Immunology of the Human Nail Apparatus: The Nail Matrix Is a Site of Relative Immune Privilege

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The nail apparatus is constantly exposed to environmental damage. It requires effective immune responses to combat infection, while avoiding the loss of nail production and regeneration by autoaggressive immunity. By immunohistology, we define here previously unknown characteristics of the normal human nail immune system (NIS). Compared with other regions of nail epithelium, human leukocyte antigen (HLA)-A/B/C expression is prominently downregulated on both keratinocytes and melanocytes of the proximal nail matrix (PNM), whereas HLA-G$^+$ is upregulated here. Together with the expression of macrophage migration inhibitory factor in PNM, this may serve to inhibit an natural killer (NK) cell attack on major histocompatibility complex (MHC) class Ia-negative PNM. PNM also displays strong immunoreactivity for potent, locally generated immunosuppressants such as transforming growth factor-β1, α-melanocyte stimulating hormone, insulin-like growth factor-1, and adrenocorticotropic hormone, exhibits unusually few CD1a$^+$, CD4$^+$, or CD8$^+$, NK, and mast cells. Finally, MHC class II and CD209 expression on CD1a$^+$ cells in and around the PNM is reduced, indicating diminished antigen-presenting capacity. Thus, the NIS strikingly differs from the skin immune system, but shows intriguing similarities to the hair follicle immune system, including the establishment of an area of relative immune privilege in the PNM. This nail immune privilege may offer a relative safeguard against autoimmunity. But, the localized intraepithelial defect of innate and adaptive immunity in the PNM revealed here also may impede effective anti-infection defense.

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The nail apparatus and its evolutionary ancestors (claws, hooves) are essential components of the haptile, tactile, mechanic, defensive, and/or predatory instrumentarium used by vertebrates, and is particularly exposed to environmental damage and infection (de Berker et al, 2001; Paus and Peker, 2003; Tosti and Piraccini, 2003). In fact, bacterial and fungal infections of the nail apparatus (paronychia, onychomycoses) rank among the more frequently encountered diseases (Hanno, 2000), and are notoriously difficult to treat. Also, chronic inflammatory skin diseases target the nail apparatus, damaging it substantially and often irreversibly (Hanno, 2000; de Berker et al, 2001; Rockwell 2001; Tosti et al, 2001; Tosti and Piraccini, 2003). Thus, the nail apparatus is faced with the dilemma of having to combine a system of effective anti-infection defenses and damage repair with safeguards against the loss of nail production and regenerating by autoaggressive/destructive immunity at each part of nail tissue.

As our knowledge of *normal* nail immunology is still very poor and fragmentary, it is as yet unknown how the nail apparatus addresses this dilemma. In normal human nail apparatus, only the presence of Langerhans cells, the absence of intracellular adhesion molecule 1 (ICAM-1) immunoreactivity, and local expression of antimicrobial peptides (human cathelicidin LL-37) have been reported so far (Cameli et al, 1994; Picardo et al, 1994; Tosti and Piraccini, 2003; Dorschner et al, 2004). In the context of studying inflammatory diseases that affect the nail, large numbers of Langerhans cells (i.e., CD1a$^+$ cells) and intraepithelial T lymphocytes (with a reported T4/T8 ratio of 2:1) were found, e.g., in the nail matrix of trachyonychia associated with alopecia areata (Tosti et al, 1991, 1995). Lichen planus, one of the best-investigated inflammatory nail diseases, reportedly shows dense lichenoid lymphocytic infiltrates with apoptosis of keratinocytes (Tosti et al, 1993; Hanno, 2000).

This lack of detailed information on even basic immunological characteristics of the normal human nail apparatus largely results from difficulty in obtaining fresh samples of uninfected, uninflamed, non-tumor-bearing human nail

apparatus for cryosectioning and immunohistological analysis. We obtained access to rare cryospecimen of normal, uninflamed, uninfected human nail apparatus, obtained with informed consent during elective surgery of three 3-mo-old infants with polydactyly (Kozin, 2003), who underwent amputation of excess digits.

In view of the developmental and biological similarities of the nail and the pilosebaceous apparatus (Chuong and Noveen, 1999; Paus and Peker, 2003; Wu *et al*, 2004), we were most interested in drawing parallels between the better-characterized hair follicle immune system (HIS) (Paus *et al*, 1999), in particular, the striking relative immune privilege of the growing (anagen) hair follicle (Christoph *et al*, 2000; Paus *et al*, 2003, 2005; Ito *et al*, 2004), and the evolutionarily older nail apparatus.

Results and Discussion

CD4$^+$ or CD8$^+$ T cells are differentially distributed in and around the nail apparatus In the epithelial tissue compartments of the infant human nail apparatus, CD4$^+$ cells were only rarely detected. Interestingly, most of these few detectable intraepithelial CD4$^+$ cells were located in the basal layer of the proximal nail fold (PNF) (Fig 1*A*) [7.75 ± 0.75 cells per microscopic field (MF)] (Mean ± SEM). Significantly fewer CD4$^+$ T cells were detected in the nail matrix epithelium (3.33 ± 0.33 cells per MF) (Fig 1*B–D*).

This highly differential distribution of intraepithelial T cells in the nail apparatus was mirrored in the nail mesenchyme (= dermis of nail tissue): A high density of CD4$^+$ cells was observed in the dermal mesenchyme in direct vicinity of the PNF (22.3 ± 1.8 cells per MF) (Fig 1*A, C, D*), nail bed (Fig 1*B*), and hyponychium (data not shown). Intradermally located T cells focally accumulated in dense cell clusters around the infantile nail apparatus (Fig 1*A, B*), This phenomenon is absent in normal human epidermis or dermis (Curry *et al*, 2003; Bos, 2005). As in nail epithelium, the lowest density of CD4$^+$ was found in nail mesenchyme around nail matrix (7.43 ± 1.17) (p < 0.01) (Fig 1*B–D*). Therefore, nail CD4$^+$ T cells show a rather differential distribution

pattern, with the nail matrix epithelium and its surrounding mesenchyme displaying the lowest CD4$^+$ T cell density.

Essentially, the same observation was made for CD8$^+$ cells, even though their total number was substantially lower than that of CD4$^+$ T cells (Fig 1*E–K*). Clusters of CD8$^+$ cells were not observed in any of the examined nail sections (Fig 1*E–K*).

