Maintenance of Hair Follicle Immune Privilege Is Linked to Prevention of NK Cell Attack

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Hair follicles (HFs) enjoy a relative immune privilege (IP) that is characterized by downregulation of major histocompatibility complex (MHC) class I and local expression of potent immunosuppressants. Normally, natural killer (NK) cells attack cells with absent/low MHC class I expression. However, because few perifollicular NK cells are found around healthy human anagen HFs, we asked how HFs escape from NK cell attack. This study suggests that this happens via an active NK cell suppression. Alopecia areata (AA), an organ-specific autoimmune disease thought to result from a collapse of HF-IP, in contrast, shows striking defects in NK cell inhibition/containment. We show that the NK cell inhibitor macrophage migration inhibitory factor is strongly expressed by the HF epithelium, and very few $CD56^+/NKG2D^+$ NK cells are observed in and around normal anagen HFs compared to AA with prominent aggregations of $CD56^+/NKG2D^+$ NK around AA-HFs. By flow cytometry, many fewer NK function-activating receptors (NKG2D, NKG2C) and significantly more killer cell Ig-like receptors-2D2/2D3 were found to be expressed on peripheral blood $CD56^+$ NK cells of healthy controls than on those of AA patients. In addition, only weak immunoreactivity for MHC class I chain-related A gene was observed in normal anagen HFs compared to AA. To our knowledge, this defect is previously unreported and must be taken into account in AA pathogenesis and its management.

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INTRODUCTION

Immunoprivileged sites are observed in a few, well-defined tissue compartments in the mammalian body. These sites include the anterior chamber of the eye, the testis, the central nervous system behind the blood–brain barrier, and the hamster cheek pouch (Head and Billingham, 1985; Niederkorn, 2002; Paus *et al.*, 2003; Mellor and Munn, 2006; Simpson, 2006). Immunoprivileged tissues need to suppress a cytotoxic immune attack on cells and antigens harbored inside these sites. This is achieved by a range of different mechanisms, among which the downregulation of major histocompatibility complex (MHC) class I antigens and the local expression of potent immunosuppressants are chief strategies (Fijak and Meinhardt, 2006; Marie *et al.*, 2006; Niederkorn, 2006; Simpson, 2006; Wahl *et al.*, 2006; Yu *et al.*, 2006).

Hair follicles (HFs) in the growth stage of the hair cycle (anagen) also are a recognized site of immune privilege (IP) (Billingham and Silvers, 1971; Westgate *et al.*, 1991; Paus *et al.*, 1994, 1998; Rückert *et al.*, 1998), and collapse of this IP is thought to initiate the loss of hair as seen in patients with the autoimmune disease alopecia areata (AA) (Paus *et al.*, 2003, 2005; Gilhar and Kalish, 2006). Like its classical counterparts, HF-IP is characterized by downregulation of MHC class I expression, dysfunction of Langerhans cells, and strong local expression of immunosuppressants (for example, α-MSH, TGF-β) (Harrist *et al.*, 1983; Paus *et al.*, 2003, 2005; Marie *et al.*, 2006; Yu *et al.*, 2006) and may serve mainly to sequester anagen- and/or melanogenesis-associated autoantigens from immune recognition by autoreactive $CD8^+$ T cells (Paus *et al.*, 2003, 2005; Gilhar and Kalish, 2006).

The absence or low expression of MHC class I expression in immunologically privileged HF compartments constitutes a basic problem in self/nonself discrimination and self-tolerance (Boehm, 2006), since, normally, natural killer (NK) cells are primed to recognize and eliminate such cells (Janeway, 2005; Andoniou *et al.*, 2006; Borrego *et al.*, 2006; Bryceson *et al.*, 2006; Gasser and Raulet, 2006; Johansson and Hoglund, 2006; Khakoo and Carrington, 2006; Yokoyama and Kim, 2006; Vivier, 2006). The fact that very few perifollicular NK cells are found around healthy human

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Abbreviations: AA, alopecia areata; CCD, charge coupled device; DP, dermal papilla; HF, hair follicle; IP, immune privilege; IR, immunoreactivity; KIR, killer cell Ig-like receptor; MHC, major histocompatibility complex; MICA, MHC class I chain-related A gene; MIF, macrophage migration inhibitory factor; NHS, normal human scalp skin; NK, natural killer; ORS, outer root sheath; PBMC, peripheral blood mononuclear cells

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anagen HFs (Christoph *et al.*, 2000) suggests that HFs indeed inhibit or contain NK functions within tightly controlled limits of activity. Therefore, we studied such mechanisms on NK cells and IP that have until now been completely unknown in the context of HF-IP.

NK cells express inhibitory receptors, such as killer cell Ig-like receptors (KIRs) and heterodimer CD94/NKG2A (Long, 1999; Janeway, 2005), and if target cells express MHC class I molecules, NK cell activation can be prevented through interaction with KIRs by phosphorylation of the immunoreceptor tyrosine inhibitory motif followed by binding to phosphatases, including SHP-1 and SHP-2 (Src homology region 2-containing protein tyrosine phosphatase) (Long, 1999; Vivier *et al.*, 2002). On the other hand, target cells lacking MHC class I expression (for example, cells that suffer viral infection or significant transformation) do not inhibit NK cells triggered by a signal from an activating receptor, such as NKG2D and CD94/NKG2C (Bauer *et al.*, 1999; Wu *et al.*, 1999; Janeway, 2005).

NKG2D is expressed not only in NK cells but also in $CD8^+$ T cells and recognizes the MHC class I chain-related A (MICA) molecule on target cells, which stimulates these immune cells to attack the target cells (Bauer *et al.*, 1999; Carosella *et al.*, 1999; Wu *et al.*, 1999; Menier *et al.*, 2002; Middleton *et al.*, 2002; Pende *et al.*, 2002). MICA is a heat-shock- or oxidative-stress-induced antigen (Groh *et al.*, 1996; Yamamoto *et al.*, 2001) belonging to the MIC gene family. MICs have been shown to be broadly expressed on human tumors of epithelial origin and intestinal epithelial cells (Bahram, 2000).

