

Natural Killer and Dendritic Cell Contact in Lesional Atopic Dermatitis Skin – *Malassezia*-Influenced Cell Interaction

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The regulation of dendritic cells is far from fully understood. Interestingly, several recent reports have suggested a role for natural killer cells in affecting dendritic cell maturation and function upon direct contact between the cells. It is not known if this interaction takes place also *in vivo*, or if a potential interaction of natural killer cells and dendritic cells would be affected by allergen exposure of the dendritic cells. The yeast *Malassezia* can act as an allergen in atopic eczema/dermatitis syndrome, and induce maturation of dendritic cells. Our aims were to study the distribution of natural killer cells in the skin from atopic eczema/dermatitis syndrome patients with the emphasis on possible natural killer cell–dendritic cell interaction, and to assess whether the interaction of *Malassezia* with dendritic cells would affect subsequent interaction between dendritic cells and natural killer cells. A few scattered natural killer (CD56⁺/CD3[−]) cells were found in the

dermis of healthy individuals and in nonlesional skin from atopic eczema/dermatitis syndrome patients. In lesional skin and in biopsies from *Malassezia* atopy-patch-test-positive skin, however, natural killer cells were differentially distributed and for the first time we could show close contact between natural killer cells and CD1a⁺ dendritic cells. Dendritic cells preincubated with *Malassezia* became less susceptible to natural-killer-cell-induced cell death, suggesting a direct effect imposed by *Malassezia* upon interaction of dendritic cells with natural killer cells. These findings indicate that natural killer cells and dendritic cells can interact in the skin and that *Malassezia* affects the interaction between natural killer cells and dendritic cells. Our data suggest that natural killer cells may play a role in regulating dendritic cells in atopic eczema/dermatitis syndrome. **Key words:** cell communication/cell death/eczema. *J Invest Dermatol* 119:850–857, 2002

The chronic inflammatory skin disease atopic dermatitis, now referred to as atopic eczema/dermatitis syndrome (AEDS) (Johansson *et al*, 2001), is increasing in prevalence, and AEDS symptoms have been recorded for as many as 20% of children throughout the world (ISAAC, 1998; Leung, 2000). A complex interplay between genetic predisposition, immune dysregulation, skin barrier dysfunction, lifestyle, and environmental factors is thought to be important for the development of AEDS (Cooper, 1994; Werfel and Kapp, 1998; Alm *et al*, 1999). Evidence is accumulating that the opportunistic yeast *Malassezia*, formerly known as *Pityrosporum*, can contribute to the inflammatory reaction in AEDS (Faergemann, 1999; Scheynius *et al*, 2002). The presence of IgE specific to *Malassezia* has been reported in 32%–68% of AEDS patients, and positive atopy patch test (APT) reactions in more than half of the patients (Tengvall Linder *et al*, 2000; Scheynius *et al*, 2002). A correlation between positive APT and Th2-like response

in peripheral blood mononuclear cells has been described (Johansson *et al*, 2002).

Dendritic cells (DCs) are recognized as the most potent of the antigen-presenting cells due to their ability to activate naive T cells, and they act as conductors of immune responses by functioning as a link between the innate and acquired immune systems (Banchereau *et al*, 2000; Granucci *et al*, 2001). Immature DCs can efficiently take up antigens in the periphery and, following activation, differentiate into professional antigen-presenting cells expressing CD83 and high levels of costimulatory molecules. These mature DCs have strongly reduced ability to take up antigen, but are instead excellent at presenting antigen (Banchereau *et al*, 2000). Langerhans cells are a subset of immature CD1a⁺ DCs found in the epidermis of the skin. DCs with a Langerhans-cell-like phenotype can be generated *in vitro* by culturing the CD14⁺ cell fraction of peripheral blood mononuclear cells (Romani *et al*, 1994; Sallusto and Lanzavecchia, 1994) to gain monocyte-derived dendritic cells (MDDCs). As DCs initiate adaptive immune responses, it is likely that they play an important role in skewing the reaction to allergens towards a Th2-like response in atopic individuals. We have shown previously that interaction of *Malassezia* and human immature MDDCs leads to uptake, maturation, and cytokine production, possibly promoting a Th2-like immune response (Buentke *et al*, 2000; 2001).