Antigen-presenting capacity of Langerhans cells and macrophages in/around the nail matrix may be impaired Next, we examined Langerhans cells in the human nail apparatus. As reported before (Tosti and Piraccini, 2003), the localization of Langerhans cells was similar to that of normal human epidermis in PNF (Fig 1*I*), nail bed, and hyponychium (data not shown). CD1a$^+$ cells, however, were very rare in the proximal nail matrix (PNM) (Fig 1*J*). This resembled the extremely low number of CD1a$^+$ cells seen in human hair follicle matrix (Christoph *et al*, 2000). Numerous CD68$^+$ cells (i.e., dermal macrophages) were detected in the dermal tissue surrounding all parts of the nail epithelium, with no apparent differences in this distribution (Fig 1*L, M*).

To assess the ability of Langerhans cells and macrophages to execute their antigen-presenting function via major histocompatibility complex (MHC) class II expression (Janeway *et al*, 2001; Goldsby *et al*, 2003; Bos, 2005), double-immunolabeling for CD1a or CD68 and human leukocyte antigen (HLA)-DP/DQ/DR was performed. Interestingly, intraepithelial Langerhans cells in dorsal nail matrix and dermal macrophages surrounding the entire nail matrix showed absent or very low immunoreactivity for HLA-DP/DQ/DR (Fig 1*J, M*).

In contrast, Langerhans cells and macrophages in/around PNF (Fig 1*I, L*) showed the expected strong HLA-DP/DQ/DR immunoreactivity, suggesting these CD1a$^+$ or CD68$^+$ cells normally perform their well-recognized "professional" antigen-presentation functions via MHC class II (Fig 1*K, N*).

Even though this remains to be corroborated by functional studies, this suggests that both dendritic cell populations in and around the nail matrix are functionally impaired in their antigen presenting-capacity.


Figure 1
Localization of immune cells in human infantile nail apparatus. (*A*) High density of CD4$^+$ cells is seen in proximal nail fold (PNF). Accumulated CD4$^+$ cells are seen in epithelium and mesenchyme (a: high power) that are not observed in normal human skin. (*B*) CD4$^+$ T cells are rarely detected in adjacent proximal nail matrix (PNM) resembling hair bulb. Some cells are also seen in epithelium. (*C*) The schematic drawing of CD4$^+$ cell distribution in nail apparatus. The density of CD4$^+$ T cells around the PNM is lower than that in the PNF. (*D*) Semiquantitative analysis of CD4$^+$ cells in epithelium and mesenchyme. The density of CD4$^+$ T cells is significantly decreased in PNM [3.33 ± 0.33 cells per microscopic field (MF)] compared with epithelium of PNF (7.75 ± 0.75 cells per MF) (n = 3). (*E* and *F*) CD8$^+$ T cells are rarely seen in and around PNF, nail bed, and PNM. Some CD8$^+$ cells are observed in PNF, although the number of CD8$^+$ cells is less than that of CD4. (*G*) The schematic drawing of distribution of CD8$^+$ T cells in nail apparatus. The density of CD8$^+$ T cells is much less than that of CD4$^+$ T cells. (*H*) Semiquantitative analysis of CD8$^+$ T cells in mesenchyme. Higher density of CD8$^+$ cells is detected in around PNF compared with around PNF (n = 3 nails). (*I*) Double staining is demonstrated using CD1a (red) and major histocompatibility complex (MHC) class II (green) antibodies on PNF. MHC class II (green) positive Langerhans cells (CD1a$^+$) (red) are seen in PNF (*I*, high power) (yellow). The density of Langerhans cells may be higher in epithelium of PNF and nail bed compared within matrix. (*J*) MHC class II negative Langerhans cells (CD1a$^+$) are detected in human dorsal nail matrix (red) but not in PNF (*J*, high power). This result implicates that Langerhans cells in matrix may be functionally impaired for antigen presentation. (*K*) Schematic drawing of immunoreactivity on CD1a (red), MHC class II (green) and double positive cells (yellow). (*L*) Double staining are demonstrated using CD68 (red) and MHC class II (green) antibodies on PNF. MHC class II$^+$ macrophages are seen in PNF (yellow). (*M*) MHC class II negative macrophages (CD68$^+$) are detected in/around PNM (red). Not only Langerhans cells but also macrophages are functionally impaired to present antigens in/around PNM. (*N*) Schematic drawing of immunoreactivity on CD68 (red), MHC class II (green), and double positive cells (yellow). (*O*) Double-staining is demonstrated using CD209 (red) and MHC class II (green) antibodies on PNF. (*P*) CD209 negative MHC class II + cells (red) are detected in/around PNM. This suggests that, in the PNM, the ability of dendritic cells and/or macrophages to activate resting T lymphocytes and/or dendritic cell trafficking may be partially impaired by insufficient expression of CD209. (*Q*) The schematic drawing of immunoreactivity on CD209 (red), MHC class II (green), and double positive cells (yellow). *Scale bar*: 50 μm, for all pictures.

Capacity of nail matrix dendritic cells to associate with T cells via DC-SIGN (CD209) may be impaired Next, we asked whether dendritic cells around nail matrix also show defects in expression of the human dendritic cell-specific adhesion receptor DC-SIGN (CD209), a C-type lectin that is highly expressed on the cell surface of immature dendritic cells in peripheral tissues (Geijtenbeek *et al*, 2000a, b). CD209 plays an important role in establishing the initial contact between dendritic cells and resting T lymphocytes via interaction with ICAM-3 and in dendritic cell trafficking through interaction with endothelial ICAM-2 (Geijtenbeek *et al*, 2000a, b). DC-SIGN is expressed by cells with an immature dendritic cell phenotype (Soilleux and Coleman, 2001), and

the DC-SIGN$^+$ cells are HLA-DR$^{+/low}$ (Soilleux and Coleman, 2001). Moreover, DC-SIGN$^+$ cells appear to be negative for CD1a (Soilleux and Coleman, 2001). Normal skin shows positive immunostaining for CD1a on dendritic cells within a DC-SIGN-negative epidermis (Soilleux and Coleman, 2001).