CD94/NKG2A heterodimers, Ig-like transcript-2 receptors, and KIRs are the three major types of MHC class I-specific inhibitory or activating receptors expressed on NK cells (Borrego *et al.*, 2005). KIRs belong to the Ig superfamily and have two or three Ig domains (denoted 2D or 3D) (Trowsdale *et al.*, 2001). They can be further subdivided into inhibitory and stimulatory receptors. The inhibitory forms are longer (L) and have intracellular immunoreceptor tyrosine inhibitory motifs in the cytoplasmic domain (Vivier and Daeron, 1997; Vivier, 2006) (KIR-3DL2, KIR-3DL1, KIR-2DL1–3; Colonna *et al.*, 1993; Moretta *et al.*, 1993; Gumperz *et al.*, 1995; Döhring *et al.*, 1996; Pende *et al.*, 1996). The stimulatory receptors (KIR-2DS1 through KIR-2DS5 and KIR-3DS1) lack immunoreceptor tyrosine inhibitory motifs (S: short) but have the charged residue in the membrane comparable to the noninhibitory forms of CD94/NKG2 (Griffiths, 2006). The antibody used in this study recognizes the inhibitory KIRs, KIR-2DL2/2DL3.

In addition to NK cell receptors and their ligands, other secreted factors should be considered. In the anterior chamber of the eye, macrophage migration inhibitory factor (MIF) suppresses NK cell activity and contributes to IP (Apte *et al.*, 1998). MIF is a pleiotropic cytokine that is also present in the aqueous humor and potently inhibits NK cell-mediated cytolysis *in vitro* (Gilhar and Kalish, 2006). However, any contribution of MIF to HF-IP and the connection between NK cell activity and HF-IP remain to be investigated.

Previous research on AA pathogenesis has focused on the role of $CD8^+$ T cells in IP collapse, likely with help received from $CD4^+$ T cells and an unclear contribution of dendritic cells (Gilhar and Kalish, 2006), but has largely ignored the possible contribution of NK cells to the pathogenesis of AA. To study this has become even more important, since the NK cell activating receptor NKG2D has recently been suspected as being involved in the pathogenesis of other prototypic autoimmune diseases, such as rheumatoid arthritis and autoimmune type I diabetes (Groh *et al.*, 2003; Jie and Sarvetnick, 2004; Caillat-Zucman, 2006), and since bidirectional interactions between NK cells and autoreactive T cells have become a recent focus of interest in autoimmunity research (Linsen *et al.*, 2005; Liu *et al.*, 2006; Shi and Van Kaer, 2006).

One would expect that MHC class I-negative or MHC class I ''low'' anagen HFs are under constant attack by NK cells, which is clearly not the case (Christoph *et al.*, 2000). This begs the question of how IP-anagen HFs manage to escape NK attack and whether a defect in these mechanisms may be linked to HF autoimmunity (Paus *et al.*, 2005; Gilhar and Kalish, 2006).

To answer this question, we investigated the mean intensity (MI) of NKG2A, C, and D on peripheral blood NK or $CD8^+$ T cells and MIF expression from healthy controls, patients with atopic dermatitis, and AA patients.

This report provides new data, which allow one to synthesize a rational scenario for how normal anagen HFs may escape an NK cell attack and for what may go wrong in this respect when patients develop AA.

RESULTS

$CD56^+$, $CD4^+$, and $CD8^+$ cells are rarely seen in and around normal hair follicles, while the opposite is true for AA hair follicles

By routine histology, lymphocytes are only infrequently seen around the normal human anagen HFs obtained from healthy controls. As previously reported, using different immunostaining protocols and analyzing more individual patients than before (Christoph *et al.*, 2000), $CD56^+$ cells (NK cells), $CD4^+$ T cells, and $CD8^+$ T cells were rarely seen around scalp anagen HFs in healthy controls (Figure 1a and b). In particular, $CD56^+$ cells were seen very rarely (Figure 1c). In contrast, $CD4^+$ or $CD8^+$ T cells were clustered at a high density around the anagen hair bulb in AA lesions (Figure 1d and e). In addition, many perifollicular $CD56^+$ cells were observed in AA lesions. However, the total number of these cells was much lower than that of perifollicular $CD4^+$ or $CD8^+$ cells (Figure 1f).

As assessed by quantitative immunohistochemistry, in all examined skin samples, the number of perifollicular $CD4^+$, $CD8^+$, or $CD56^+$ cell infiltrates was highly significantly increased in lesional AA skin compared to both anagen and catagen HFs in healthy control skin (Figure 1g–i). There were no significant differences in $CD4^+$, $CD8^+$, or $CD56^+$ cell numbers between normal anagen and normal catagen HFs (Figure 1g–i). This shows that the increase in the number of $CD56^+$, $CD4^+$, or $CD8^+$ cells was not just a reflection of hair cycle-dependent changes in skin immune parameters (Paus *et al.*, 1998, 1999), but likely represented an AA-associated phenomenon.

