^{+}MHCII^{+}CD11c^{+}$ cells) in epidermal cells prepared from 2-, 5-, and 10-mo-old *K5/Pros1^{fl/fl}* and *Cre⁻* mice. Representative flow cytometry plots, as well as bar graphs, present the fold change in LC frequencies (normalized to *Cre⁻* mice) or absolute LC numbers in the epidermis of these mice. Data are representative of four independent experiments, and each experiment included at least five separately analyzed mice. (D and E) Whole-mount immunofluorescence staining on epidermal layers prepared from adult *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice, stained for langerin (green) and MHCII (red). Representative fluorescent images are presented representing one of three independent experiments ($n = 3$ mice in each experiment). ** $P < 0.01$. (Scale bars: A and E, 50 μ m; D, 500 μ m.)

Postnatal Differentiation of LCs Is Not Impaired in *K5/Pros1^{fl/fl}* Mice.

We next checked postnatal development of LCs using flow cytometry at postnatal day 9 (P9) in *K5/Pros1^{fl/fl}* and *Cre⁻* mice. The frequencies of MHCII⁺ LC precursors and langerin expression levels were similar in both groups (Fig. 5A and B). Of note, a considerable reduction in the mRNA levels of *Pros1* was already evident at this time point (Fig. 5C), indicating that Cre is expressed by keratinocytes early after birth, as previously reported (22). Since the reduction of LCs in adult mice was accompanied with an altered expression of various cytokines, we examined whether a similar phenomenon occurs also during postnatal differentiation. In contrast to adult mice, no differences were found in the expression of the tested cytokines at P9 in *K5/Pros1^{fl/fl}* and *Cre⁻* mice (Fig. 5D). Because TAM signaling regulates the expression of these cytokines in adult mice (23), we compared the expression of TAM molecules in the epidermis of adult and P9 *Cre⁻* mice. The comparison revealed low mRNA levels of all TAM molecules in P9 mice compared with adult mice (except for *Mertk*, which is not expressed in the adult epidermis), suggesting that TAM signaling might have less impact on keratinocytes early after birth (Fig. 5E). These results suggest that early differentiation of LCs is not altered in *K5/Pros1^{fl/fl}* mice presumably because PROS1 ablation minimally affects epidermal function postnatally.

Altered Proliferation and Increased Apoptosis of LCs Following PROS1 Ablation in Keratinocytes.

Since postnatal development of LCs is normal in *K5/Pros1^{fl/fl}* mice, we examined whether the reduced LC

levels observed in adult mice are a result of impaired homeostatic proliferation. To address this issue, we treated 21-d-old *K5/Pros1^{fl/fl}* mice and littermate controls with BrdU for 3 wk or 8 wk and analyzed the incorporation of BrdU into LCs during cell proliferation. Of note, 21-d-old mice were chosen for this assay since we found that, at this age, the frequencies of LCs are normal in *K5/Pros1^{fl/fl}* mice. This allows tracking alterations in LC proliferation until adulthood, a time in which LCs are significantly reduced. Exposing the mice to BrdU for 3 wk resulted in comparable levels of BrdU-labeled LCs in *K5/Pros1^{fl/fl}* mice and littermate controls (Fig. 6A). However, following an 8-wk treatment, whereas the percentages of BrdU-labeled LCs were similar in both groups, the mean fluorescence intensity (MFI) of BrdU-labeled LCs was lower in *K5/Pros1^{fl/fl}* mice compared with littermates (Fig. 6A). This suggests that, in *K5/Pros1^{fl/fl}* mice, some of the LCs failed to undergo additional rounds of proliferation, which could be explained by increased apoptosis of LCs. To measure apoptosis, we prepared epidermal sheets from 6- to 8-wk-old *K5/Pros1^{fl/fl}* mice and littermate controls and performed whole-mount immunofluorescence staining against cleaved-caspase 3 and langerin. Indeed, we found, in *K5/Pros1^{fl/fl}* mice, a four- to fivefold increase in the numbers of cleaved-caspase 3-positive LCs compared with littermate controls (Fig. 6B). We also measured the frequencies of dead LCs in the epidermis by flow cytometry using propidium iodide (PI) and detected elevated percentages of PI⁺ LCs in *K5/Pros1^{fl/fl}* mice compared with control (Fig. 6C). Taken together,