CD1a$^+$ Langerhans cells did not express CD209 (Fig 1*J*, *M*), as reported before (Soilleux and Coleman, 2001). HLA-DP/DQ/DR$^+$ cells, however, showed strong IR for CD209 in dermis beneath the PNF (Fig 2*R*). Compared with dermis beneath the PNF, in dermis adjacent to dorsal nail matrix, the signal intensity of MHC class II IR on CD209$^+$ cells was substantially weaker (Fig 1*O*). This suggests that, in PNM, the ability of dendritic cells and/or macrophages to activate
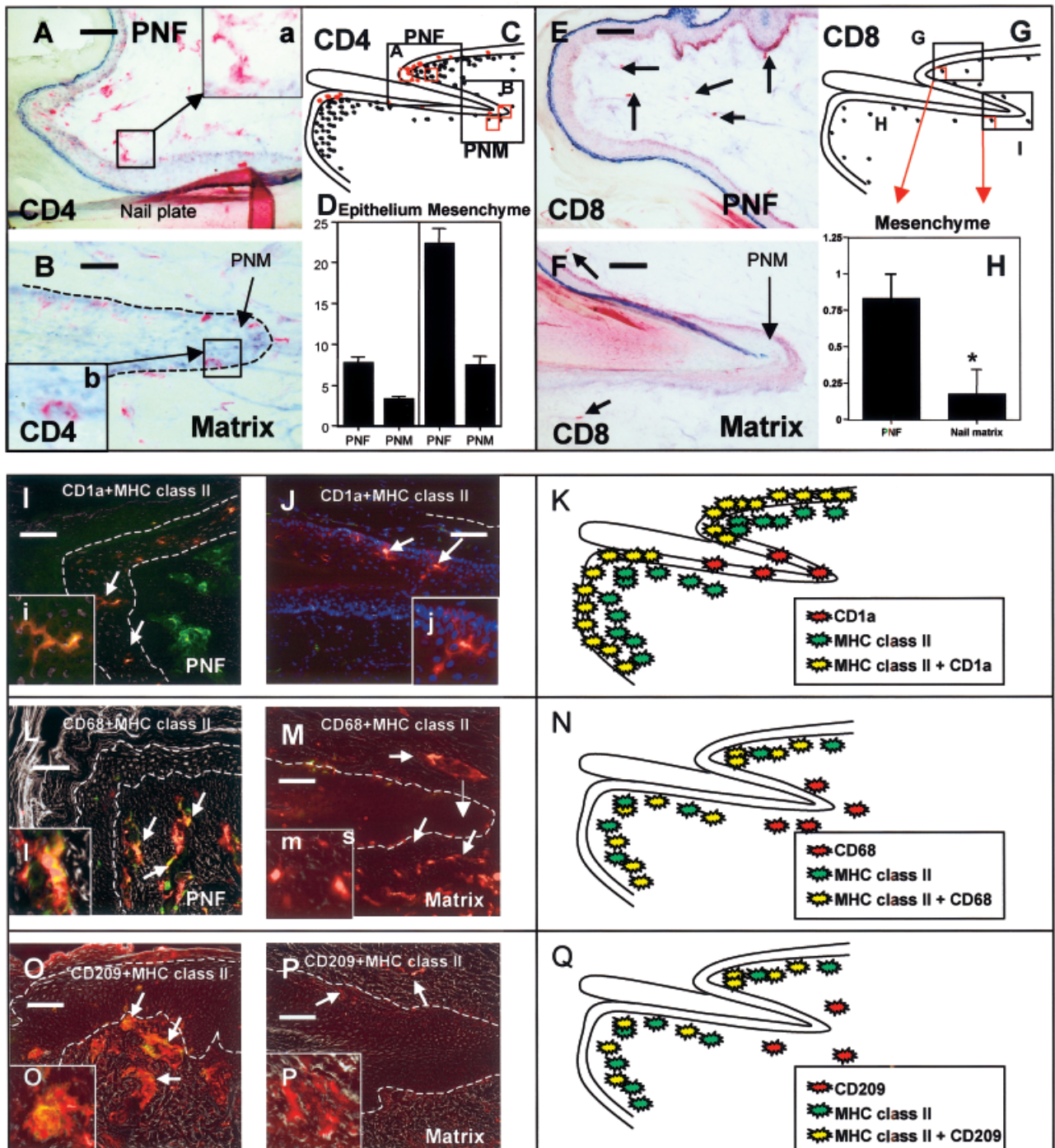
resting T lymphocytes and/or dendritic cell trafficking may be partially impaired by insufficient expression of CD209 (compared with the PNF).

Also, if CD209 expression is indeed a marker of dendritic cell immaturity (Soilleux and Coleman, 2001), it is interesting to note that surprisingly many of such "immature" CD209$^+$/MHC class II$^+$ cells were detected in the nail mesenchyme below the PNF (Fig 1*Q*) and below the nail bed (not shown). These immunostaining results are summarized in Fig 1*Q*.

Natural killer (NK) cells and mast cells are unusually scarce in the vicinity of the nail matrix It is well appreciated that chronic infectious paronychia and periungual human papilloma virus-induced verrucae are difficult to treat and therapy-resistant (de Berker *et al*, 2001; Tosti *et al*, 2001). This may be related to the apparent defect in professional antigen presentation in and around the nail matrix revealed above (Fig 1*J, M*). Alternatively, or in addition to this, NK cells and mast cells, as key cellular mediators of innate immunity (Janeway *et al*, 2001; Goldsby *et al*, 2003; Maurer *et al*, 2003; Bos, 2005), may be reduced in number and/or function in this location.

Using CD56 as a marker for NK cells (Head *et al*, 1985), CD56$^+$ cells were detected at low density in human digital

dermis at some distance to the nail apparatus (data not shown). CD56$^+$ cells, however, were only very rarely detected in the nail mesenchyme immediately adjacent to PNF (Fig 2*A, B, D–F*) (5.67 ± 0.41 cells per MF) or nail bed (data not shown). Interestingly, the number of CD56$^+$ cells further declined significantly in mesenchyme associated with nail matrix (1.18 ± 0.32 cells per MF) (p < 0.01) (Fig 2*C, E, F*). This suggests the human nail matrix mesenchyme, especially around PNM, is only very poorly equipped with NK cells.