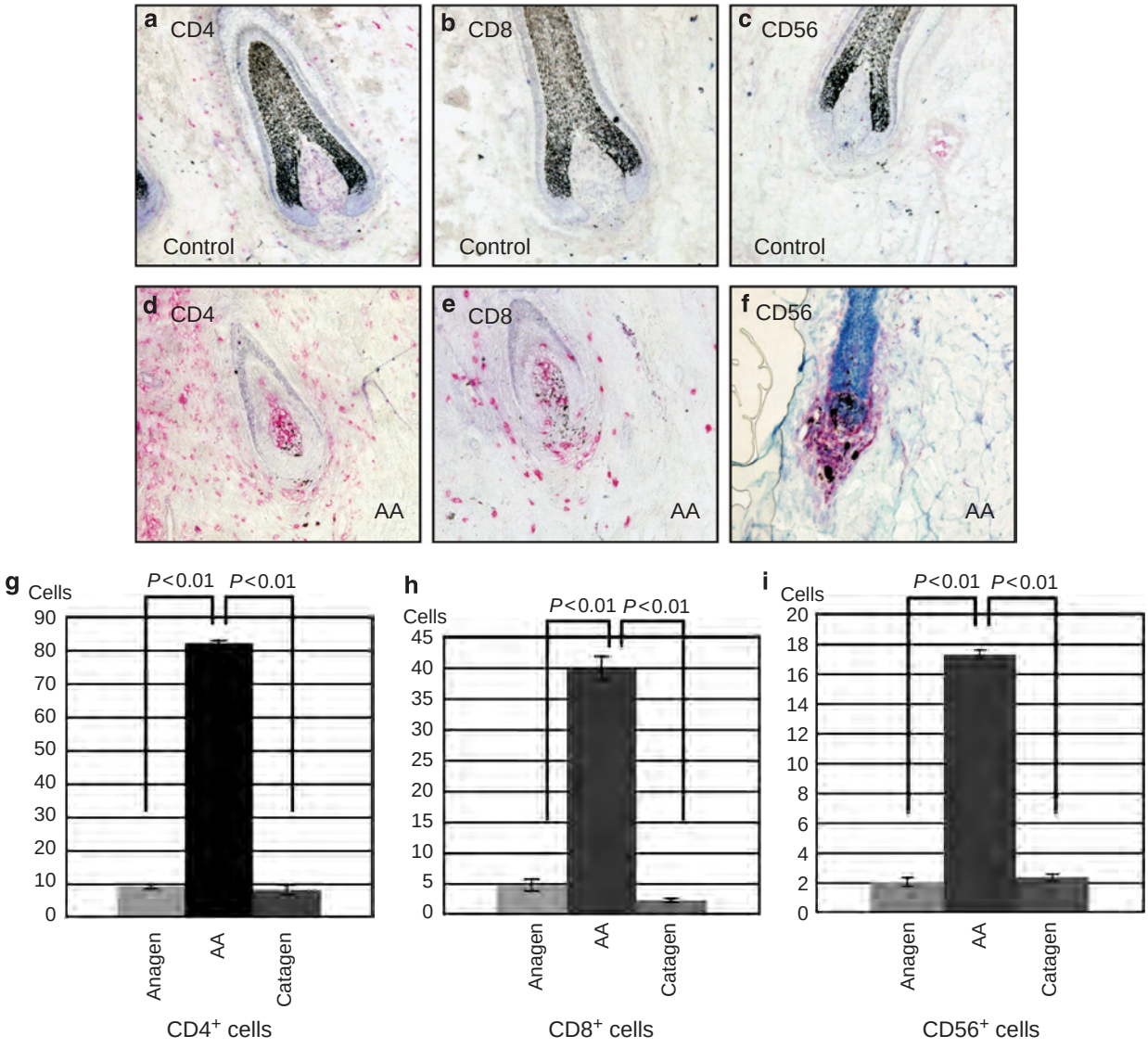

Figure 1. Perifollicular NK cells and T lymphocytes are scarce in normal skin, but accumulate around HFs in lesional skin of AA patients. In healthy controls, $CD8^+$ T cells, $CD4^+$ T cells, and $CD56^+$ cells (NK cells) are rarely seen around anagen HFs (**a**–**c**, respectively; APAAP immunohistology). (**d**–**f**) However, these cells prominently infiltrate in and around HFs in AA lesions. (**i**) The number of infiltrating $CD56^+$ cells in and around AA lesions (17.3 ± 1.03) is significantly higher than around normal anagen (2 ± 0.3) or catagen (2.3 ± 0.19) HFs ($P<0.01$). The same is true for (**g**) $CD4^+$ or (**h**) $CD8^+$ T cells.

In AA, NKG2D$^+$ cells infiltrate HFs that express strongly increased MICA immunoreactivity

In this study, we found that normal human skin and its appendages show essentially no specific MICA immunoreactivity (IR), with the notable exception of some MICA IR in the anagen hair matrix of healthy control HFs (Figure 2a). In contrast, lesional AA skin was found to display massive and widespread MICA IR, with a maximum seen in the proximal outer root sheath (ORS), the dermal papilla (DP), and the connective tissue sheath of AA HFs (Figure 2b). Analysis of the mean IR intensity (MI) revealed that the ORS MI for MICA was significantly lower in the ORS of normal human scalp HFs (27.82 ± 3.33) than in lesional AA HFs (115.0 ± 2.2), while the observed difference in MICA MI failed to reach the level of significance in the hair matrix (normal human scalp skin (NHS): 71.4 ± 8.28, AA: 87.6 ± 0.38) (Figure 2c).

$NKG2D^+$ NK cells and $CD8^+$ T cells also were found to aggregate in and around (atrophic) HFs in AA lesions (Figure 2d and e). These results suggest that upregulation of the NKG2D ligand MICA on AA greatly enhances the susceptibility of these HFs to an attack by $NKG2D^+$ cells, which may then promote anagen termination and AA progression. They also suggest that at least the proximal hair matrix of normal scalp HFs has a constitutively higher risk of activating the NKG2D NK cell receptor via MICA expression.

MIF is strongly expressed by anagen scalp HFs and downregulated in AA-HFs

Since MIF is considered to play an important IP maintenance role in the anterior eye chamber—for example, by suppression of NK cell activity (Apte *et al.*, 1998; Taylor, 2003; Caspi, 2006)—we next studied MIF expression in anagen HFs