Fig. 4. LCs are not activated in *K5/Pros1^{fl/fl}*. (A) Epidermal cells were prepared from adult *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice. Representative flow cytometry plots present the expression of AXL and TYRO3 on LCs and keratinocytes. Data of one of three independent experiments are provided, and each experiment included at least five separately analyzed mice. (B and C) Relative expression of various genes in epidermal layers of adult *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice were analyzed by RT-qPCR ($n = 5$ mice). RNA expression data of one of two independent experiments are presented as the mean values $\pm$ SD. Representative FACS plot demonstrates MHCII expression levels on keratinocytes of adult *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice. (D) Expression levels of MHCII, langerin, and CD86 on epidermal LCs of adult *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice ($n = 5$). Representative FACS plots of one of three independent experiments are presented. (E) Relative expression of genes involved in LC maturation and migration to the LNs as measured by RT-qPCR. Data are representative of two independent experiments, and each experiment included five separately analyzed mice. (F) *K5/Pros1^{fl/fl}* and littermate *Cre⁻* mice were sensitized with 1% TNCB on the shaved abdomen, and, 5 d later, the mice were challenged on one ear with 0.5% TNCB while the unchallenged ear was used as control. The graph presents the change in ear swelling at the indicated time points and represents the mean values $\pm$ SD ($n = 5$). Data of one of two independent experiments are provided. Ctrl, control. * $P < 0.05$.

these results suggest that the reduced numbers of LCs in *K5/Pros1^{fl/fl}* mice is due to increased apoptosis of these cells.

Excision of PROS1 in *LysM⁺* Cells Affects BM-Derived LCs but Not Embryonic LCs. To examine whether intrinsic expression of PROS1 by LCs is required for their development, we employed *LysM-cre* mice and crossed them with *Pros1^{fl/fl}* mice. The *LysM* promoter is active during embryonic development of LCs (24), allowing one to study the role of intrinsic PROS1 expression on LCs during embryonic development and throughout adulthood. Moreover, the *LysM* promoter is expressed by monocytes (25) which also express PROS1 (SI Appendix, Fig. S5) and give rise to LCs in adult mice under certain conditions (7, 8, 26). To visualize LCs that were also deleted for PROS1, we crossed the *LysM-cre* mice to both *Pros1^{fl/fl}* mice and *Rosa26-lslEYFP* mice to generate *LysM/Pros1^{fl/fl}EYFP* mice. In these mice, YFP⁺ cells are expected to have an excision of the *Pros1* gene as shown in SI Appendix, Fig. S6. In agreement with a previous study (24), analysis of *LysM/Pros1^{fl/fl}EYFP* mice revealed that only 45 to 60% of LCs were YFP⁺ and, by inference, also *Pros1*-ablated (Fig. 7A). Examining LC precursors in the skin epidermis and in the blood of E18 embryos demonstrated similar levels of partial YFP labeling, confirming that ablation of the *Pros1* gene in LCs occurred during embryogenesis (Fig. 7B). Since the epidermis of *LysM/Pros1^{fl/fl}EYFP* mice contains both *Pros1*-excised LCs and intact LCs, we used this phenotype as an internal control to examine the role of *Pros1* in LC development. First, time-course analysis revealed that the percentages of YFP⁺ LCs in the epidermis remain constant, suggesting that PROS1 does not affect homeostatic proliferation of LCs (Fig. 7C). Additionally, PROS1 deletion did not alter the radioresistant feature of LCs as the fre-

quencies of YFP⁺ and YFP^{neg} LCs remained similar after lethal irradiation (Fig. 7D).