By toluidine blue histochemistry, dermal mast cells were found at low numbers in vicinity of nail matrix (2.67 ± 0.58 cells per MF) (Fig 2*G, H*), whereas mast cell density was significantly higher in dermis adjacent to PNF (7.25 ± 0.85 cells per MF) (p < 0.01) (Fig 2*I–L*). This low number of periungual mast cells is in apparent contrast to high density of mast cells found in mesenchyme surrounding the human hair matrix (Christoph *et al*, 2000). Given that mast cells are important cellular elements of innate immunity (Maurer *et al*, 2003), this poor equipment of human nail matrix mesenchyme with fully differentiated (i.e., Toluidine blue$^+$) mast cells may further compromise the innate immune defense capacity of this region of nail mesenchyme. The more distally located, terminally differentiated nail epithelium, in
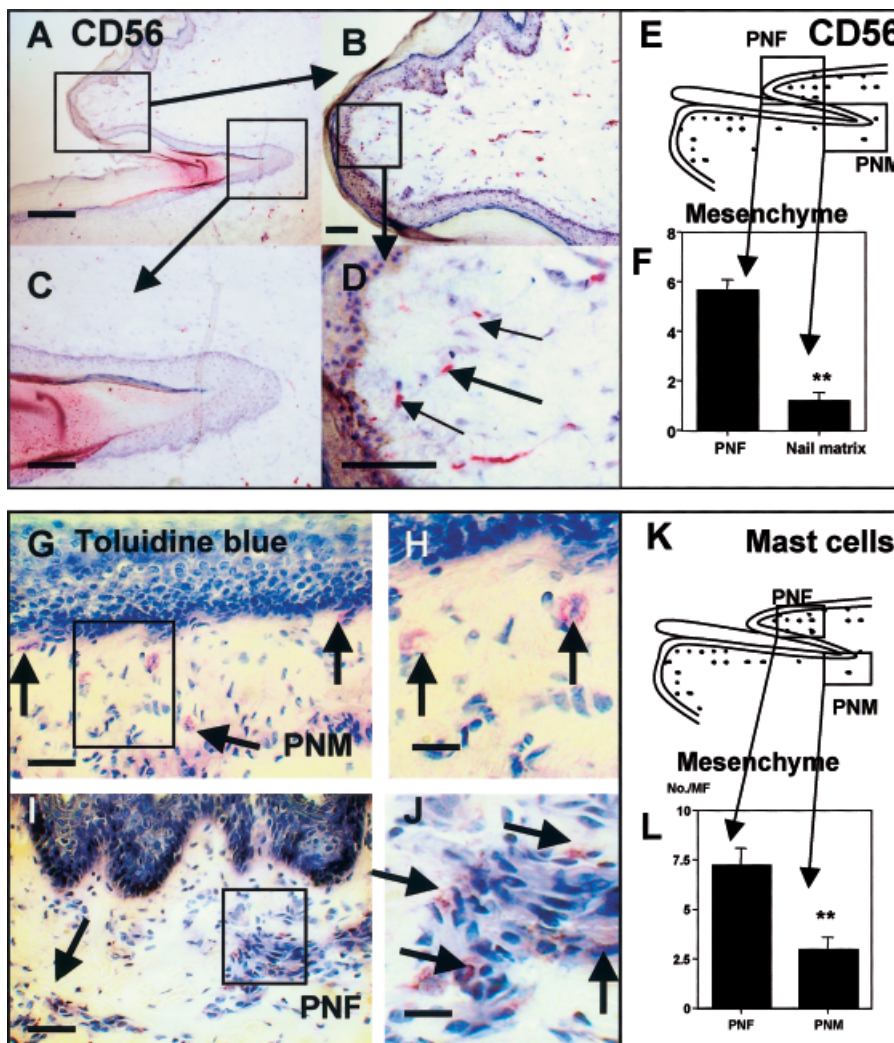

Figure 2
Distribution of NK cells and mast cells in human infantile nail apparatus. (*A*) The number of CD56$^+$ cells is low but significantly higher than that of nail matrix. (*B*) Although the number of CD56 positive cells is extremely low in nail tissue, CD56 positive cells are significantly higher in proximal nail fold (PNF) than around nail matrix. (*C*) CD56$^+$ cells are rarely detected in the vicinity of proximal nail matrix (PNM). (*D*) High power of Fig 3*B* (PNF). (*E*) The schematic drawing of CD56$^+$ cell distribution in nail apparatus. (*F*) Semiquantitative analysis of CD56$^+$ cells in mesenchyme. Higher CD56$^+$cells (5.67 ± 0.41 cells per microscopic field (MF)) are detected in around PNF compared with around nail matrix (1.18 ± 0.32 cells per MF) (p < 0.01). (*G*) Toluidine blue staining shows reddish deposition (mast cells) in mesenchyme just around PNM. Lower number of mast cells is seen in the mesenchyme around the nail matrix compared with that in PNF. (*H*) The high power photograph from Fig 3*G*. (*I*) Higher number of mast cells are observed around PNF compared with mesenchyme just around PNM. (*J*) High power of Fig 3*I*. (*K*) The schematic drawing of mast cell distribution in nail apparatus. (*L*) Semiquantitative analysis of mast cells in mesenchyme. Higher number of mast cells is detected in PNF (7.25 ± 0.85 cells per MF) (p < 0.01) compared with around nail matrix (3.00 ± 0.58 cells per MF). *Scale bars*: 100 μm in (*A*); 50 μm in (*B–D*); 25 μm in (*G* and *I*); 12.5 μm in (*H* and *J*).

