Phenotypic Characterization of Leukocytes in Prenatal Human Dermis

Christopher Schuster[1], Christine Vaculik[1], Marion Prior[1], Christian Fiala[2], Michael Mildner[3], Wolfgang Eppel[4], Georg Stingl[1] and Adelheid Elbe-Bürger[1]

The adult human skin harbors a variety of leukocytes providing immune surveillance and host defense, but knowledge about their ontogeny is scarce. In this study we investigated the number and phenotype of leukocytes in prenatal human skin (dermal dendritic cells (DDCs), macrophages, T cells (including FoxP3$^+$ regulatory T cells), and mast cells) to unravel their derivation and to get a clue as to their putative function *in utero*. By flow cytometry and immunofluorescence, we found a distinction between CD206$^+$CD1c$^+$CD11c$^+$ DDCs and CD206$^+$CD209$^+$CD1c$^-$ skin macrophages by 9 weeks estimated gestational age (EGA). T cells appear at the end of the first trimester, expressing CD3 intracytoplasmatically. During midgestation, CD3$^+$FoxP3$^-$ and CD3$^+$FoxP3$^+$ cells can exclusively be found in the dermis. Similarly, other leukocytes such as CD117$^+$ (c-kit) mast cells were not identified before 12–14 weeks EGA and only slowly acquire a mature phenotype during gestation. Our data show at which time point during gestation antigen-presenting cells, T cells, and mast cells populate the human dermis and provide a step forward to a better understanding of the development of the human skin immune system.

INTRODUCTION

The dermis harbors a variety of leukocytes, which interact to provide immune surveillance and host defense (Bangert *et al.*, 2011; Teunissen *et al.*, 2012). Recently, we reported that CD45$^+$HLA-DR$^+$ leukocytes are already present by 9 weeks estimated gestational age (EGA) in human embryonic skin and that their development and attraction depends on various growth and differentiation factors present in developing skin, leading to the sequential acquisition of the phenotypic repertoire, exemplified by the development of epidermal Langerhans cells (Schuster *et al.*, 2009). Despite these recent advances in the understanding of the development of the skin immune system, the leukocytes of the embryonic and fetal human dermis have remained poorly characterized. In particular, our understanding of the diversity, origin as well

as function of antigen-presenting cells (APCs) and mast cells along with the development of cells of the adaptive immunity in skin is still scarce and needs further elucidation.

Dermal dendritic cells (DDCs) and skin macrophages are APCs that share a similar surface marker profile. They express HLA-DR and various monocyte/macrophage markers (e.g., CD11c, CD14, CD36, CD68, FXIIIa, or CD206 (mannose receptor)) to a varying extent (Meunier *et al.*, 1993; Nestle *et al.*, 1993; Ebner *et al.*, 2004; Angel *et al.*, 2007; Zaba *et al.*, 2009; Geissmann *et al.*, 2010). Yet, these markers have failed to unambiguously distinguish between DDCs and macrophages. Recently, Zaba *et al.* (2007) proposed CD1c (BDCA-1) as a reliable marker for resident skin DCs. Controversy exists about the expression of CD209 (DC-SIGN), which has been described by some groups as a specific marker for skin macrophages (Ochoa *et al.*, 2008; Canard *et al.*, 2011), while other groups find co-expression with DDC subsets (Geijtenbeek *et al.*, 2000; Zaba *et al.*, 2007). With regard to the distinction of macrophages and dermal DCs in prenatal skin, we and others have shown that CD36$^+$, CD1c$^+$, FXIIIa$^+$, and HLA-DR$^+$ cells can be identified during the first trimester, but detection of co-expression and inclusion of other markers is necessary to gain better insight into their development (Foster *et al.*, 1986; Fujita *et al.*, 1991; Gibran *et al.*, 1996; Schuster *et al.*, 2009).

As part of the adaptive immune system, T cells fulfill important roles in immunosurveillance (Foster and Elbe, 1997; Clark *et al.*, 2006; Clark, 2010). In adult skin, a large pool of trafficking memory T cells allows for a rapid response in case of antigenic challenge. The entry of T cells into skin is

[1]Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Vienna, Austria; [2]Gynmed-Ambulatorium, Vienna, Austria; [3]Research Division of Biology and Pathobiology of the Skin, Department of Dermatology, Medical University of Vienna, Vienna, Austria and [4]Department of Gynaecology and Obstetrics, Medical University of Vienna, Vienna, Austria

This study was performed in Vienna, Austria.

Correspondence: Adelheid Elbe-Bürger, Laboratory of Cellular and Molecular Immunobiology of the Skin, Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Währinger Gürtel 18-20, Room 4P904, Vienna 1090, Austria. E-mail: adelheid.elbe-buerger@meduniwien.ac.at

Abbreviations: APC, antigen-presenting cell; CLA, cutaneous lymphocyte-associated antigen; DDC, dermal dendritic cell; EGA, estimated gestational age

a multistep process, initialized by the binding of cutaneous lymphocyte-associated antigen (CLA)-positive T cells to E-selectin on post capillary venules (Chong *et al.*, 2004). CCL17/thymus and activation-regulated chemokine (Hijnen *et al.*, 2004; Saeki and Tamaki, 2006) and CCL27/cutaneous T-cell-attracting chemokine (Morales *et al.*, 1999) have a central role for the attraction of T cells. Of note, CCL27, a chemokine constitutively produced in skin by epidermal keratinocytes, selectively attracts CLA^+CCR10^+ memory T cells into skin (Morales *et al.*, 1999). In contrast to the well-studied ontogeny of T cells in fetal liver, spleen, and thymus, the phenotype of T cells in human prenatal skin has been poorly investigated. While ultrastructural studies failed to detect T cells in developing skin (Breathnach, 1977), a recent report identified naive T cells ($CD4^+ > CD8^+$) at 18 weeks EGA using immunohistochemistry (Di Nuzzo *et al.*, 2009). In the course of the development of the immune system, T cells are selected or regulated to become tolerant to self-antigens and reactive against foreign antigens. In addition to the deletion of self-reactive cells, it has been demonstrated that regulatory T cells increase in numbers during the very early stages of pregnancy and help to suppress fetal immune responses in humans (Sasaki *et al.*, 2004; Somerset *et al.*, 2004; Jasper *et al.*, 2006; Mold *et al.*, 2008; Tilburgs *et al.*, 2008; Yang *et al.*, 2008).

Information about mast cell ontogeny in human prenatal skin is scarce (Fujita *et al.*, 1969; Breathnach, 1978; Omi *et al.*, 1991). Mast cells are bone marrow–derived cells expressing the pan-leukocyte marker CD45 as well as CD117 (c-kit; Metcalfe *et al.*, 1997). Their function in skin is essentially unknown, but probably involves both homeostatic regulation of nerves and blood vessels, as well as host defense (Galli *et al.*, 2008). The most characteristic feature of mature mast cells is cytoplasmic granules, which contain numerous preformed mediators that are released at the time of stimulation. Proteoglycans within the mast cell granule permit the binding of a variety of cationic dyes, such as toluidine blue or Giemsa, which stain the mast cell metachromatically. Additionally, antibodies specific for two granule-associated serine proteases, tryptase and chymase, can be used to identify skin mast cells (Irani *et al.*, 1989). Ultrastructural studies performed in the seventies and eighties of the last century identified unanimous mast cells at around 20–26 weeks EGA, containing specific granules as well as immature pre-stages (Omi *et al.*, 1991). During the first trimester, only cells containing small vesicles with no clearly recognizable granules were rarely observed ultrastructurally representing either melanoblasts or immature mast cells (Breathnach, 1978).