We then asked whether the repopulation of epidermal LCs from BM-derived precursors is altered due to *Pros1* excision. For this, we lethally irradiated langerin-DTR (diphtheria toxin receptor) mice and adoptively transferred them with BM cells purified from *LysM/Pros1^{fl/fl}EYFP* or *LysM/Pros1^{+/+}EYFP* mice as a control. Eight weeks later, epidermal LCs were depleted by diphtheria toxin (DT), and LC repopulation was tracked in the epidermis (Fig. 8A). Two to 3 mo after LC depletion, a small population of LCs repopulated the epidermis of both groups with comparable levels (Fig. 8B). In this experimental paradigm, circulating BM-derived precursors gave rise to epidermal LCs, and YFP⁺ cells were derived only from the transplanted donor BM cells. Nevertheless, the percentages of YFP⁺ LCs in mice receiving BM of *LysM/Pros1^{fl/fl}EYFP* were considerably higher in comparison with the control group (Fig. 8C). This phenomenon cannot be explained by the presence of increased YFP⁺ circulating precursors in the donor since comparable frequencies of YFP⁺ Ly6C^{lo} and Ly6C^{hi} monocytes were found in the blood of *LysM/Pros1^{fl/fl}EYFP* and *LysM/Pros1^{+/+}EYFP* mice (Fig. 8D). Interestingly, in a separate experiment, we examined the capacity of PROS1-deficient monocytes to repopulate the epidermis under inflammatory conditions induced by tape stripping. In contrast to the above results, we found that PROS1 was crucial for infiltration of monocytes to the epidermis, resulting in a higher differentiation to MHCII⁺CD11c⁺ cells (SI Appendix, Fig. S7). These suggest that lack of PROS1 in monocytes facilitates their differentiation into LCs only during steady-state or low inflammatory conditions. We next hypothesized that the excision of *Pros1* might facilitate the differentiation of BM precursors in

Fig. 5. Intact postnatal differentiation of LCs in *K5/Pros1^{fl/fl}*. Epidermal cells were prepared from 9-d-old (P9) *K5/Pros1^{fl/fl}* mice and littermate *Cre⁻* controls. (A) Representative flow cytometry plots and graph present the frequencies of CD45⁺MHCII⁺ cells in the epidermis. (B) Frequencies and absolute numbers (per a half ear) of LCs (CD45⁺MHCII⁺langerin⁺) in the epidermis. Representative flow cytometry histogram demonstrates langerin expression in P9 *K5/Pros1^{fl/fl}* and *Cre⁻* mice, as well as adult *Cre⁻* mice. Data are representative of two independent experiments, and each experiment included at least three separately analyzed mice. (C–E) Relative expression of *Pros1* and various genes in epidermal layers of (C and D) P9 *K5/Pros1^{fl/fl}* and *Cre⁻* mice, or (E) adult vs. P9 *Cre⁻* mice were analyzed by RT-qPCR ($n = 5$ mice). RNA expression data of one of two independent experiments are presented as the mean values $\pm$ SD. Ctrl, control. * $P < 0.05$.

the epidermis, leading to increased numbers of YFP⁺ LCs in mice receiving BM from *LysM/Pros1^{fl/fl}Eyfp* mice. To address this issue, we cultured BM cells purified from both mice with GM-CSF and TGF- β 1 to drive their differentiation into LC-like cells. Indeed, BM cells heterozygously or homozygously deleted for *Pros1* developed larger percentages of MHCII⁺CD11c⁺CD205⁺ EpCAM⁺ cells, representing LC-like cells (27), in a dose-dependent manner (Fig. 8E). In line with these results, LC-like cells generated by these BM cells presented higher percentages of YFP compared with intact BM cells (SI Appendix, Fig. S8). We further demonstrated that AXL-expressing cells in the culture, representing LCs (9), also express PROS1 (Fig. 8F). Taken together, in contrast to PROS1 expressed by keratinocytes, intrinsic expression of PROS1 by embryonic-derived LCs is suggested to be dispensable for their development and maintenance. Expression of PROS1 in BM precursors, however, inhibits their differentiation into epidermal LCs under steady-state conditions.

Discussion

This study examined the role of TAM ligands on the development of epidermal LCs. The negligible impact of GAS6 on murine LC development was unexpected since its predominant receptor AXL was proposed to play a major role in the differentiation and activation of human LCs (9). Whereas it is possible that the reported impact of AXL on LCs is mediated by PROS1 rather than GAS6, previous studies have shown that binding of PROS1 to AXL fails to induce intracellular signaling (13, 15). Alternatively, PROS1 may modify the interaction of AXL with a non-TAM tyrosine kinase receptor, which could affect LCs. However, it is also possible that AXL is dispensable for LC differentiation since, in *Axl^{-/-}* mice, the LC network is intact (9). In fact, the LC network was reported to be intact also in *Tyro3^{-/-}* or *Mertk^{-/-}* mice, and only ablation of all three TAM receptors displayed reduced LC frequencies (9). Whereas the inability of AXL to control LC differentiation in *Axl^{-/-}* mice was explained by functional redundancy among the various TAM receptors, our

study proposes an alternative explanation. We demonstrate here that PROS1 and its predominant receptor TYRO3 are expressed by keratinocytes, and specific ablation of PROS1 in keratinocytes dysregulated cytokine expression in these cells. As keratinocytes play an instructive role in LC differentiation and homeostasis, it is likely that TRYO3/PROS1 signaling in keratinocytes is required to maintain epidermal homeostasis and prevent apoptosis of LCs. The lack of effect of PROS1 on embryonic-derived LCs in *LysM/Pros1^{fl/fl}Eyfp* mice further suggests that PROS1 expressed by keratinocytes regulates epidermal LCs.