Figure 3
The expression of MHC class I and II in human infantile nail apparatus. (*A*) Human leukocyte antigen (HLA)-A/B/C immunoreactivity is prominently downregulated on keratinocytes of proximal nail matrix (PNM). This expression pattern is very similar to that in hair follicle. (*B*) HLA-A/B/C expression on NM. The expression is weaker than on epithelium of PNF. High power photographs from Fig 2*A*. (*C*) HLA-A/B/C is strongly expressed on the epithelium of PNF. High power photographs from Fig 2*A*. (*D*) The schematic drawing of HLA-A/B/C immunoreactivity pattern on nail. (*E* and *F*) β_2 microglobulin is moderately expressed both in nail matrix and PNF. This expression pattern is not paralleled with HLA-A/B/C. (*G* and *H*) Double staining is demonstrated using NHC-A/B/C (red) and NKI/beteb (green) antibodies on PNF and PNM. Major histocompatibility complex (MHC) class I (red) positive melanocytes are seen in PNF (*G*, high power) (yellow). HLA-A/B/C protein expression is prominently down regulated on NKI/beteb (melanocytes) of PNM compared with that of PNF by TSA (*H*) (*H*, high power). (*I*) The schematic drawing of MHC class I immunoreactivity of melanocytes. (*J* and *K* (*V–W*)) Compared with PNF (*J*), HLA-G is strongly expressed in PNM (*K*). (*L* (*X*)) The schematic drawing of HLA-G immunoreactivity on nail. *Scale bars*: 50 μm for all pictures.
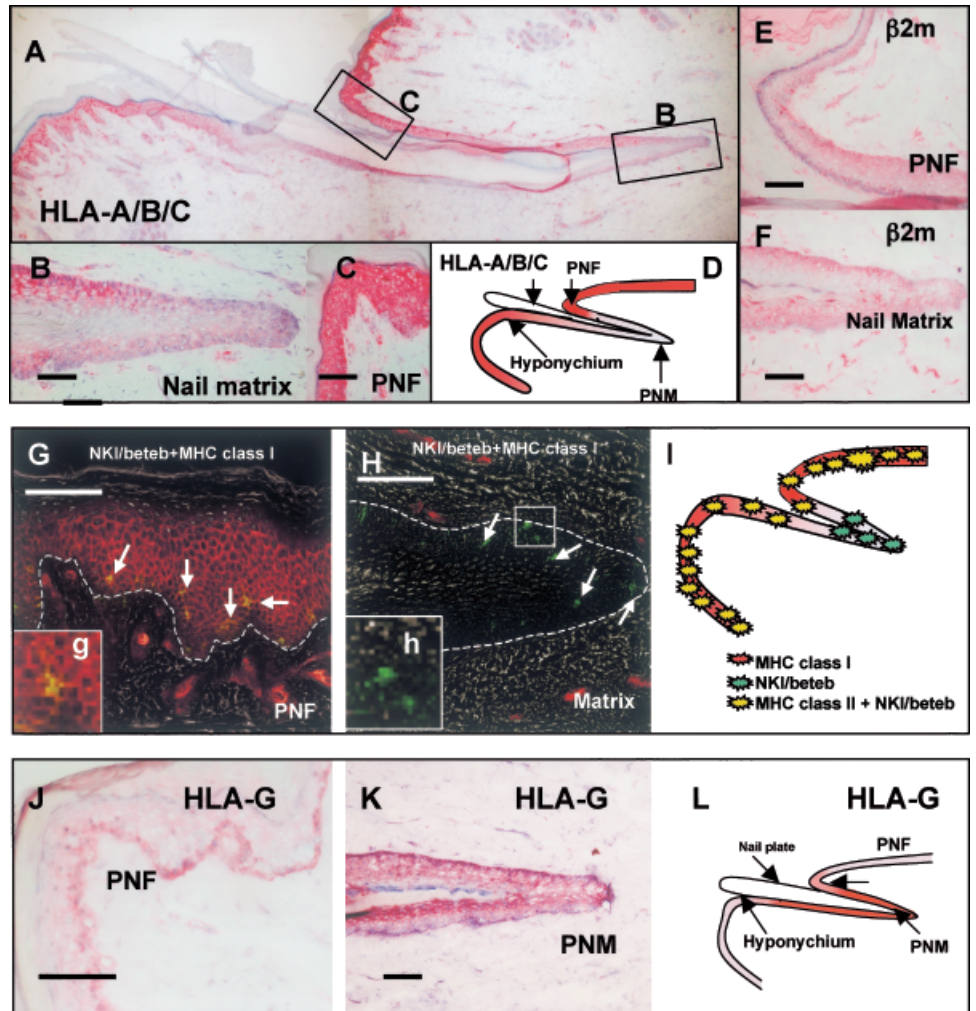
contrast, may show more satisfactory innate immune defenses, e.g., by production of antimicrobial peptides (Dorschner *et al*, 2004).

MHC class I expression is prominently downregulated on PNM keratinocytes HLA-A/B/C is expressed on all nucleated cells except for immunoprivileged tissues (e.g., brain, cornea, anterior chamber of the eye, testis, liver, fetotrophoblast, hamster cheek pouch, and hair matrix) (Head and Billingham, 1985; Streilein, 1993, 2003; Mellor and Munn, 2000; Erlebacher, 2001; Niederkorn, 2002; Paus *et al*, 2003, 2005). As the nail apparatus shares its embryonal and evolutionary origin as well as many structural and functional features with the hair follicle (Chuong and Noveen, 1999; Paus and Peker, 2003; Wu *et al*, 2004), we explored whether defined compartments of the nail apparatus also display a relative down-modulation of MHC class I as a key feature of immunoprivileged tissue sites (Streilein, 1993, 2003; Niederkorn, 2002).

Indeed, the nail matrix epithelium, and most prominently the PNM, displayed downregulated HLA-A/B/C immuno reactivity (Fig 3*A*, *B*, *D*), compared with PNF (Fig 3*A*, *C*, *D*), nail bed, and hyponychium (Fig 3*A*, *D*). This low or absent MHC class I expression in nail matrix epithelium was mirrored in the immediately adjacent nail mesenchyme (Fig 3*A*, *D*). In contrast, the functionally important MHC class I-as-

sociated molecule, β2-microglobulin, which stabilizes MHC class I-antigen interactions (Janeway *et al*, 2001; Bos, 2005), was expressed throughout the human nail apparatus, even though the most PNM appeared to show slightly lower β2-microglobulin immunoreactivity than the rest of the nail epithelium (Fig 3*E*, *F*).

PNM melanocytes are MHC class I negative Normal nail matrix contains melanocytes (Higashi, 1968; Higashi and Saito, 1969; Hashimoto, 1971; Tosti *et al*, 1994). As melanocytes of the human pigmentary unit (Tobin and Paus, 2001) show deficient classical MHC class I expression (Moseley *et al*, 1997, Ito *et al*, 2004), we investigated the HLA-A/B/C expression on melanocytes in nail matrix. Melanocytes in the PNF and nail bed were strongly HLA-A/B/C$^+$ (Fig 3*G*, *H*). In contrast, PNM melanocytes displayed no HLA-A/B/C immunoreactivity (Fig 3*I*). This reflects the situation in the (MHC class I-negative) human hair follicle matrix (Moseley *et al*, 1997; Ito *et al*, 2004) and further supports the notion that the human nail matrix is a site of relative immune privilege.

Comparable with the hair follicle (Paus *et al*, 2005), lack of MHC class I expression on nail melanocytes may prevent attacks by autoreactive CD8$^+$ T cells on melanocyte-associated antigens in PNM. Collapse of nail immune privilege and a concomitant ectopic upregulation of MHC class

I-expression on nail melanocytes, e.g., in patients with alopecia areata may expose melanocytes to immune recognition and cytotoxic autoimmune attack (Paus *et al*, 1994, 2005), thus leading to onychodystrophy/trachyonchia, as it is frequently associated with alopecia areata (Tosti *et al*, 1991).