Given that our understanding of multiple aspects of the development of the skin immune system is incomplete in humans and that the situation in mice differs in significant aspects (Fadel and Sarzotti, 2000; Adkins *et al.*, 2004; Levy, 2007; Elbe-Bürger and Schuster, 2010), we believe that the study of the ontogeny of skin leukocytes has the potential to yield valuable information about the origin and relationship of skin leukocytes and the general status of the skin immune system *in utero*.

RESULTS

Embryonic skin contains significant populations of DDCs and macrophages

To assess whether macrophages in prenatal human dermis express important lineage molecules, skin of defined gestational age groups and, in parallel, of adults was immunostained for CD45, CD206, and CD209. Similar to adult skin, $CD45^+CD206^+CD209^+$ cells (Figure 1, arrows), most probably macrophages, as well as $CD45^+CD206^+CD209^-$ cells (Figure 1, arrowheads) are identified already in embryonic dermis. The staining intensity of CD206 and CD209 in embryonic skin is mostly comparable to adult skin, yet some cells show only weak expression (compare Figure 2, left column, arrowhead), implying that the upregulation of these markers occurs in the skin. Of note, CD206 staining of linear structures, most likely blood vessels, is observed occasionally in prenatal but not in adult skin (Figure 1, middle column, double arrow). Epidermal $CD45^+$ cells at all age groups investigated lack CD206 and CD209. To evaluate whether CD1c and CD209 are mutually exclusive, triple immunofluorescence staining was performed. We found virtually nonoverlapping populations of $CD206^+CD1c^+$ DDCs (Figure 2, arrowhead) and $CD206^+CD209^+$ macrophages (arrow) already in prenatal dermis, similar to what has been reported in adult dermis, with the exception that $CD1c^+$ cells more often lack or weakly express CD206. Statistical analysis revealed that the frequency of $CD1c^+$ leukocytes significantly increases with gestational age (Figure 2b) and that $CD209^+$ cells outnumber $CD1c^+$ cells between 9 and 14 weeks EGA, reaching adult-like ratios already by midgestation (Figure 2c). By flow cytometry, we found that in all age groups investigated $CD11c^+$ cells are detectable and that CD11c expression is restricted to $CD45^+$ leukocytes (Figure 3a). In depth analysis of HLA-$DR^{high}CD45^+$ cells reveals that $CD1c^+CD11c^+$ cells are already present in the developing skin. The HLA-$DR^{high}CD45^+$ population contains a similar percentage of $CD1c^-CD11c^+$ cells but, in contrast to adult skin, a higher percentage of $CD1c^-CD11c^-$ cells (Figure 3b, R1). In addition, HLA-$DR^{low/neg}CD45^+$ cells express CD11c to a similar extent at all time points investigated (Figure 3b, R2 and R3).

FoxP3$^+$ T cells are present in fetal human skin

$CD45^+CD3^+$ T cells were not detectable in skin during the first trimester in six out of eight samples (Figure 4a-c), whereas two samples (11 and 14 weeks EGA) show a minute population, comprising 0.01% of total skin single cell suspensions. In these embryonic samples, CD3 was rarely found by immunofluorescence and seemed to be expressed only intracellulary (Figure 4b). During midgestation, a moderate increase in T cells takes place, but numbers are approximately 15- to 20-fold lower than in adult skin. $CD3^+CD45^+$ cells are exclusively found in the dermis during skin development.

Given the prominent role of CCL27 in the cutaneous recruitment of memory T cells, we analyzed skin cell culture supernatants from embryonic, fetal, and adult single cells, and found that CCL27 concentrations are significantly higher
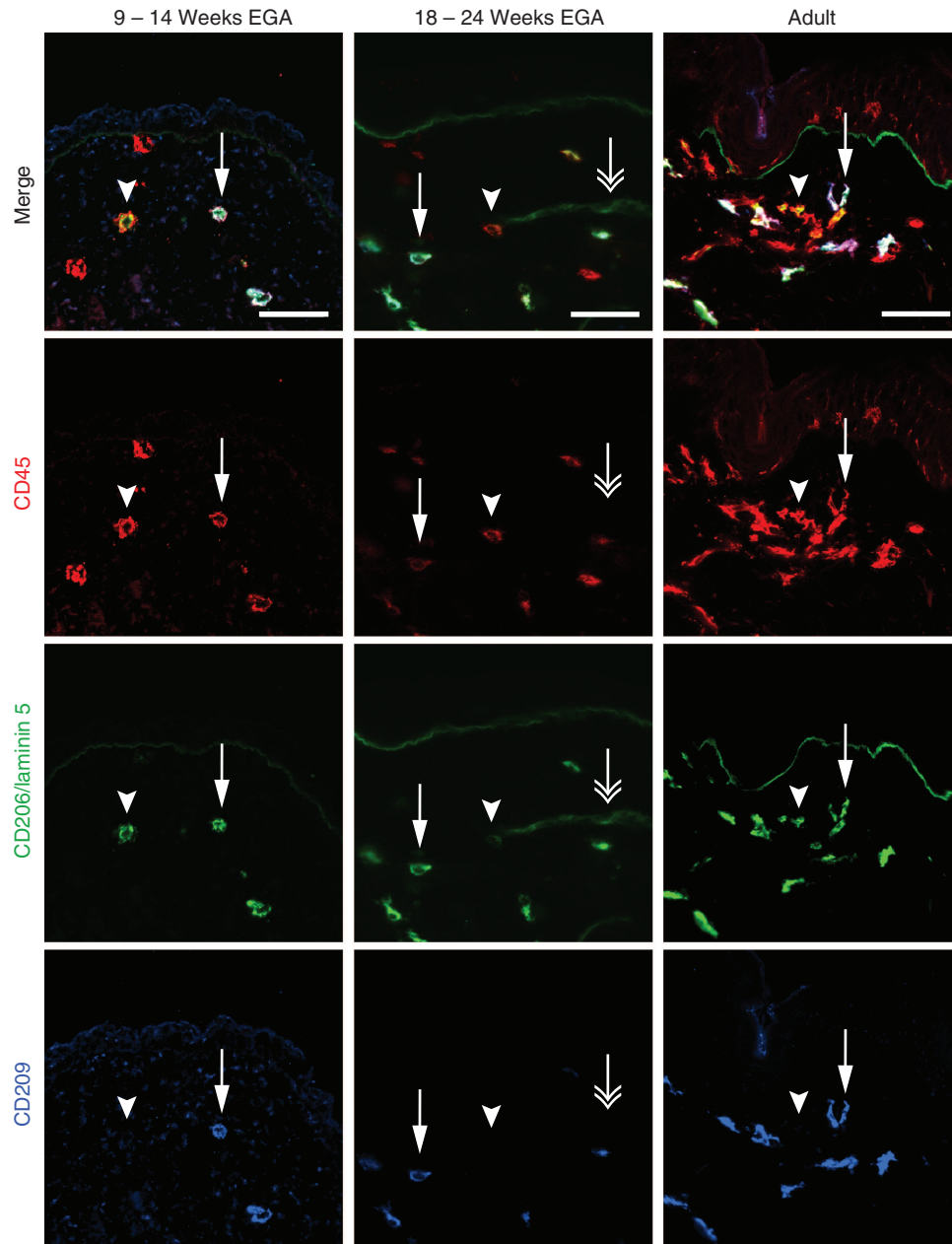
Figure 1. **CD45$^+$CD206$^+$CD209$^+$ and CD45$^+$CD206$^+$CD209$^-$ cells are present in embryonic dermis.** Immunofluorescence triple staining was performed on cryostat sections of embryonic, fetal, and adult skin. Arrows indicate triple positive cells, arrowheads CD45$^+$CD206$^+$ double-positive cells, and double arrows indicate CD206$^+$ vascular structures. One of at least three experiments per group is shown. Bars = 50 μm. EGA, estimated gestational age.