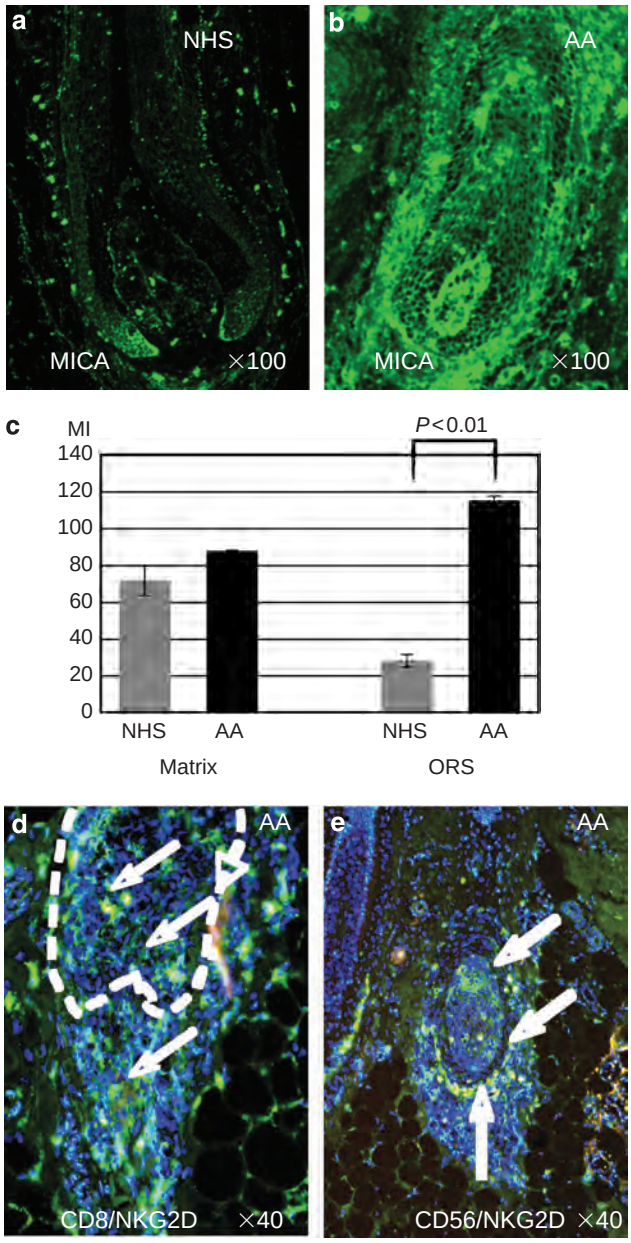

Figure 2. AA-HFs prominently and aberrantly express the NK cell activator MICA. (**a**) MICA IR was found to be largely absent in healthy control skin, with the exception of some MICA IR in the hair matrix of some normal anagen HFs. (**b**) Instead, MICA IR was massive throughout lesional skin HF from AA patients and was seen prominently in the inner root sheath, ORS, and connective tissue sheath. The MI of MICA IR was analyzed using ImageJ software. (**c**) The MI in the ORS was significantly lower in normal human scalp skin (27.82 ± 3.33) than in lesional AA skin (115.0 ± 2.2) ($P<0.01$), while there was no significant difference in hair matrix (control: 71.4 ± 8.28, AA: 87.6 ± 0.38). (**d** and **e**) NKG2D$^+$ NK cells and CD8$^+$ lymphocytes, which recognize MICA, prominently accumulate around HFs in AA (note the perifollicular aggregation of NKG2D$^+$/CD56$^+$ and NKG2D$^+$/CD8$^+$ cells around lesional AA-HFs).

by immunohistology. This revealed strong, widespread MIF IR throughout most of the epithelium of normal anagen scalp HFs, particularly in the area of the proximal inner root sheath and ORS (Figure 3a and b). Instead, the epithelium of lesional

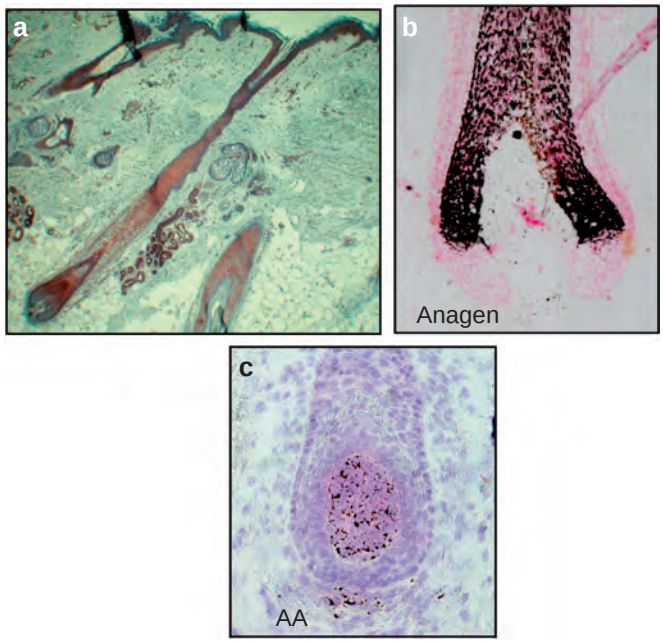

Figure 3. MIF is strongly expressed on the proximal inner root sheath and matrix cells of anagen HFs. HF cells are compared with epidermal cells in healthy control skin (**a** by avidin–biotin complex and **b** by EnVision). (**a**) Avidin–biotin complex immunohistochemistry revealed strong IR of MIF in the proximal inner root sheath and matrix keratinocytes of anagen HFs in healthy control skin. (**b**) In contrast, MIR IR was greatly reduced in lesional AA HFs (not shown) and even a considerably more sensitive immunostaining technique (EnVision) revealed only weak MICA-associated IR in lesional AA-HFs (**c**).

skin AA HFs displayed greatly reduced or absent MIF IR (Figure 3c). This suggests that HFs in established AA exhibit a decreased capacity for suppressing undesired NK cell functions.

NKG2D expression on NK and CD8$^+$ T cells is abnormally high in AA

Next, we compared NKG2D expression on CD56$^+$ NK cells and CD8$^+$ T cells in peripheral blood mononuclear cells (PBMCs) from AA patients and healthy controls. Flow cytometry analysis revealed that the MI of NKG2D expression on CD56$^+$ NK (213.5 ± 23.63) (Figure 4a and b) and CD8$^+$ cells (183.5 ± 9.15) (Figure 4c and d) was significantly higher in AA patients than in normal controls (90.06 ± 19.06 on NK cells, 97.50 ± 9.72 on CD8$^+$ T cells) (*$P<0.05$, **$P<0.01$) (Figure 4a–c). Instead, no significant differences from healthy controls in NKG2D MI were seen in atopic dermatitis patients (90.92 ± 30.13 on NK cells, 69.61 ± 9.15 on CD8$^+$ T cells) (Figure 4a–c). Therefore, this abnormally high expression of NKG2D is unlikely to represent a general phenomenon of chronic skin inflammation.

IFN-γ strongly upregulates NKG2D expression on CD56$^+$ NK cells

The NK cell is a major source of IFN-γ, which plays an important role in host defense mechanisms against tumors and viruses. IFN-γ also activates NK cells (Novelli *et al.*,