HLA-G is most prominently expressed in the nail matrix In the fetotrophoblast (Table S1: used as positive control), downregulation of classical MHC class I molecules (MHC class Ia, i.e., HLA-A/B/C) is paralleled by an upregulation of non-classical MHC class I molecules (MHC class Ib), such as HLA-G, which can interact with killer inhibitory receptors (KIR) on NK cells to downregulate NK and T cell cytotoxic functions (Pazmany *et al*, 1996; Rouas-Freiss *et al*, 1997; Carosella *et al*, 1999; Janeway *et al*, 2001). The infantile human nail apparatus indeed expresses MHC class Ib molecules and that, compared with the PNF (Fig 3*J*), HLA-G-like IR is strongly upregulated in PNM (Fig 3*K*, *L*). HLA-G expression plays an important role for establishing fetal immunotolerance (Weetman, 1999; Fuzzi *et al*, 2002) and may rank among the escape strategies of malignant tumors from immunosurveillance via inhibition of NK cell functions (Rouas-Freiss *et al*, 1997; Rjagopalan and Long, 1999; Aractingi *et al*, 2003). Therefore, HLA-G expression in PNM may serve to inhibit an attack of NK cells on this HLA-A/B/C-negative tissue.

NKG2D and major histocompatibility complex class I chain-related gene A (MICA) are expressed in/around the nail matrix HLA-A/B/C molecules are ligands for members of the KIR expressed on NK cells, whose stimulation by MHC class I prevents NK cell-mediated lysis of target cells (Diefenbach *et al*, 2000). NK cells are thought to eliminate cells that have lost MHC class I surface expression because of viral infection or malignant transformation (Ljunggren and Karre 1990; Goldsby *et al*, 2003). Therefore, the loss of MHC class I expression in the PNM (Fig 3*A*, *B*, *D*) makes it vulnerable to attack by NK cells. As has previously been reported, however, in other immunoprivileged tissues (Apte *et al*, 1998; Christoph *et al*, 2000), NK cell activity in and around the normal nail matrix appears to be rather reduced than increased, as judged by the unusually low number of NK cells detected here (Fig 2*C–F*).

Besides the expression of HLA-G, NK cell-activating systems might be downregulated in and around the PNM so as to ward off an NK cell attack. Therefore, we studied the expression of MICA and NKG2D. The MHC-encoded protein MICA binds to NKG2D as its receptor (Bauer *et al*, 1999). MICA is expressed on a subset of intestinal epithelial cells, and is upregulated by many human carcinoma cells (Table S1: breast carcinoma used as positive control) and under conditions of stress (Groh *et al*, 1996, 1999). NK cells, γδT cells, macrophages, and CD8^{+} T cells express NKG2D, and these cells are activated by stimulation with MICA (Bauer *et al*, 1999).

Within the obvious limits of immunohistology, we could not detect a defect in this NK-stimulating system in the nail matrix. On the contrary, whereas the (MHC class I[+]) PNF and epidermis both showed only weak MICA expression

(Fig 4*A*, *B*), the MHC class I-negative nail matrix displayed significantly stronger MICA IR (Fig 4*C*), confirmed by semiquantitative immunohistomorphometry (Fig 4*D*). Furthermore, the MICA receptor, NKG2D, was expressed in the nail matrix (Fig 4*E*, *F*) and on isolated cells in the adjacent mesenchyme (Fig 4*E*), which likely reflected NK cells, CD8^{+} cells, and/or macrophages (Bauer *et al*, 1999).

Therefore, the immunoprivileged human nail matrix still seems to be capable of NK cell activation via the MICA/NKG2D system. Strong MICA expression in the nail matrix may help to protect it against viral infection (e.g., human papilloma virus) and/or malignant epithelial transformation so as to counter-balance the enhanced susceptibility to viral infection that comes along with the generation of an area of immune privilege.

The nail matrix expresses macrophage migration inhibitory factor (MIF) Next, we investigated the local expression of secreted immunosuppressive factors that are classically associated with areas of relative immune privilege (Streilein 1993, 2003; Niederkorn 2003; Paus *et al*, 2005). As in ocular immune privilege, MIF inhibits an attack of NK cells on MHC class Ia-negative corneal cells (Table S1: used as positive control) (Apte *et al*, 1998; Niederkorn, 2002, 2003), we also checked MIF expression in the human nail apparatus.

Here, we show that the entire human nail epithelium expresses MIF-like IR. Compared with the PNF and the epidermis (Fig 4*I*, *J*), however, MIF-IR Is upregulated in the nail matrix (Fig 4*K*), as confirmed by semiquantitative immunohistomorphometry (Fig 4*L*). MIF may inhibit NK cell-mediated cytotoxicity in PNM by preventing the release of cytolytic perforin granules from NK cells (Fig 4*K*) (Apte *et al*, 1998). Thus, comparable with the anterior eye chamber, MIF expression (in addition to HLA-G expression) may protect the nail matrix from NK cell-mediated injury.

PNM expresses potent immunosuppressants Next, we investigated whether other potent immunosuppressive agents that are recognized as key mediators of relative immune privilege (Taylor, 1999; Niederkorn, 2003; Paus *et al*, 2003, 2004; Ito *et al*, 2004) are expressed locally in the MHC class I-negative human nail matrix.

Prominent IR for insulin-like growth factor-1 (IGF-1) (Fig 4*M*), α-melanocyte stimulating hormone (α-MSH) (Fig 4*N*), adrenocorticotropic hormone (ACTH) (Fig 4*O*), transforming growth factor-β1 (TGF-β1) (Fig 4*P*) was detected by tyramide signal amplification (TSA) immunofluorescence, most prominently in the PNM compared with the epithelium beside PNF, which show slight IR of these immunosuppressive molecules (data not shown). Thus, as in ocular and/or hair follicle immune privilege (Paus *et al*, 1997, 2003, 2005; Taylor, 1999; Niederkorn, 2003; Ito *et al*, 2004), TGF-β1, ACTH, α-MSH, and IGF-1 may join ranks in the PNM of the human nail apparatus with MIF (Apte, 1998) in order to serve as "guardians" of nail immune privilege.