in adult than in embryonic and fetal skin (Figure 4d). In line with these data, immunohistochemical analysis of CCL27 expression revealed a lower staining intensity in fetal compared with adult epidermis (Figure 4e). Of note, skin appendages in fetal skin showed a similar staining intensity as adult skin (data not shown). Taken together, these findings indicate that fetal keratinocytes produce less CCL27 than adult keratinocytes. As the majority of skin-homing T cells in fetal skin are naive (Di Nuzzo *et al.*, 2009) and, thus, not attracted by CCL27 (Morales *et al.*, 1999), our finding suggests that the influx of predominantly naive T cells during skin development might be independent of CCL27.

Given the key role of regulatory T cells controlling and maintaining tolerance to self-antigens and the linkage of FoxP3 to this function, we further investigated whether these cells may already be present in prenatal skin. Indeed, FoxP3 expression was found in 20.0% (SD 6.5%; $n = 5$) of all T cells (5.9 CD3$^+$ cells cm^{-1}, SD 2.9; $n = 5$), a frequency comparable to what is found in the adult skin (Figure 5; Clark and Kupper, 2007; Agius *et al.*, 2009).

Immature mast cells appear during the first trimester
By flow cytometry, CD45$^+$CD117$^+$ mast cells are not detectable in human prenatal skin until 11 weeks EGA
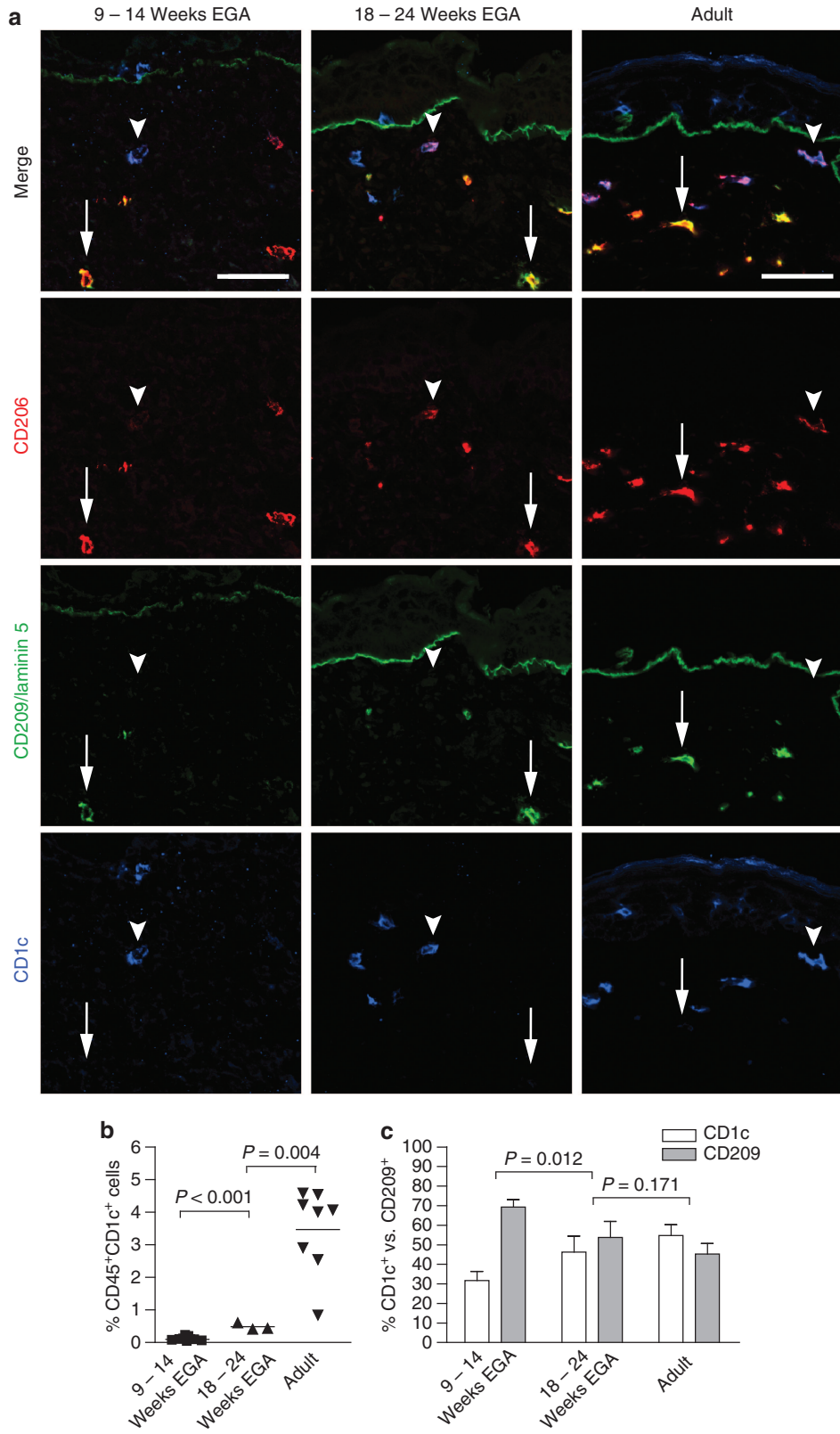
Figure 2. **CD206$^+$CD1c$^+$ DDCs can be distinguished from CD206$^+$CD209$^+$ skin macrophages in embryonic skin.** (**a**) Arrows indicate CD206$^+$CD209$^+$ cells, and arrowheads indicate CD1c$^+$ DCs in all indicated age groups. One of at least four experiments per group is shown. Bars = 50 μm. (**b**) Dot graph shows the increase of CD45$^+$CD1c$^+$ cells in developing skin using flow cytometry. (**c**) Shown are relative numbers of CD1c$^+$ and CD209$^+$ dermal cells at selected developmental time points analyzed by immunoflourescence (n = 4–5 per group). DC, dendritic cell; DDC, dermal dendritic cell; EGA, estimated gestational age.
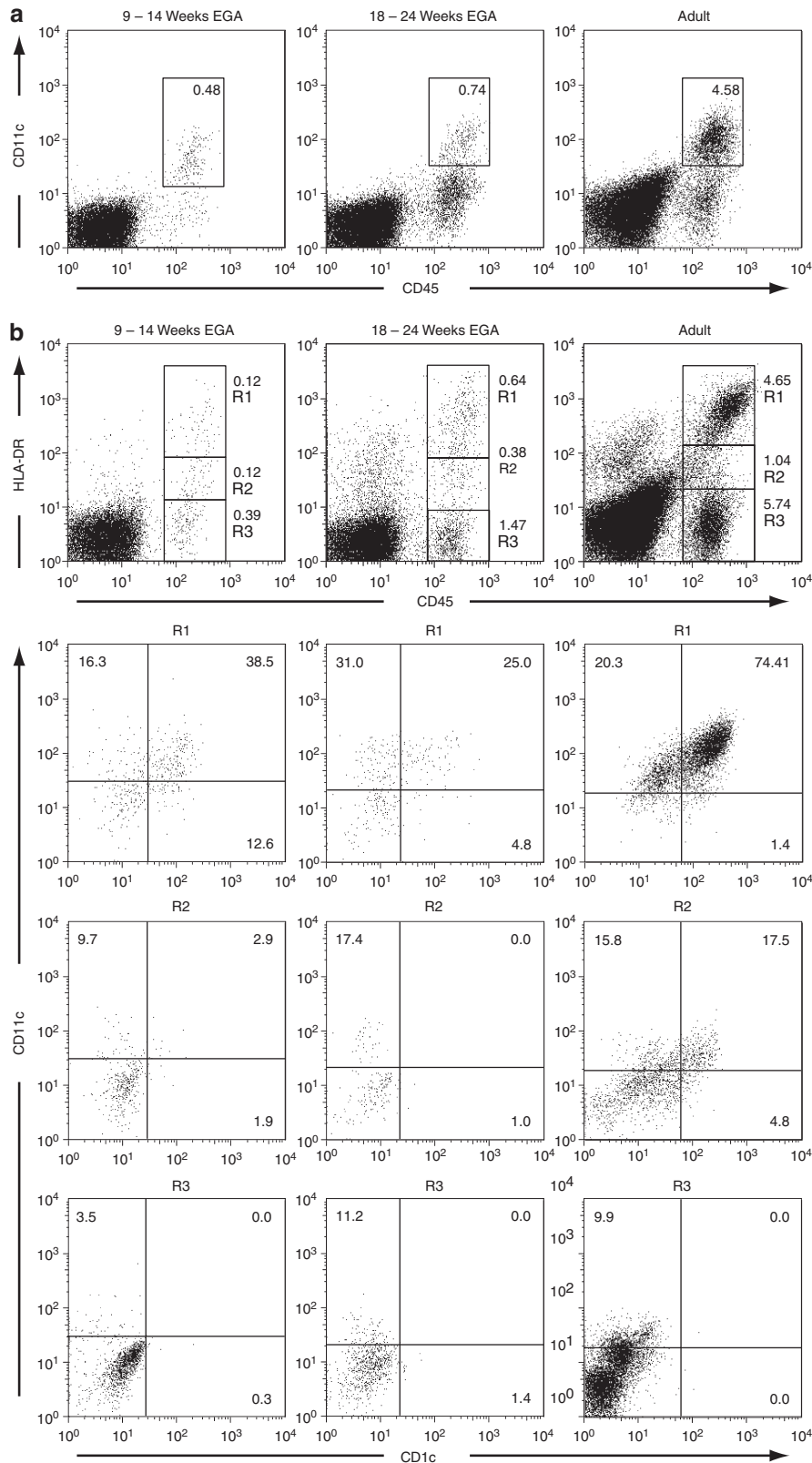