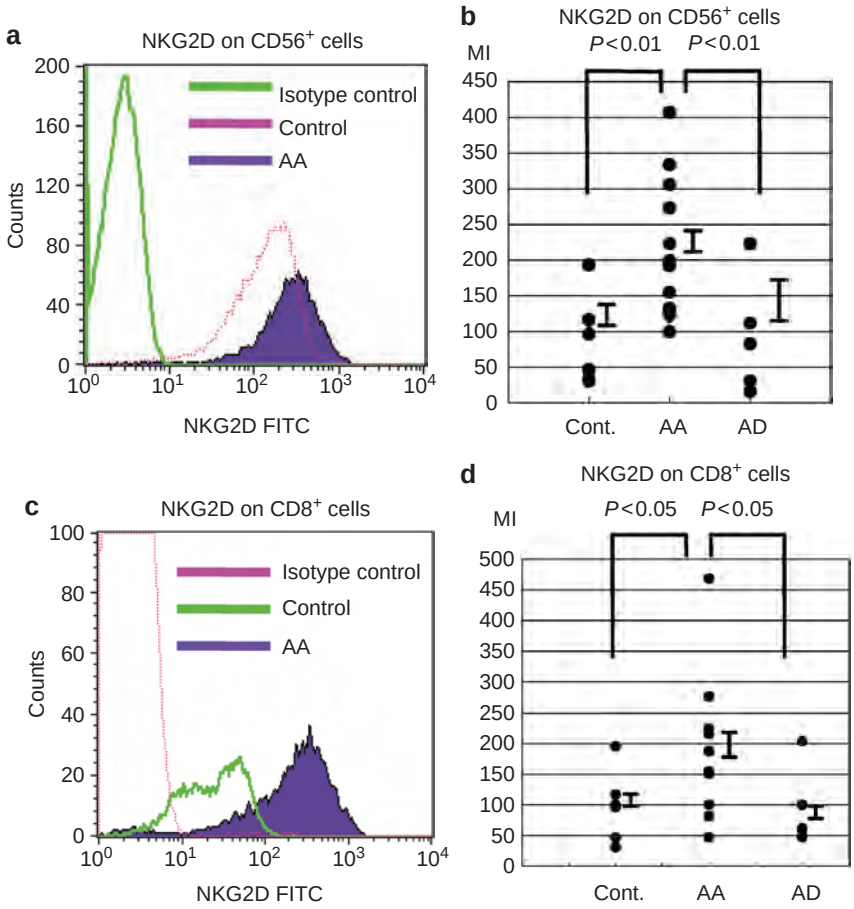

Figure 4. In AA patients, the NK cell-activating receptor NKG2D is significantly upregulated on CD56$^+$ NK cells and CD8$^+$ T cells. By flow cytometry, peripheral blood CD56$^+$ NK cells and CD8$^+$ T cells of AA patients show significantly higher MI of NKG2D compared to normal controls ($P<0.01$, $P<0.05$, respectively): NKG2D MI is 90.06 ± 19.06 on NK cells (**a** and **b**) and 97.50 ± 9.72 on CD8$^+$ T cells (**c** and **d**) in normal control and 90.92 ± 30.13 on NK cells (**a** and **b**) and 69.61 ± 9.15 on CD8$^+$ T cells (**c** and **d**) in AD patients. In AA patients, MI is 213.5 ± 23.63 on NK cells (**a** and **b**) and 183.5 ± 9.15 on CD8$^+$ T cells (**c** and **d**).

1991; Bryceson *et al.*, 2006). On the other hand, IFN-γ is now appreciated as a key cytokine in AA pathogenesis, in particular in mediating the underlying IP collapse of anagen HFs (Rückert *et al.*, 1998; Paus *et al.*, 2003; Ito *et al.*, 2004; Freyschmidt-Paul *et al.*, 2006; Gilhar and Kalish, 2006). CD56$^+$ cells treated with 10 IU ml^{-1} IFN-γ that were obtained from healthy controls showed no significant differences in their NKG2D expression, compared to phosphate-buffered saline-treated CD56$^+$ cells (Figure 5a). However, 100 and 1×10^3 IU ml^{-1} IFN-γ significantly increased NKG2D MI (Figure 5b). This result from the FACS analysis is also supported by real-time PCR (Figure 5c). This IFN-γ-induced upregulation of NKG2D on normal NK cells corresponded to the much higher constitutive level of NKG2D expression on NK cells from AA patients. This greatly elevated baseline level of NKG2D expression in AA patients was further upregulated by 1×10^3 IU ml^{-1} IFN-γ (Figure 5b).

NK cells of AA patients also show higher expression of other NK-activating receptors

In addition to NKG2D expression on NK cells, the MI of other NKG2 family members, such as NKG2A (Long, 1999) and NKG2C (Lanier *et al.*, 1998), was studied by flow cytometry. While there was no significant difference in the MI of the inhibitory receptor NKG2A between AA patients (97.21 ± 7.27) and controls (90.84 ± 14.7), the expression level of the NK-activating receptor NKG2C (Lanier *et al.*, 1998) in AA patients (81.89 ± 5.6) was significantly higher than that of controls (39.22 ± 14.7) ($P<0.05$) or atopic dermatitis (42.46 ± 2.76) ($P<0.05$) (Figure 6). This suggests that NK cells in AA patients are considerably more susceptible to activating stimuli than those of healthy controls or of patients with pathogenetically distinct, common inflammatory diseases.

KIR-2D2/2D3 expression on CD56$^+$ cells is reduced in AA patients

Finally, we analyzed the expression of the NK cell inhibitory receptor, KIR-2D2/2D3. By flow cytometry, the ratio of KIR-2D2/2D3$^-$CD56$^+$/KIR-2D2/2D3$^+$CD56$^+$ was significantly higher in PBMC preparations of AA patients (3.85 ± 0.18) compared to normal controls (2.58 ± 0.12) and atopic dermatitis patients (2.43 ± 0.27) ($P<0.05$) (Figure 7). This suggests that NK cells in AA patients have a decreased sensitivity toward NK cells' inhibitory stimuli, while, under physiological circumstances, the risk of NK activation by

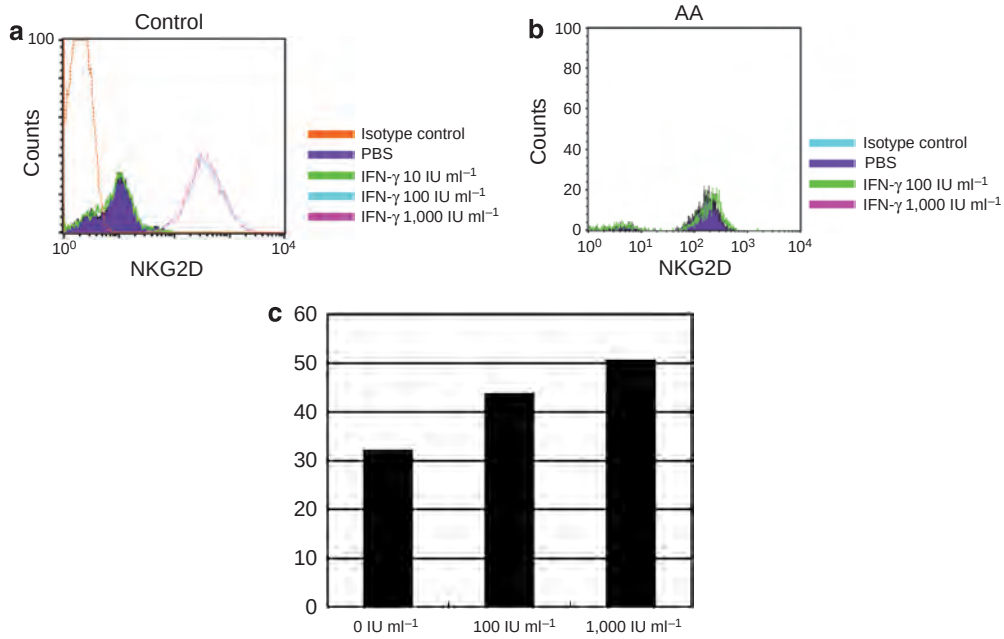

Figure 5. IFN-γ treatment potently upregulates NKG2D expression on NK cells of healthy controls to the abnormally high constitutive expression level of AA patients. (**a**) NKG2D MI is strongly upregulated by 100 and 1000 IU ml^{-1} IFN-γ *in vitro*. (**b**) HFs from AA patients show a high constitutive NKG2D MI, which is comparable to that induced by 100 IU ml^{-1} IFN-γ in normal controls, and cannot be further upregulated by IFN-γ administration. (**c**) Real-time PCR reveals up-regulation of NKG2D by IFN-γ.