Nail epithelium does not express ICAM-1 Finally, we explored the constitutive level of ICAM-1 (CD54) expression in the human nail apparatus, as ICAM-1 is a key cell adhesion molecule involved in the upregulation of a wide range of

Figure 4
The immunoreactivity of NK cell–associated molecules and immunosuppressants in human infantile nail apparatus.
(*A–C*) The epidermis (*A*) and epithelium of proximal nail fold (PNF) (*B*) show the faint expression of major histocompatibility complex class I chain-related gene A (MICA). On the other hand, the major histocompatibility complex (MHC) class I-negative nail matrix displayed significantly stronger MICA immunoreactivity. (*D*) The schematic drawing of MICA expression in nail apparatus. Semiquantitative analysis of MICA in nail matrix shows significant higher mean intensity of MICA expression compared with epithelium of PNF. Mean intensity is measured by NIH image software. (*E*) NKG2D positive cells are infiltrated around the PNM (*F*, High power of Fig 4*E*). (*G*) intracellular adhesion molecule 1 (ICAM-1) positive cells are extremely rare in the vicinity of PNM. (*H*) ICAM-1 positive cells are easily detected in vascular endothelial cells in the vicinity of epidermis. (*I–K*) Compared with epidermis (*I*) and PNF (*J*), nail matrix substantially expressed macrophage migration inhibitory factor (MIF) (*K*). (*L*) The schematic drawing of MIF expression in nail apparatus. Semiquantitative analysis of MIF in nail matrix shows significant higher expression of MIF compared with epithelium of PNF. (*M–P*) Immunoreactivity of immunosuppressant (IGF-1, α-MSH, ACTH, and TGF-β1) is strongly detected in the PNM. These immunoreactivities are substantially stronger than in epithelium of PNF (data not shown). *Scale bars*: 50 μm for all pictures. IGF-1, insulin-like growth factor-1; α-MSH, α-melanocyte stimulating hormone; ACTH, adrenocorticotropic hormone; TGF-β1, transforming growth factor.
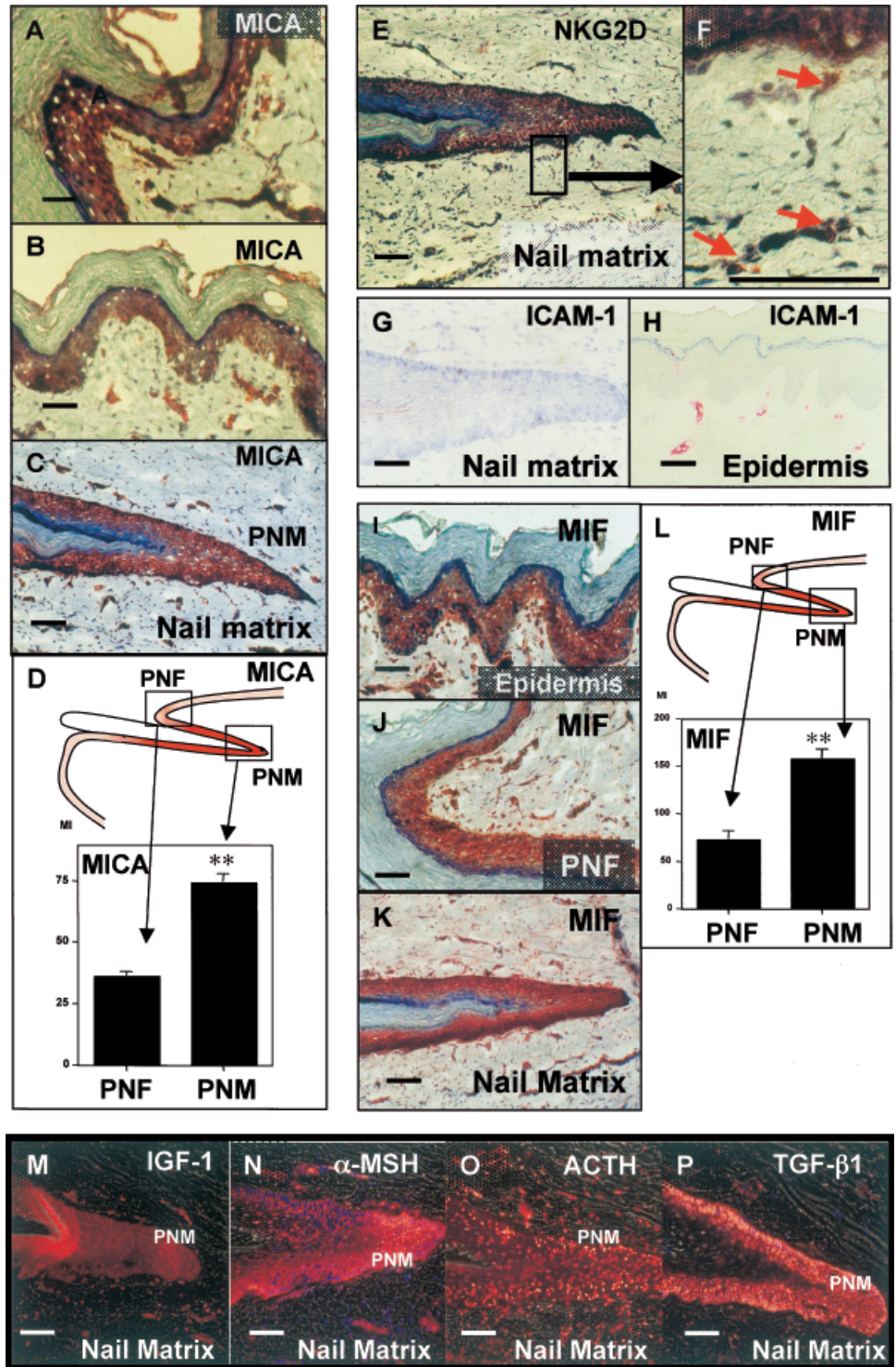
pro-inflammatory immune responses (Cameli *et al*, 1994; Janeway *et al*, 2001; Goldsby *et al*, 2003; Bos, 2005). In addition, human nail matrix cells reportedly express a low level of ICAM-1 *in vitro* (Picardo *et al*, 1994).

But, *in situ*, the infantile human nail apparatus does not show ICAM-1 IR in any epithelial compartment (Fig 4*G*, *H*), and only the expected constitutive ICAM-1 expression on blood vessels was detected in the periungual mesenchyme (Fig 4*H*). Therefore, the reported upregulation of ICAM-1 on cultured human nail matrix keratinocytes (Picardo *et al*, 1994) likely represents an experimental artifact, e.g., as

reflection of a wounding response of these keratinocytes during tissue dissection, cell dissociation, and culture.