Figure 3. CD11c⁺ leukocytes are present throughout skin development. (**a**, **b**) Multiparameter flow cytometry of freshly isolated single cell suspensions of embryonic, fetal, and adult skin was performed by incubation with mAbs against the cell surface markers indicated. Gates in dot plots were set according to isotype-matched control staining. Dead cells were excluded by 7-amino-actinomycin-D uptake. Dot plots are representative of 3–6 experiments. (**b**) CD45⁺HLA-DR^high (R1), CD45⁺HLA-DR^weak (R2), and CD45⁺HLA-DR⁻ (R3) cells from the indicated age groups are compared with regard to expression of CD11c and CD1c (lower panels). EGA, estimated gestational age.
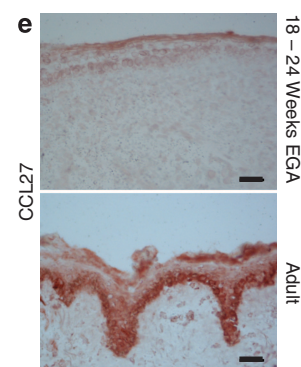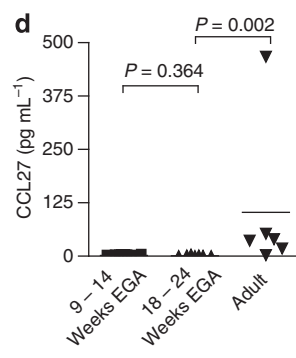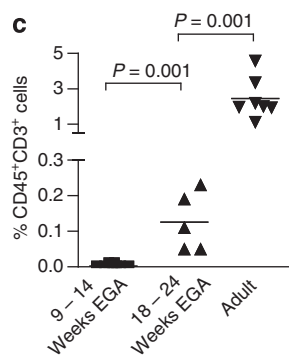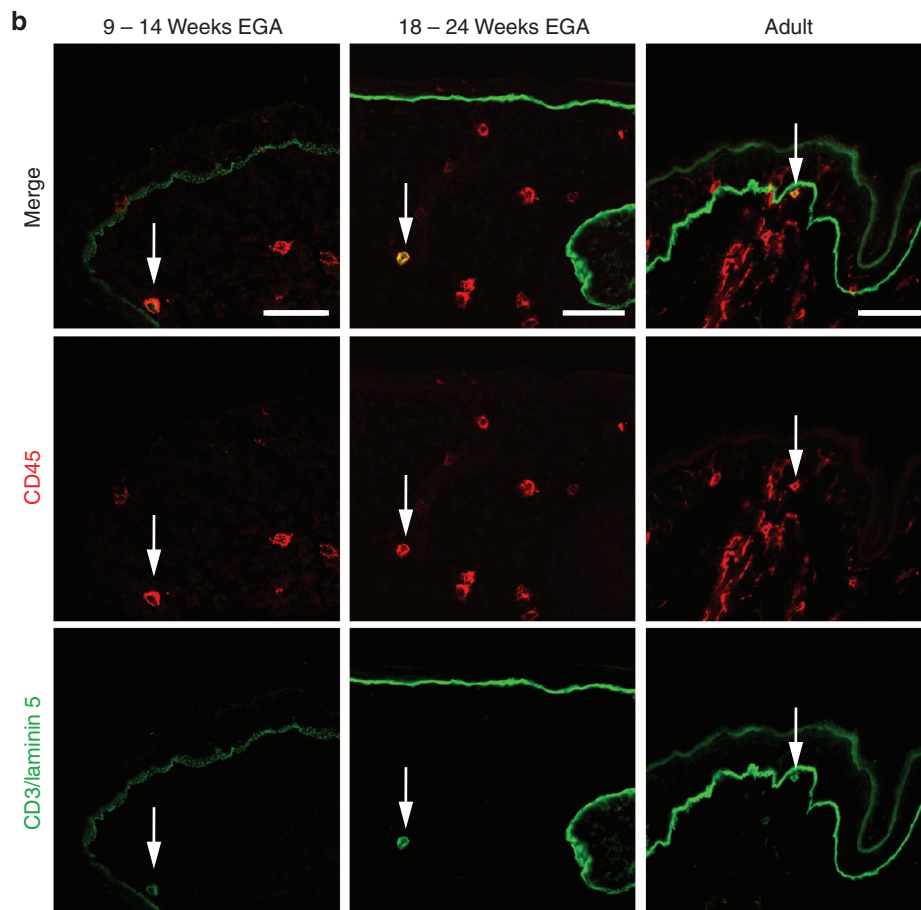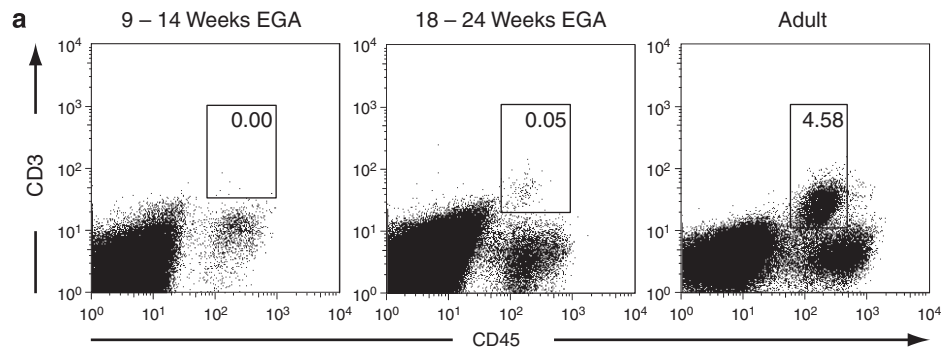
(Figure 6a and b). Coinciding with the beginning of bone marrow hematopoiesis a minute, though distinct, population of mast cells can be first identified at 12–14 weeks EGA. The frequency of CD45$^+$CD117$^+$ cells increases considerably during the second trimester, occasionally reaching adult-like levels (Figure 6a and b). A comparison of the scatter profiles
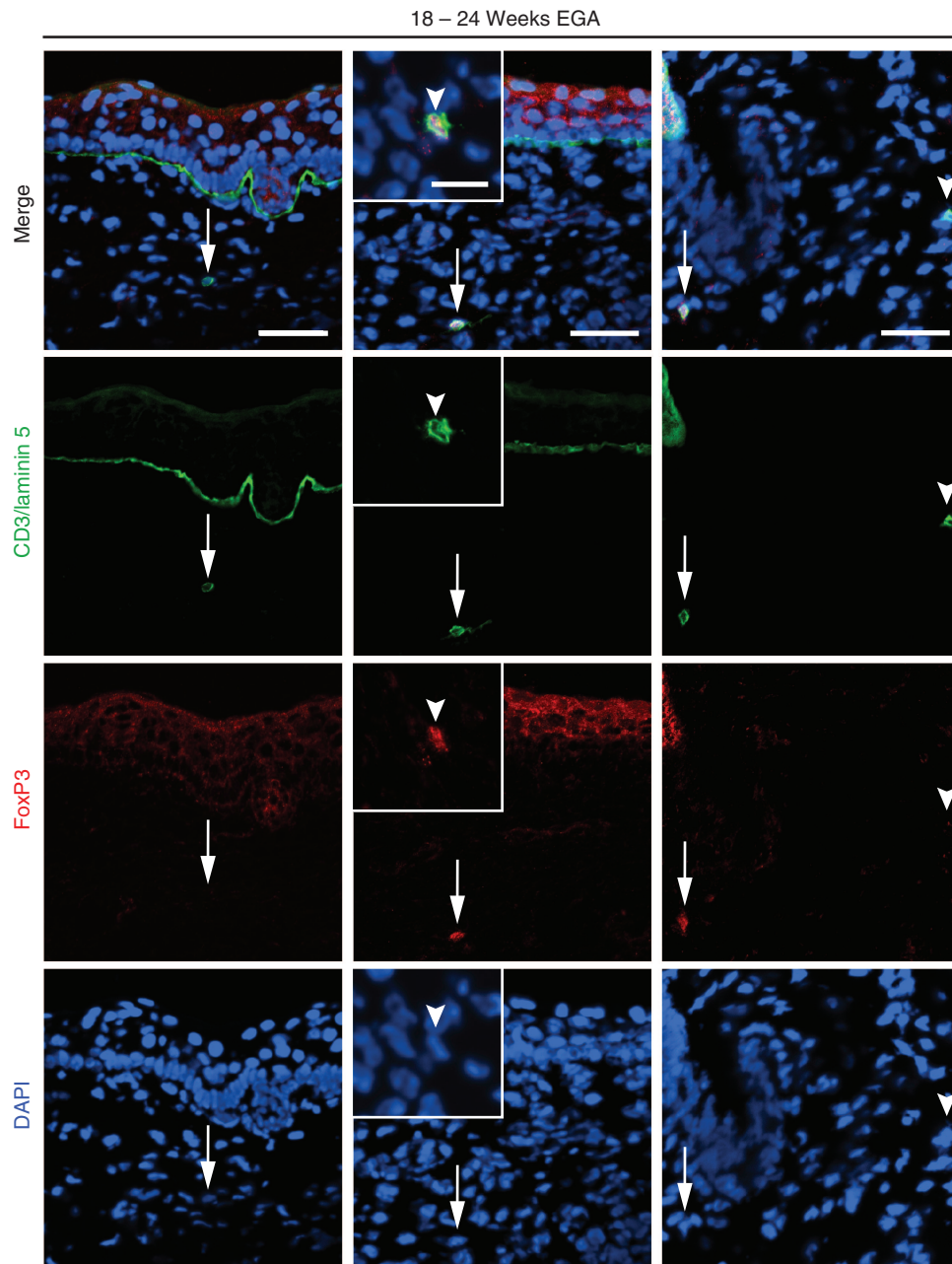