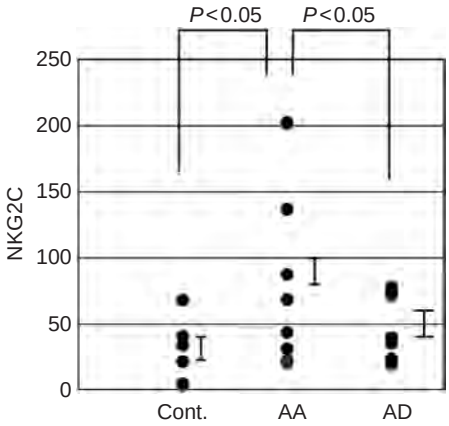

Figure 6. NKG2C expression is significantly higher on NK cells in AA patients compared to normal controls and patients with atopic dermatitis. The MI of another NK-activating receptor, NKG2C, is significantly higher on NK cells from PBMCs in AA patients (81.89 ± 5.6) compared to normal controls (39.22 ± 14.7) and AD patients (42.46 ± 2.76) ($P<0.05$).

anagen HFs is relatively low, due to a low constitutive expression level of the NK-activating receptors, NKG2C and NKG2D, and a high basic expression level of NK-inhibitory KIR-2D2/2D3.

DISCUSSION

To our knowledge, the association between NK cells and the collapse of HF-IP has not previously been reported, and it calls attention to the general role of NK cells in organ-specific autoimmune disorders characterized by IP collapse, for which AA offers an excellent disease model (Paus *et al.*, 2003; Gilhar and Kalish, 2006). We report evidence suggesting that a finely tuned balance in the mechanism normally collaborates to suppress intracutaneous NK cell activities, and this balance is disturbed in AA. This study provides important new pointers to a role for NK cells in AA pathogenesis, thus following earlier, indirect immunogenetic hints of such an involvement (that is, association of the nonclassic MHC gene MICA with AA (Barahmani *et al.*, 2006; Martinez-Mir *et al.*, 2007).

In our attempts to understand better why the immunoprivileged, MHC class I-negative HF epithelium is not constantly subjected to NK cell attack (Karre, 1997), we confirm—first in multiple samples of normal human scalp skin—that indeed there is no sign of an NK attack on normal anagen VI HFs (Christoph *et al.*, 2000). Second, we show that stimulation-sensitive (that is, NKG2D$^+$) CD56$^+$ NK cells prominently aggregate around AA HFs. This is in line with the previous study by Imai *et al.* (1994), who found that patients with severe multifocal AA, AA totalis, or AA universalis had significantly more CD57$^-$CD16$^+$ NK cells in the PBMCs compared to normal controls. This suggests that, under physiological conditions, the immunoprivileged HF escapes from NK cell attack, and this cannot be prevented any longer when HF-IP collapses.

Third, we show that human anagen HFs *in situ* largely lack MICA expression, just like other healthy tissues (Groh *et al.*, 1998, 1999; Deng and Mariuzza, 2006). As an exception to this rule, the proximal hair matrix is constitutively MICA$^+$, even in healthy human scalp skin, which designates this HF area as a ''high-risk zone'' for an attack by cells that express a

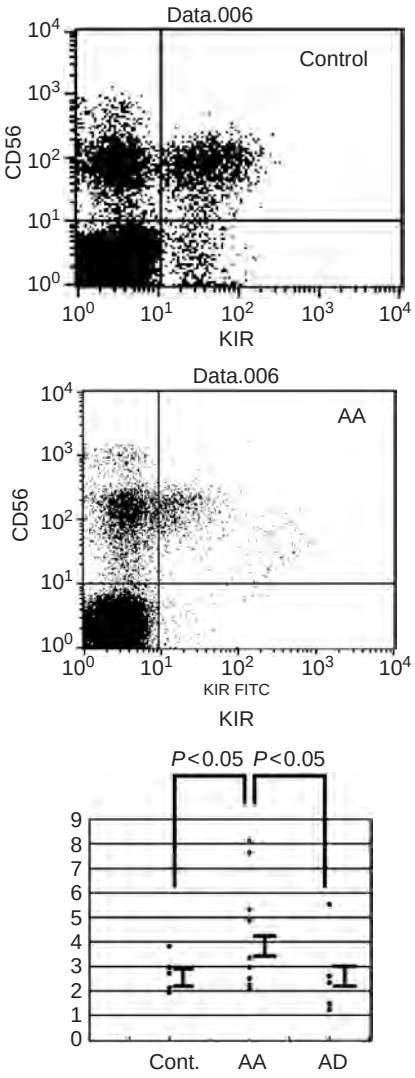

Figure 7. NK cell expression of the inhibitory receptor, KIR-2D2/2D3, is lower in AA patients. The ratio of KIR-2D2/2D3^{-}CD56^{+} cells/KIR-2D2/2D3^{+}CD56^{+} cells is significantly higher in AA patients (3.85 ± 0.18) compared to normal controls (2.58 ± 0.12) ($P<0.05$) and patients with atopic dermatitis (2.43 ± 0.27) ($P<0.05$).

corresponding receptor (for example, NKG2D^{+} NK cells). This corresponds well to the fact that it is exactly this HF region that is the most frequently attacked in patients with AA (Todes-Taylor *et al.*, 1984; Messenger and Simpson, 1997; Bodemer *et al.*, 2000; McDonagh and Messenger, 2001).

In striking contrast to normal HFs, AA skin abounds in strong, widespread, extra- and intrafollicular MICA IR. This is at least suggestive of the concept that excessive NK cell stimulation may play a role in AA pathogenesis.