As for AXL, careful examination of recent data supported a role for AXL in regulating LC activation rather than LC differentiation (9), which is in line with the immunoregulatory roles of AXL in other systems (21). This function of AXL is likely to involve binding to GAS6 since GAS6 is known to be up-regulated upon activation in both APCs and epithelial cells (16, 20, 21). Of note, ablation of PROS1 in keratinocytes resulted in an up-regulation of AXL in both LCs and keratinocytes. AXL was reported to be up-regulated in LCs upon stimulation to limit their activation (9). It is thus likely that, in *K5/Pros1^{fl/fl}* mice, AXL was up-regulated to compensate for the reduced levels of TGF- β 1 known to down-regulate the activation of LCs and keratinocytes (28–30). Indeed, elevated expression of type I interferons known to regulate tissue homeostasis was detected in the epidermis of adult *K5/Pros1^{fl/fl}* mice (31). Nevertheless, no augmentation in the expression of proinflammatory cytokines, such as TNF- α and IL-1 β , was found, indicating that keratinocyte activation is under control in *K5/Pros1^{fl/fl}* mice and the epidermis is not inflamed. A previous study demonstrated that type I IFN production by APCs is modulated by AXL upon interaction of AXL with IFN α receptor 1 (INFA1) (21). We reported recently that similar mechanisms take place also in stratified epithelia to regulate epithelial cell activation and to maintain tissue homeostasis (16). It is thus possible that ablation of PROS1 in keratinocytes induces temporary activation of keratinocytes, leading to up-regulation of AXL to prevent further production of type I interferons. Such activity is likely to take place only

Fig. 6. Increased apoptosis rate of LCs in *K5/Pros1^{fl/fl}* mice. (A) Three-week-old *K5/Pros1^{fl/fl}* and *Cre⁻* mice were treated with BrdU in the drinking water for 3 or 8 wk. Representative flow cytometry plots demonstrate the frequencies of CD45⁺MHCII⁺ (LCs) in the epidermis, as well as the frequencies of BrdU-labeled LCs at the end of the treatments. Bar graphs present the frequencies of BrdU-labeled LCs and the mean fluorescence intensity (MFI) of BrdU staining in LCs ($n = 5$). Data of one of two independent experiments are provided and present the mean values $\pm$ SD. (B) Epidermal sheets of 6- to 8-wk-old mice were stained against langerin and anti-caspase 3 to detect LCs undergoing apoptosis. Representative images of whole-mount immunofluorescence staining and graph demonstrating the numbers of active-caspase 3⁺ langerin⁺ cells are presented and present the mean values $\pm$ SD. Data are representative of three independent experiments. Each experiment included at least three separately analyzed mice, and 20 fields per mouse were analyzed. White circles exemplify langerin⁺ cells stained positively to active-caspase 3. (C) Representative flow cytometry plots and graph present the frequencies of dead PI⁺ LCs in the epidermis of *K5/Pros1^{fl/fl}* and *Cre⁻* mice ($n = 5$). Results of one of two independent experiments are provided and present the mean values $\pm$ SD. ctrl, control. * $P < 0.05$.

in adult mice as we demonstrated that TAM signaling has minimal effect on keratinocytes and LCs early after birth. This proposes the establishment of new homeostatic conditions in the epidermis of *K5/Pros1^{fl/fl}* mice, which involves higher apoptotic rates of LCs. Interestingly, a study by Sparber et al. (32) recently reported a reduced density of LCs in adulthood caused by reduced postnatal proliferation of LCs and increased apoptosis. Collectively, these observations suggest that homeostasis of LC in adulthood is shaped during the early weeks after birth.