Conclusions and Perspectives

Here, we show that the distribution and functional markers of key protagonists of acquired and innate cutaneous immunology differ between the human skin immune system (SIS) (Bos, 2005) and the nail immune system (NIS) (Table S2). Namely, number and function of antigen-presenting

cells are substantially lower in the NIS than in the epidermis (Fig 1*I–N*), with a striking downregulation of MHC class II and CD209 expression by Langerhans cells in the PNM (Fig 1*O, P*), Also, key protagonists of innate immunity (NK and mast cells) are found at reduced numbers and/or function around the human nail apparatus (Fig 2). Furthermore, whereas MIF and MICA expression has not been described in normal human epidermis (Fig 4*A–D, I–L*), these important regulators of NK cell function are prominently expressed in the PNM (Fig 4*C, K*). Most importantly, our data suggest that the human nail matrix represents a site of relative immune privilege, not only because of the downregulation of MHC class I expression, and the low number of (functionally impaired) dendritic cells, but also because of the prominent expression of potent immunosuppressants (ACTH, α-MSH, IGF-1, TGF-β1, MIF) (Fig 4*M–P*).

The normal NIS of humans infants shows intriguing similarities to (evolutionarily younger) human HIS (Paus *et al*, 1997, 2003, 2005; Christoph *et al*, 2000; Ito *et al*, 2004)—namely that HIS and NIS both exhibit defined compartments that represent as the sites of relative immune privilege (Table S2). For this immune tolerance, immune privilege is reasonable for infant nail matrix same as adult. If PNM is significantly attacked, the nail structure will not be complete, and nail is vulnerable from many kinds of microorganism. Our study, however, also reveals important immunological differences between the nail apparatus and the pilosebaceous unit in man (Table S2):

- β2 microglobulin is not detected in the proximal epithelium of human hair follicle (Ito *et al*, 2004), but is positive in the PNM (Fig 3*E, F*).
- In contrast to the strongly MHC class I$^+$ and β2 microglobulin + mesenchyme of the human hair follicle (Christoph *et al*, 2000; Ito *et al*, 2004), the nail immune privilege appears to extend to the periungual mesenchyme, especially around the PNM, as the latter is also MHC class I-negative and shows a greatly reduced number of T, Langerhans, and NK cells (Fig 3*A, B*, Table S2).
- HLA-G is strongly expressed in the nail matrix (Fig 3*K, X*), but has not been reported in human hair matrix and was also not found by us in sections of normal human scalp skin (Ito *et al*, unpublished observations).

The next challenge is to obtain *functional* evidence that defined compartments of the nail apparatus really are immunoprivileged, and to understand which potential benefits may have arisen from the peculiar arrangement of the NIS. For example, the well-developed immune system of the more distally located components of the NIS (along with the expression of antimicrobial peptides here (Dorschner *et al*, 2004)) offers reasonable anti-infection defenses. Hyponychium and PNF are—often forgotten, but clinically highly relevant—ports of entry for microbial invasion into the mammalian organism, perhaps best comparable with the follicular canal (Paus *et al*, 1999; Christoph *et al*, 2000; Paus and Peker, 2003). Therefore, it makes perfect sense that many more CD1a, CD68$^+$, or CD4$^+$ cells are stationed in and around PNF and hyponychium than in the PNM (Fig 1*A–N*).

On the other hand, chronic and acute inflammatory nail disorders because of infection or irritation (maceration, chemical damage) affect primarily the PNF not the PNM (de Berker *et al*, 2001; Rockwell 2001; Mallet, 2002). Therefore, it is conceivable that the relative immune privilege of the PNM may serve to suppress inflammatory/autoimmune damage to the most critical component of the actual "nail factory" to promote the survival chances of a species by protecting it from a loss of claws, hooves, or nails because of pro-inflammatory environmental insults (maceration, trauma, chemical irritation, bacterial and fungal infection), and to quickly restore the vital use of these skin appendages by limiting swelling and pain after nail trauma or infection.

Materials and Methods

Tissue specimens Human infantile nail specimens were obtained from three different healthy male infants with polydactyly (3 mo) with informed consent from their parents during elective surgery. Before staining, specimens were embedded and processed for longitudinal cryosections. Cryosections (8 μm thick) were fixed with acetone at −20°C for immunohistochemistry, and stored at −20°C until use.

Immunohistology Among the primary antibodies listed in Table S1, antigen-antibody complexes were visualized with the highly sensitive TSA technique (Perkin Elmer, Boston, Massachusetts) (Roth *et al*, 1999) in the indicated cases, following our previously published TSA protocol (Ito *et al*, 2004). MICA, NKG2D, and MIF antigens were stained by the ABC method, using established protocols (Christoph *et al*, 2000). CD4, CD8, CD54, CD56, and β2-microglobulin were localized by the EnVision technique (DAKO, Hamburg, Germany) (Ito *et al*, 2004) using the anti-human primary antibodies indicated in Table S1 because this technique gave better immunostaining results for these antigens than the TSA technique.

Cryosections stained with antibody against human CD1a, CD68, or CD209 by the TSA technique were double stained with fluorescein isothiocyanate-conjugated antibody against HLA-DP/DQ/DR (Table S1). Cryosections TSA-stained with antibody against human NKI/beteb were double stained with rhodamine-conjugated anti-HLA-A/B/C (Table S1).

All the appropriate positive (Table S1) and negative controls for each of the used antigens were performed, and the immunostaining results confirmed the specificity and sensitivity of the corresponding immunoreactivity patterns.

Toluidine blue staining In order to localize mast cell in human nail tissue, toluidine blue staining was applied. Acetone-fixed cryosections were stained with 1% toluidine blue (MERCK, Darmstadt, Germany) (pH 8.9) for 1 min at room temperature. Mast cells were detected as red-purple granules (Haas *et al*, 1995).

Quantitative (immuno-)histomorphometry Positively stained cells were counted by quantitative (immuno-)histomorphometry in a blinded fashion following our previously described morphometry techniques (Paus *et al*, 1998; Christoph *et al*, 2000; Ito *et al*, 2004). The nail was divided into the nail matrix and the PNF anatomically as shown, and CD4$^+$, CD8$^+$, CD54$^+$, CD56$^+$, and mast cells (toluidine blue staining) in these compartments were counted in three MF (magnification × 250) from one nail (n = 3 individuals) (Student's *t*-test). The staining intensity of MICA and MIF was compared between matrix and PNF using NIH image software (NIH, Bethesda, Maryland) for semiquantitative analysis (Ito *et al*, 2004).

Statement The study was conducted according to the Declaration of Helsinki Principles. Participant's parents gave their written

informed consent. The medical ethical committee of the University Hospital Hamburg-Eppendorf approved all described studies.

Supplementary Material

The following material is available online for this article.
Table S1. The list of antibodies used for immunohistochemical staining
Table S2. Comparison of distribution and immunoreactivity between SIS, HIS, and NIS

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