Figure 5. **FoxP3$^+$ T cells are present in fetal human skin.** Immunofluorescence double staining was performed on cryostat sections of fetal skin ($n = 5$). Arrows denote CD3$^+$FoxP3$^-$ T cells (left panel) and CD3$^+$FoxP3$^+$ T cells (middle panel). Arrowheads indicate intracellular FoxP3 staining in the insert. Demonstration of a FoxP3$^+$ and a FoxP3$^-$ T cell on the same section (right panel). Bars = 50 μm, bar in inset = 20 μm. DAPI, 4,6-diamidino-2-phenylindole; EGA, estimated gestational age.

Figure 4. **Skin T cells can be identified at midgestation.** (**a**) Multiparameter flow cytometric analysis was performed after incubation with mAbs against the cell surface markers indicated. Gates in dot plots were set according to isotype-matched control staining. Dead cells were excluded by 7-amino-actinomycin-D uptake. Dot plots are representative of 5–8 experiments. (**b**) Immunofluorescence double staining was performed on cryostat sections of embryonic, fetal, and adult skin. One of at least three experiments per group is shown. Arrows denote CD45$^+$CD3$^+$ T cells. (**c**) Graphs show an increase of T-cell numbers in developing prenatal skin analyzed by flow cytometry ($n = 5$–8). (**d**) Single cell suspensions of embryonic, fetal, and adult skin were cultured for 48 hours, supernatants were harvested, and CCL27 levels were analyzed by ELISA. Bars represent the mean. (**e**) Immunohistochemical staining was performed on cryostat sections of fetal and adult skin, and CCL27 binding was visualized using amino-ethyl-carbazole. Bars = 50 μm. EGA, estimated gestational age.
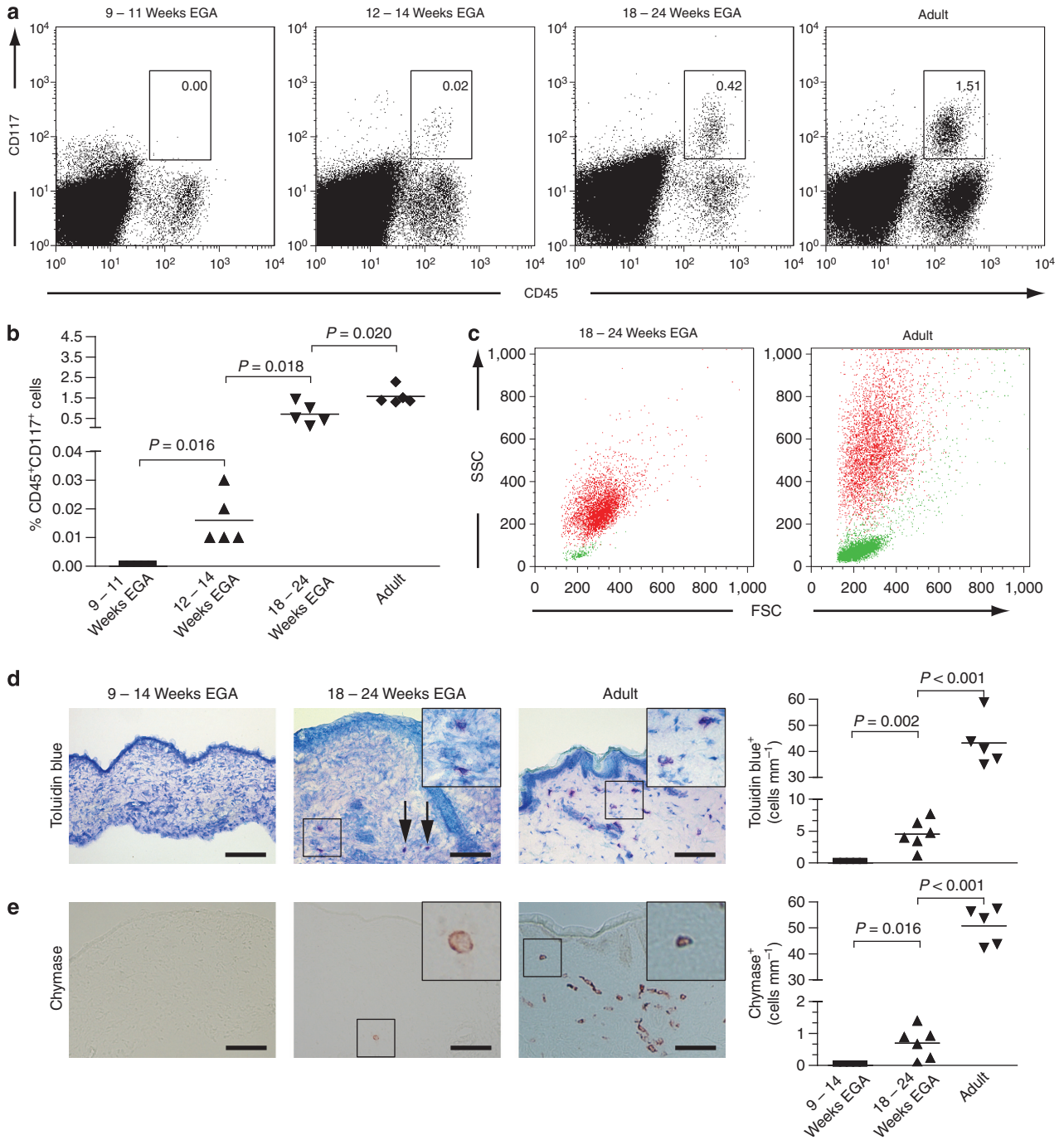
Figure 6. Mast cells appear during the first trimester but are not fully mature at midgestation. (a) Flow cytometric analysis of freshly isolated single cell suspensions of embryonic, fetal, and adult skin revealed the presence of immature mast cells in fetal skin. Shown are representative dot plots of five experiments per group. (b) Graphs show the increase in mast cell numbers of developing skin analyzed by flow cytometry. Five specimens were investigated per age group. Bars represent the mean of investigated groups. (c) The size, granularity, and numbers of $CD45^+CD117^+$ cells (red dots) and $CD45^+CD3^+$ cells (green dots) between fetal and adult skin are compared. (d, e) Toluidin blue (d) and chymase (e) stainings were performed on cryostat sections of embryonic, fetal, and adult skin (left panel). Arrows denote toluidine blue-expressing cells in fetal dermis. Bar = 100 μm. The numbers of metachromatic cells (d, right panel) and chymase$^+$ cells (e, right panel) were determined and bars represent the mean of investigated groups ($n = 5$–6 per group). EGA, estimated gestational age; FCS, forward scatter; SSC, side scatter.

of fetal and adult $CD45^+CD117^+$ skin mast cells reveals a substantially weaker granularity of fetal than adult mast cells (Figure 6c, red dots), indicating immaturity of fetal mast cells due to the lack of specific granules. By contrast, $CD45^+CD3^+$ T cells in fetal and adult skin have a similar forward and side scatter profile, but significantly differ in numbers (Figure 6c, green dots). In line with the suspected mast cell immaturity, we could not detect mast cells by their

metachromatic staining with toluidin blue up to 14 weeks EGA (Figure 6d). Toluidin blue–expressing cells were first identified in the second trimester, but their numbers were approximately 8-fold lower than in adult skin. Similar to adult skin, these cells are preferentially found around vessels and in vicinity to skin appendages. Mature, chymase-expressing mast cells are rarely found in fetal skin (Figure 6e), but can be easily identified in adult skin. The ratio of toluidin blue/chymase-positive cells changes from 6.4:1 in fetal skin to 0.9:1 in adult skin.

DISCUSSION

We have shown that human embryonic skin contains almost mutually exclusive, committed $CD209^+$ skin macrophages and $CD1c^+$ DDCs. In addition, we have found that important cells of the skin immune system colonize the developing skin at different time points, i.e., APC by 9 weeks EGA (the earliest time point investigated), T cells during the second trimester, and mast cells at 12 weeks EGA.