Unlike LC differentiation from embryonic precursors, ablation of PROS1 facilitated the differentiation of LCs from adult BM precursors both in vitro and in vivo. Pre-DCs are not labeled in LysM-driven cre recombination (33), and thus monocytes represent the origin of YFP⁺ LCs upon DT treatment. Interestingly, monocyte-derived LCs were detectable in the epidermis up to 5 mo after LC depletion, demonstrating their capacity to generate long-lived LCs. This result contradicts a recent publication reporting that monocytes give rise to short-lived LCs following exposure to

Fig. 7. Normal development and homeostasis of LCs in *LysM/Pros1^{fl/fl}EYFP* mice. (A) Epidermal cells were prepared from adult *LysM/Pros1^{fl/fl}EYFP* and littermate *Cre⁻* mice. Representative flow cytometry plots present the percentages of YFP⁺ LCs among total epidermal LCs. Representative whole-mount immunofluorescence staining on epidermal sheet stained against langerin (red) and YFP (green). Data are representative of four independent experiments, and each experiment included at least three separately analyzed mice. (B) Blood and back skin epidermis of E18 embryos were sampled from *LysM/Pros1^{fl/fl}EYFP* and *Cre⁻* mice. Representative flow cytometry plots present the percentages of YFP⁺ cells among LC precursors in the blood (CD45⁺MHCII⁺) and epidermis (CD45⁺MHCII⁺F4/80⁺). Results of one of two independent experiments are presented. (C) YFP⁺ LCs were analyzed in the epidermis of *LysM/Pros1^{fl/fl}EYFP* mice at the indicated time points. Representative flow cytometry plots and graph present the frequencies of YFP⁺ LCs among total LCs ($n = 3-5$). Data of one of three independent experiments are provided and present the mean values $\pm$ SD. (D) Adult *LysM/Pros1^{fl/fl}EYFP* mice were lethally irradiated and, 24 h later, were administered i.v. with BM cells purified from littermate *Cre⁻* mice. The epidermis of the chimeric mice was analyzed 7, 14, and 21 d after BM transplantation to quantify the frequencies of YFP⁺ LCs. Representative flow cytometry plots and graph present the frequencies of YFP⁺ LCs among total LCs ($n = 3$). Data of one of two independent experiments are provided and present the mean values $\pm$ SD. Ctrl, control. (Scale bars; 50 μ m.)

Fig. 8. Differentiation of LCs from circulating BM precursors is augmented in *LysM/Pros1^{fl/fl}EYFP* mice. (A) Lethally irradiated langerin-DTR mice were adoptively transferred with BM cells purified from *LysM/Pros1^{fl/fl}EYFP* mice or *LysM/Pros1^{+/+}EYFP* mice as a control. Eight weeks later, LCs were ablated by a single injection of DT, and the repopulation of LCs was examined 7 and 11 wk after the depletion. (B) The frequencies and numbers of repopulating LCs in the epidermis of the chimeric mice are presented ($n = 3$). (C) FACS plots present the percentages of YFP⁺ cells among the repopulating LCs ($n = 3$). Results of one of two independent experiments are provided and present the mean values $\pm$ SD. (D) Representative flow cytometry plots demonstrate the percentages of YFP⁺ cells among Ly6C^{low}, Ly6C^{high}, and neutrophils in the blood of *LysM/Pros1^{fl/fl}EYFP* or *LysM/Pros1^{+/+}EYFP* mice before the transplantation. Data are representative of two independent experiments, and each experiment included at least three separately analyzed mice. (E) BM cells were purified from *LysM/Pros1^{+/+}EYFP*, *LysM/Pros1^{fl/fl}EYFP*, or *LysM/Pros1^{+/+}EYFP* mice and then cultured for 5 d in serum containing media supplemented with GM-CSF and TGF- β 1 to drive differentiation of LC-like cells. Representative flow cytometry plots demonstrate the percentages of MHCII⁺CD11c⁺ cells and further expression of CD205⁺EpCAM⁺, representing LC-like cells. The graph presents the frequencies of LC-like cells, as well as MHCII⁺CD11c⁺CD205⁺EpCAM⁺ cells, which are also known to differentiate to LC-like cells ($n = 3$). Data of one of four independent experiments are provided and present the mean values $\pm$ SD. (F) LCs were differentiated in vitro from BM cells of WT mice and stained for PROS1 (red), AXL (green), and DAPI (blue) for nuclear visualization at 5 d in culture. Representative immunofluorescence images taken from one of two independent experiments are shown. ** $P < 0.01$.