Fourth, we show that not only CD4^{+} and CD8^{+} T cells but also CD56^{+} NK cells massively accumulate around AA HFs. Both infiltrating CD56^{+} NK cells and CD8^{+} T cells show prominent expression of the NK cell-activating receptor, NKG2D. This suggests that a (as yet unknown) stimulus associated with AA pathogenesis induces MICA expression on HFs, thus facilitating or even inviting an attack of infiltrating NKG2D^{+} cells on MICA^{+} HFs, accelerating disease progression.

Fifth, our data demonstrating that AA patients show an increased NKG2D expression on both peripheral blood CD56^{+} NK cells and CD8^{+} T cells compared to normal controls and patients with another chronic inflammatory skin disease, atopic dermatitis, suggest that AA patients have a systemic (and possibly constitutive) heightened sensitivity of NK cells and CD8^{+} T cells toward MICA stimulation. Although our corresponding quantitative data were obtained from peripheral blood human CD56^{+} NK and CD8^{+} T cells and may thus not necessarily reflect the intracutaneous NKG2D expression patterns on these cells, they suggest that anagen HFs, under physiological conditions, may escape from NK cell attack, for example, due to a low constitutive expression level of NKG2D. In addition, normal scalp HFs may also provide a poor stimulatory milieu for NK cells by low constitutive MICA expression. This subtle balance appears to be constitutively disturbed in patients at risk of developing AA or may reflect a stage of disease progression in established AA pathogenesis.

Our observations are in line with previously reported associations between NKG2D, MICA, and autoimmune diseases: For example, MICA is aberrantly expressed on rheumatoid arthritis synoviocytes, which are attacked by NKG2D^{+}CD4^{+}CD28^{-} T cells (Groh *et al.*, 2003), and the blockade of NKG2D signaling appears to protect from insulin-dependent diabetes mellitus in mice (Ogasawara *et al.*, 2003). Interestingly, MICA polymorphisms are significantly associated with defined clinical varieties of AA (Menier *et al.*, 2002; Barahmani *et al.*, 2006; Martinez-Mir *et al.*, 2007), and this corresponds well with our immunohistological findings.

Taken together, this suggests that normal human anagen HFs maintain their IP and escape the induction of autoimmune diseases at least in part by reducing the chance of stimulation of NKG2D-mediated NK cell activation. Instead, AA patients show opposite phenomena and may have a constitutively increased sensitivity of NK cells and CD8^{+} cytotoxic T cells to be stimulated via NKG2D (that is, the two immunocyte populations that are most relevant in terms of HF-IP, since they can destroy either MHC class I-negative HF cells or cells that present MHC class I-presented HF autoantigens (Paus *et al.*, 2005)).

Sixth, our finding that AA patients have a significantly higher percentage of NK cells that do not express NK cell-inhibitory KIR-2D2/2D3 compared to controls suggests that this further contributes to rendering the NK cells of AA patients constitutively hypersusceptible to activation. Interestingly, a number of autoimmune disorders, such as rheumatoid arthritis and scleroderma, have recently been associated with specific KIR genes (Yen *et al.*, 2001; Momot *et al.*, 2004; Williams *et al.*, 2005).

Seventh, our finding that IFN-γ upregulates and induces not only ectopic MHC class I expression (Ito *et al.*, 2004) but also NKG2D expression on NK cells (Freyschmidt-Paul *et al.*, 2005b) is in line with the observation that the expression of NKG2D by macrophages can be induced by IFN-γ and

IFN-α/β (Jamieson *et al.*, 2002) and underscores the role of IFN-γ as a key cytokine in AA pathogenesis (Hoffmann *et al.*, 1994; Rückert *et al.*, 1998; Paus *et al.*, 2003; Ito *et al.*, 2004; Freyschmidt-Paul *et al.*, 2006; Gilhar and Kalish, 2006).

Eighth, we found that normal anagen HFs also strongly express the potent NK cell inhibitor MIF, while AA HFs show dramatically decreased MIF expression. Since MIF plays a key role in suppressing NK-mediated cytolysis in other immunoprivileged sites—such as the anterior eye chamber, where MIF inhibits the NK-mediated lysis of corneal endothelial cells via preventing the release of perforin granules by NK cells—the strong follicular expression of MIF resembles that of the corneal epithelium (Apte *et al.*, 1998; Taylor, 2003; Caspi, 2006). Constitutive or induced downregulation of MIF, therefore, represents another potentially important, previously unknown mechanism by which human HFs become more prone to suffer an NK cell attack.

The new data provided by this study already invite the hypothesis that IFN-γ-induced collapse of HF-IP simultaneously facilitates an attack of $NKG2D^+$ NK cells on $MICA^+$ HFs. Our data also suggest that normal anagen HFs escape from NK cell attack by downregulating MICA and upregulating MIF, along with a low level of constitutive expression of NKG2D on NK cells and a high number of $KIR\text{-}2D2/2D3^+$ NK cells. Thus, although the exact role of NK cells in AA pathogenesis remains to be clarified, these experiments strongly suggest a key role for NK cells in autoimmune diseases associated with IP collapse. Therefore, our study provides interesting new pointers for related autoimmune diseases such as autoimmune hepatitis, uveitis, orchitis, and fetal rejection, which highlight the importance of keeping NK cells in check in order to maintain IP.

MATERIALS AND METHODS

Human tissues and cells

Human frontotemporal scalp skin specimens were obtained with patients' informed consent during routine face-lift surgery from five female patients aged 18–60 years (mean age 43 years) and used as control tissue. Lesional scalp skin from AA patients ($n=8$) was also biopsied. Cryosections (8 μm thick) were fixed in acetone at −20°C for immunohistochemistry and stored at −20°C until use. PBMCs were taken from healthy controls ($n=10$), atopic dermatitis (AD) patients ($n=10$), and AA patients ($n=15$). Because AD is recognized as a Th2 disease (Hamid *et al.*, 1994; Kakinuma *et al.*, 2002), in contrast to the predominance of Th1-type immune response in AA (Freyschmidt-Paul *et al.*, 2005a, b, 2006), AD patients were chosen as a control group.

Informed consent was obtained from all participants, and study approval was granted by the ethical committee of the Hamamatsu University School of Medicine. The study was conducted according to the Declaration of Helsinki Principles.