The distinction of DDCs and skin macrophages by phenotypic terms can be difficult, owing to their high plasticity and/or different lineage affiliation (Meunier *et al.*, 1993; Nestle *et al.*, 1993; Ebner *et al.*, 2004; Angel *et al.*, 2007; Zaba *et al.*, 2009; Geissmann *et al.*, 2010). Recently, various groups proposed new markers for the identification of DDCs and skin macrophages, namely CD1c and CD209, respectively (Ochoa *et al.*, 2008; Zaba *et al.*, 2009; Schäkel and Hänsel, 2011). We show in this study that already by 9 weeks EGA skin macrophages and DCs can be phenotypically separated by the almost distinct expression of CD1c and CD209. Of note, both populations express CD206, making this marker suitable for detecting dermal APCs. On the basis of the expression of these molecules and on earlier results one may reason that both DCs and skin macrophages possess the ability to recognize and ingest foreign antigen in case the epithelial barrier has been breached, e.g., under the condition of microbial invasion of the amniotic fluid. The weak expression of CD206 on some $CD1c^+$ cells as well as the fact that CD206 expression is not found on potential blood precursors (Shepherd *et al.*, 1982) suggest an upregulation of this marker *in situ*, which can be induced by high levels of M-CSF and IL-6, cytokines already found in embryonic skin (Gersuk *et al.*, 2005; Schuster *et al.*, 2009).

The elucidation of the origin of DCs and skin macrophages is generally complicated by the diversity and plasticity of DDCs. Similar to epidermal Langerhans cells, they co-express CD11c and CD1c, yet, also a subset of $CD11c^+CD1c^-$ DDCs positive for CD141 has been described (Zaba *et al.*, 2007). In developing skin, $HLA-DR^{high}CD45^+$ cells are more heterogeneous with regard to the expression of CD11c and CD1c. From an ontogenetic point of view, it is conceivable that $CD11c^-CD1c^-$ cells act as precursors of $CD11c^+CD1c^+$ via $CD11c^+CD1c^-$ cells, especially given the higher percentage of $CD11c^-CD1c^-HLA-DR^{high}$ leukocytes in prenatal skin. Whether these cells express CD141 remains to be investigated.

T cells are the prototypical cells of the adaptive immune system. In adult, noninflamed skin, the majority of resident T cells are $CLA^+CD45RO^+$ memory cells, which are recruited into skin mainly by two chemokines, CCL27 and CCL17 (Morales *et al.*, 1999; Clark *et al.*, 2006; Saeki and Tamaki, 2006). During *in utero* development, T-cell precursors are first detectable in the fetal liver at 7 weeks of gestation and are found in the developing thymus and circulation by 8–9 weeks EGA (Campana *et al.*, 1989; Holt and Jones, 2000). With regard to function, alloreactivity is found in the fetal liver at 7 weeks EGA and T cells are believed to be immunocompetent by midgestation (Fadel and Sarzotti, 2000; Holt and Jones, 2000; Hermann *et al.*, 2002; Michaelsson *et al.*, 2006; Mold *et al.*, 2008; Vermijlen *et al.*, 2010). By immunofluorescence, we occasionally identified $CD45^+CD3^+$ cells in the first trimester, which appear to have intracellular CD3 expression and were, thus, not identified by conventional flow cytometry. $CD3^+$ T cells are detectable in the fetal circulation at about 15–16 weeks of gestation (Haynes *et al.*, 1988). In line with this we found that by midgestation, $CD45^+CD3^+$ cells are present in higher numbers, yet, still in numbers much smaller than in adult skin. Two possibilities—or a combination thereof—may explain this observation. First, as almost no exogenous antigen is presented in secondary lymphatic tissues *in utero*, skin-homing $CD45RO^+$ memory cells simply do not develop. This is corroborated by the recent finding that T cells in fetal skin have a largely naive $(CD45RA^+)$ phenotype and do not express CLA, suggesting that T cells may nonspecifically be recruited into prenatal skin (Di Nuzzo *et al.*, 2009). Second, it appears that developing skin contains lower levels of CCL27 than adult skin, thus, preventing the influx of memory T cells from the circulation (Morales *et al.*, 1999). The expression of other cytokines critical for the attraction of lymphocytes in the steady state, such as CCL17, remains to be investigated. With regard to the phenotype of skin lymphocytes, we found that the majority of $CD3^+$ cells expressed CD4, thus, confirming previous results described by Di Nuzzo *et al.* (2009). We exclusively observed TCR $\alpha\beta^+CD3^+$-expressing cells in fetal human skin (C Schuster, unpublished data) making the existence of a human analog of murine dendritic epidermal T cells (Steiner *et al.*, 1988) and the recently described murine TCR $\gamma\delta$ dermal T cells (Gray *et al.*, 2011; Sumaria *et al.*, 2011) in prenatal skin quite unlikely. Whether skin-homing fetal T cells have a particular function, e.g., in inducing or maintaining tolerance, is currently not known (Mold *et al.*, 2010). Of note, fetal dermatitis after microbial invasion of the amniotic fluid results in inflammatory infiltrates including $CD3^+$ T cells (Kim *et al.*, 2006). This finding suggests that circulating T cells can be attracted under certain conditions.

It has been shown that fetal tissues and blood contain higher numbers of regulatory T cells to control and at least partially maintain tolerance to self-antigens (Somerset *et al.*, 2004; Mold *et al.*, 2008). We were surprised to find similar numbers of $FoxP3^+$ cells in fetal and adult skin, supporting the concept for the existence of various tolerance mechanisms.

The importance of mast cells in skin immunity and homeostasis is increasingly recognized (Metcalfe, 2008). Early electron microscopy studies failed to detect definitive mast cells in human first trimester skin. At the end of the first

trimester, Breathnach (1977) identified cells of unclear affiliation containing vesicles compatible with both melanoblasts and mast cells. Using flow cytometry, we showed that mast cells are not present in embryonic skin until 11 weeks but appear after 12 weeks EGA. Yet, we also observed $CD45^-CD117^+$ cells, probably representing melanoblasts and/or melanocytes. Of note, the appearance of $CD45^+CD117^+$ cells coincides with the beginning of bone marrow hematopoiesis (Tavian and Peault, 2005). Whether these $CD45^+CD117^+$ cells are derived from the newly formed bone marrow or are attracted from other hematopoietic stem cell sources due to changing microenvironmental factors is not known. Also hematopoietic progenitor cells can express CD117 in certain stages of development (Tavian and Peault, 2005). It appears quite unlikely that $CD45^+CD117^+$ cells represent hematopoietic progenitor cells—either as blood contamination or as resident cells in skin—given that these cells were not observed between 9 and 11 weeks of EGA. $CD45^+CD117^+$ mast cells during the first trimester stain neither for toluidin blue nor for chymase, suggesting that no specific granules are present and thus confirming earlier assumptions (Fujita *et al.*, 1969). During midgestation, toluidin blue$^+$ and chymase$^+$ cells are first detectable, although flow cytometric analysis using the side scatter profile as well as the density of toluidin blue- and chymase-staining indicates that the mast cells are immature with regard to both granule numbers as well as granule content. Of note, just a minority of mast cells express chymase, whereas all cells in adult skin stain for this marker (Irani *et al.*, 1989). To date, nothing is known about the functional potential of mast cells in human fetal skin. Interestingly, we occasionally observed degranulation, probably resulting from tissue preparation and cutting. This might indicate that mast cells already in fetal skin possess the capacity to exert their ancestral function. In addition, it is not known whether distinct developmental pathways or environmental factors contribute to the maturation of mast cells. The most important cytokine for mast cell development, stem cell factor, is found at highest concentration in embryonic skin, perhaps providing an important chemoattractant for blood-borne precursors (Schuster *et al.*, 2009).