UV (8). Nevertheless, since depletion of LCs is not considered to induce severe inflammation as UV treatment, it is likely that such differences are related to the different inflammatory milieu within the epidermis upon each treatment. Indeed, the necessity of PROS1 for efficient infiltration of monocytes to the epidermis

upon tape stripping-induced inflammation further highlights the impact of the inflammatory milieu on this process.

Finally, we demonstrate in this study that PROS1, but not GAS6, plays a role in LC differentiation in a cell-specific manner. Considering the previously reported function of AXL on the

activation of these cells, the current work further highlights the multifaceted involvement of TAM signaling on epidermal LCs.

Experimental Procedures

Mice. CD45.2⁺ C57BL/6 mice were purchased from Harlan. CD45.1⁺ C57BL/6, *LysM-cre* mice (25) and *Rosa26-IsfYfp* mice (34) were purchased from The Jackson Laboratory. Langerin (CD207)-DTR mice were obtained from Björn E. Clausen, Mainz University, Mainz, Germany (35). *Gas6*^{-/-} mice and *Pros1*^{fl/fl} mice were previously described (19, 36). *K5-cre* mice were kindly provided by Angel Ramirez, Centro de Investigaciones Energéticas, Medio Ambientales y Tecnológicas (CIEMAT), Madrid, Spain (22). *K5/Pros1*^{fl/fl} mice were generated by crossing *K5-cre* mice with *Pros1*^{fl/fl} mice. To generate the *LysM/Pros1*^{fl/fl} *Eyfp* mice, we crossed the *LysM-cre* mice to both *Pros1*^{fl/fl} mice and *Rosa26-IsfYfp* mice. The mice were maintained under specific pathogen-free (SPF) conditions and analyzed between 8 and 12 wk of age unless otherwise described in the text. All animal protocols were approved by the Hebrew University Institutional Animal Care and Use Committee.

Antibodies and Reagents. Antibodies and reagents are described in [SI Appendix](#).

Chimeric Mice. *LysM/Pros1*^{fl/fl} *Eyfp* recipient mice were lethally irradiated with 900 rad, and, 24 h later, the mice were injected i.v. with 5×10^6 BM cells obtained from donor *Cre*⁻ mice. The frequencies of YFP⁺ among total epidermal LCs were analyzed at various times as explained in [SI Appendix](#).

Ablation of Langerin-Expressing Cells. Mice were injected once intraperitoneally with 1 μ g of DT (Sigma-Aldrich) in 150 μ L of PBS per mouse. Control mice received PBS only.

Isolation and Identification of LCs. Ear skin was excised and separated into two halves, and epithelial sheets were prepared as explained in [SI Appendix](#).

BrdU Incorporation. Three- to 4-wk-old mice were injected once intraperitoneally with BrdU (2 mg per mouse; Sigma) and subsequently received BrdU (0.8 mg/mL) in the drinking water as explained in [SI Appendix](#).

CHS Assay. Adult *K5/Pros1*^{fl/fl} mice and littermate controls were sensitized with 1% TNBC (2,4,6-trinitrochlorobenzene) (Sigma) in acetone/olive oil (4:1) on the shaved abdomen and, 5 d later, was challenged as explained in [SI Appendix](#).

LC-Like Cell Differentiation Cultures. The femur was isolated, cleaned from soft tissues in RPMI 1640, and soaked in 70% ethanol for 1 min for sterilization as explained in [SI Appendix](#).

RNA Extraction and RT-qPCR. For RNA isolation, the ear epidermis was homogenized in 300 μ L of TRI reagent (Sigma) using an electric homogenizer as explained in [SI Appendix](#).

Western Blot. Ear epidermis was isolated and homogenized in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 0.1% SDS, 0.5 mM EDTA) supplemented with a protease inhibitor mixture (Sigma) as explained in [SI Appendix](#).

Immunofluorescence Staining. For whole-mount staining, ear skin was separated into two halves and fixed in ice-cold 95% ethanol for 40 min and subsequently digested as described in [SI Appendix](#).

Statistics. Data are expressed as means $\pm$ SD. Statistical tests were performed using one-way ANOVA and a Student *t* test (**P* < 0.05; ***P* < 0.01).

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