Quantitative immunohistomorphometry

Cryosections were routinely stained by hematoxylin and eosin. The hair cycle stage of each HF was assessed and classified by morphological criteria (Whiting and Howsden, 1996; Sinclair *et al.*, 1999; Müller-Röver *et al.*, 2001). CD4, CD8, CD56, MIF, MICA, and NKG2D expression was studied on normal human scalp skin (including anagen and catagen HFs) and AA lesions. The $CD4^+$, $CD8^+$, and $CD56^+$ cells distributed in the HFs and around a distance of the diameter of a hair bulb from HFs were counted and statistically analyzed (three parts/HF, three HFs/specimen, three samples/group). NKG2D was double-immunostained with either CD8 or CD56.

First, NKG2D was detected by the highly sensitive tyramide signal amplification technique (Perkin Elmer, Boston, MA) (Roth *et al.*, 1999) as described previously (Ito *et al.*, 2004). Briefly, after normal human scalp skin and AA lesions were washed in TNT buffer (0.1 M Tris, pH 7.5; 0.15 M NaCl; 0.1% Tween 20), these specimens were immunostained using monoclonal anti-human NKG2D antibody (BD Bioscience PharMingen, San Jose, CA) as the primary antibody diluted 1:300 in TNB blocking buffer (TNT + 0.5% blocking buffer) for 60 minutes at room temperature. This was followed by incubation with the biotinylated anti-mouse IgG antibody (Dako, Glostrup, Denmark) (1:200 in TNB, 30 minutes, room temperature). Next, streptavidin horseradish peroxidase was applied (1:50 in TNB, 30 minutes, room temperature). Finally, the reaction was amplified by FITC-tyramide amplification reagent at room temperature for 3 minutes (1:50 in amplification diluents provided with the kit). Then, phycoerythrin-conjugated anti-human CD8 (BD Bioscience, San Jose, CA) or CD56 (BD Bioscience) antibody was applied on the specimens.

IR of MIF (Steinhoff *et al.*, 1999) (R&D Systems, Minneapolis, MN) and MICA (Santa Cruz, CA) (Xu *et al.*, 2006) was also studied on normal anagen human scalp skin and AA lesional skin using avidin–biotin complex and EnVision methods, as these molecules were difficult to detect by routine immunofluorescence techniques.

CD4 was detected by immunofluorescence. FITC-conjugated anti-human CD4 antibody (1:50 in phosphate-buffered saline) (BD Bioscience) was incubated on cryosections of human scalp skin for 60 minutes. The signals were visualized under a fluorescence microscope (Olympus, Tokyo, Japan) with a CCD (charge-coupled device) camera (Olympus), and mean intensity was measured using "Image J" software, which can be downloaded from http://rsbweb.nih.gov/ij.

Flow-cytometric analysis

The MI for cell surface expression of NKG2A, NKG2C, NKG2D, and KIR-2D2/2D3 on PBMCs was studied by flow-cytometric analysis (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) from PBMCs of healthy controls ($n=13$), atopic dermatitis patients ($n=18$), and AA patients ($n=37$). Briefly, purified mouse anti-human NKG2D antibody (BD Bioscience PharMingen) was incubated with PBMCs for 30 minutes. After the cells were washed in phosphate-buffered saline for 5 minutes three times, they were incubated with biotinylated anti-mouse IgG antibody (Dako) for 30 minutes and then were incubated with streptavidin fluorescein conjugate (Biosource, Carlsbad, CA). Monoclonal phycoerythrin-conjugated anti-human CD56 antibody (MY31, mouse IgG_1) and PerCP-conjugated monoclonal anti-human CD8 antibody (SK1, mouse IgG_1) were applied to the cells. PBMCs were triple stained with phycoerythrin (PE)-conjugated anti-human NKG2A antibody (Immunotech, Marseille, France) or PE-conjugated anti-human NKG2C antibody (R&D Systems), together with FITC-conjugated anti-human CD56 antibody (NCAM 16.2, mouse IgG_{2b}) and PerCP-conjugated monoclonal anti-human CD8 antibody (SK1, mouse IgG_1) (Becton Dickinson) for 30 minutes. PBMCs were also incubated with the

FITC-conjugated KIR-2D2/2D3-specific reagents KIR-NKAT2 (DX27, mouse IgG_{2a}) and PerCP-conjugated monoclonal anti-human CD4 antibody (SK3, mouse IgG_1) (Becton Dickinson) for 30 minutes.

PBMCs cultured with IFN-γ

IFN-γ is a potent catagen inducer and upregulates MHC class I expression on ORS and hair matrix keratinocytes *in situ* (Ito *et al.*, 2005) and likely plays a key role in triggering AA (Ito *et al.*, 2004; Paus *et al.*, 2005; Gilhar and Kalish, 2006). Therefore, we wished to explore the effects of IFN-γ on the expression of NKG2D on NK cells. PBMCs were cultured with 10, 100, and 1000 IU ml^{-1} of IFN-γ for 12 hours at 37°C. NKG2D expression was then studied on NK cells by FACSCalibur.

NK cell isolation

To isolate NK cells from human PBMCs, the MACS NK Cell Isolation Kit (Militenyi Biotec, Bergisch Gladbach, Germany) was applied in this study. Briefly, PBMCs were isolated by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden) in LeucoSep (Greiner Bio-One GmbH, Frickenhausen, Germany). Isolated PBMCs were incubated with Hapten-Antibody Cocktail (cocktail of hapten-conjugated anti-CD3, -CD14, -CD19, -CD36, and -IgE antibodies) for 10 minutes at 8°C in MACS buffer, pH 7.2, degassed phosphate-buffered saline supplemented with 0.5% BSA and 2 mM EDTA. After the cells were washed, anti-hapten microbeads were added and incubated for 15 minutes at 8°C. After another wash, the suspension of PBMCs was applied onto an LS^+/VS^+ column in the magnetic field following the manufacturer's instructions. The unlabeled cells were collected as purified NK cells confirmed by FACS analysis.

Quantitative real-time PCR

Total RNA from negatively isolated NK cells was extracted using an RNeasy mini-kit (Qiagen, Hilden, Germany). Then, first-strand cDNA was generated from 1 μg of RNA with 10 × PCR buffer (Roche, Indianapolis, IN), 25 mM $MgCl_2$ (Roche), 10 mM deoxyribonucleotide triphosphate mix (Qiagen), random hexamers (Roche), RNase inhibitor (Roche), and MuLV reverse transcriptase (Roche) on a GeneAmp PCR System 9700 for 10 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 99°C and stored at 4°C (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed following the manufacturer's instructions on an ABI Prism 7000 using TaqMan PCR primers (Hs01095635 m1, NM 007360) with generated cDNA and TaqMan Universal PCR Master Mix (all from Applied Biosystems).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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