Taken together, we found that the myeloid part of the skin-resident immune system (i.e., DDCs, macrophages, mast cells and, as described previously, epidermal Langerhans cells (Schuster *et al.*, 2009; Elbe-Bürger and Schuster, 2010)) shows a high degree of maturity already at a prenatal stage. Indeed, both *in vitro* experiments as well as *in vivo* data indicate that the human fetal skin is already capable of recognizing and responding to microbial products (Kim *et al.*, 2006).

MATERIALS AND METHODS
Skin samples
After legal termination of pregnancy, 42 specimens of human embryonic and fetal trunk skin ranging from 8 to 24 weeks EGA were studied. The age was estimated by crown-rump length and maternal history. Healthy adult (18–59 years) skin was collected after abdominal, back, and breast surgery. The study was approved by the local ethics committee and conducted in accordance with the declaration of Helsinki Principles. Parents/participants gave their written informed consent.

Preparation of skin cell suspensions
After removal of subcutaneous tissue, embryonic, fetal, and adult skin specimens were incubated on $1.2\,U\,ml^{-1}$ Dispase II (Roche Diagnostics, Indianapolis, IN) in phosphate-buffered saline overnight at $4\,^\circ C$. It was not possible to efficiently separate dermis and epidermis in embryonic skin, thus unseparated skin regardless of age was vigorously agitated in a shaking water bath in $0.53\,U\,ml^{-1}$ Liberase 3 (Roche Diagnostics) in phosphate-buffered saline at $37\,^\circ C$ for 60 to 90 min. The resulting single cell suspensions were analyzed by flow cytometry or cultured for 48 hour ($10^6\,cells\,ml^{-1}$) in RPMI 1640 medium (Invitrogen, Eugene, OR) supplemented with 10% heat-inactivated fetal calf serum (PromoCell, Heidelberg, Germany), 25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), $10\,\mu g\,ml^{-1}$ gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercapto-ethanol, and 0.002% antibiotic-antimycotic solution (all Invitrogen).

Flow cytometry
Single cell suspensions were stained with the following mAbs: PE-anti-CD117 (YB5.B8), PE-anti-CD11c (Leu-M5), PE-anti-HLA-DR (L243; all Becton Dickinson, Mountain View, CA), PE-Cy7-anti-CD45 (J.33; both Beckman Coulter, Fullerton, CA), FITC-anti-CD3 (UCHT1, An der Grub, Kaumberg, Austria), and FITC-anti-CD1c (M241; Ancell, Bayport, MN). Appropriate isotype controls were included. Dead cells were excluded with 7-amino-actinomycin-D (Calbiochem, Darmstadt, Germany). Five-color flow cytometry analyses were performed on a LSR-II (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Immunohistochemistry
Embryonic, fetal and adult skin specimens were embedded in optimum cutting tissue compound (Tissue-Tek, Sakura Finetek, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at $-80\,^\circ C$ until further processing. Six-micrometer sections were cut, air-dried, fixed in ice-cold acetone for 10 minutes and washed in phosphate-buffered saline. Sections were then incubated with an unconjugated anti-CCL27 mAb (124302, R&D Systems, Minneapolis, MN) or anti-chymase mAb (B7, Millipore, Billerica, MA) overnight at $4\,^\circ C$, followed by blocking of endogenous peroxidase activity by incubating sections for 10 minutes in methanol containing 0.03% hydrogen peroxide. Subsequently, sections were incubated for 2 hours at room temperature with biotin-conjugated goat anti-mouse IgG using the Elite mouse IgG Vectastain Kit (Vector Laboratories, Burlingame, CA). Biotinylated antibodies were detected with horseradish peroxidase–streptavidin and staining was visualized with amino-ethyl-carbazole (Dako, Glostrup, Denmark). Finally, sections were mounted with Aquatex (Merck, Darmstadt, Germany) and examined using a Nikon Eclipse 80 microscope (Nikon, Tokyo, Japan). Appropriate isotype controls (Becton Dickinson) were included.

Immunofluorescence
Fixed sections were stained with unconjugated primary antibodies anti-CD1c (L161; Serotec, Kidlington, UK), anti-CD209 (DCN46,

Becton Dickinson), or FoxP3 (236A/E7; eBioscience, San Diego, CA) overnight at 4 °C and then detected with donkey-anti-mouse NorthernLight637 (R&D Systems). Subsequently, sections were blocked with 10% mouse serum and 2% mouse IgG (Becton Dickinson) for 1 hour at room temperature and stained with the following conjugated antibodies overnight at 4 °C: Alexa Flour 555-anti-CD45 (MEM28, Exbio, Vestec, Czech Republic), Alexa Flour 555-anti-CD206 (19.2, Becton Dickinson, both labeled using the Alexa Flour 555 Monoclonal Antibody Labeling Kit, Invitrogen), FITC-anti-CD206 (19.2; Becton Dickinson), and FITC-anti-CD3 (UCHT1, An der Grub). Alexa Flour 488-anti-Laminin 5 (D4B5; Millipore) was used to visualize the dermo–epidermal junction. Slides were mounted using Vectashield (Vector Laboratories) and images were recorded using a confocal laser scanning microscope (LSM 410; Carl Zeiss, Jena, Germany) equipped with three lasers emitting lights at 488, 543, and 633 nm. For FoxP3 staining, nuclear counterstain was performed with Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories).

Cytokine determination in skin cell supernatants

Skin single cell suspensions (1×10^6 ml^{-1}) were cultured in 24-well plates (Costar, Lowell, MA) in RPMI 1640 supplemented as described above. After 48 hours, supernatants were harvested, snap-frozen and stored at −80 °C until use. CCL27 was determined by ELISA (R&D Systems) according to the manufactures instructions. Experiments were performed in duplicates.

Toluidin blue staining

Six micrometer frozen sections were cut, air-dried, stained in toluidin blue O/methanol (Sigma-Aldrich, St Louis, MI) for 15 seconds. After washing in phosphate-buffered saline, the sections were mounted with Aquatex (Merck) and examined using a Nikon Eclipse 80 microscope (Nikon).

Quantification of cells in skin sections

FoxP3$^+$CD3$^+$, chymase$^+$, and toluidin blue-reactive cells were enumerated by two independent investigators in multiple skin sections with a total length of at least 6 cm (×40 object lens, Carl Zeiss; ×20 object lens; Nikon).

Statistical analysis

Differences between groups were assessed with the Mann–Whitney *U* test or Student's *t*-test (GraphPad Software, San Diego, CA). The reported *P*-value is a result of a two-sided test. A *P*-value <5% is considered statistically significant.

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