The regulation of DCs by other cells is far from fully understood. Natural killer (NK) cells are part of the innate immune system; their ability to induce cell death in tumor cells and virus-infected cells

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Abbreviations: AEDS, atopic eczema/dermatitis syndrome; APT, atopy patch test; DC, dendritic cell; MDDC, monocyte-derived dendritic cell; NK, natural killer.

has been studied intensely. More recently a potential role in affecting DC regulation has been addressed. Several studies have demonstrated an interaction between DCs and NK cells, indicating an importance of cross-talk for their mutual regulation (Chambers *et al*, 1996; Geldhof *et al*, 1998; Carbone *et al*, 1999; Fernandez *et al*, 1999; Wilson *et al*, 1999; Spaggiari *et al*, 2001; Ferlazzo *et al*, 2002; Gerosa *et al*, 2002; Piccioli *et al*, 2002). It is not known if this interaction takes place also *in vivo*, or if a potential interaction of NK cells and DCs would be affected by allergen exposure of the DCs. In this study, we addressed these questions by investigating the presence of NK cells in the skin from AIDS patients and healthy individuals. Specifically, we studied the effect of *Malassezia* uptake by DCs on subsequent interactions with NK cells.

MATERIALS AND METHODS

Immunohistochemical and immunofluorescence staining of skin biopsy specimens Skin specimens (4 mm punch biopsies) were taken under local anesthesia from three healthy individuals, and from nonlesional, lesional, and *Malassezia* extract APT-positive skin (at 24 h and 72 h after provocation) from four AIDS patients who had serum IgE antibodies specific for *Malassezia* (Tengvall Linder *et al*, 2000). The biopsies were snap frozen on dry ice and stored at -80°C until further analysis. Acetone-fixed, 6 μm thick, cryostat sections were immunohistochemically stained with an anti-CD56 (MY31, Becton Dickinson, Sweden) monoclonal antibody (MoAb), using Vectastain ABC-Elite (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol, and then developed with 3-amino-9-ethylcarbazole (0.02% wt/vol, Aldrich-Chemie, Germany), followed by counterstaining with Mayer's hematoxylin (Sigma, Sweden). CD56⁺ cells were identified by their definite brownish-red staining and visible nuclei. For double immunofluorescence staining, the sections were incubated, first, with normal donkey or goat serum, followed by anti-CD56 MoAb overnight at 4°C . Thereafter either donkey antimouse IgG Rhodamine Red-X (Jackson ImmunoResearch, Göteborgs Termometerfabrik, Sweden) or goat antimouse IgG Alexa 546 (Molecular Probes, The Netherlands) conjugated antibody was applied for 1.5 h at room temperature. Next, normal mouse serum was added to the sections, followed by either a fluorescein isothiocyanate (FITC) conjugated anti-CD3 MoAb (SK4, Becton Dickinson) or an FITC-conjugated anti-CD1a MoAb (NA1/34, Dakopatts, Denmark) incubated overnight at 4°C . The sections were evaluated using a Leica TCS SP2 confocal laser scanning microscope system, equipped with an inverted Leica DM IRBE microscope, an argon laser, and two HeNe lasers (Leica Microsystems, Germany). Oil was used as immersion medium, and paraphenylenediamine in glycerol as mounting medium to reduce fading (Johnson *et al*, 1982). Leica confocal software was used to acquire and visualize the data. Staining was not observed when irrelevant isotype-matched antibodies, mouse IgG₁ (DAK-GO1, Dakopatts) or mouse IgG_{2a} FITC (X39, Becton Dickinson), were used or when primary antibodies were omitted.

Culturing of *Malassezia* *M. sympodialis* strain no. 42132, previously designated *M. furfur* (Mayser & Gross, 2000; Scheynius *et al*, 2002), was obtained from the American Type Culture Collection, and is referred to here as *Malassezia*. The yeast was cultured at 37°C for 4 d as previously described (Buentke *et al*, 2001), and then harvested in sterile water and counted under a light microscope before incubation with MDDCs. The endotoxin content was determined in yeast culture supernatants from three independent experiments, and was found to be less than 0.3 EU per ml (Limulus test, performed by Apoteket, Stockholm, Sweden).

Generation of MDDCs from peripheral blood Buffy coats were obtained from healthy donors from the Karolinska Hospital blood bank. All the test samples were ImmunoCapTM, m70, negative to *Malassezia* (= 0.35 kU per l) with a median total serum IgE level of 16 kU per l (range 2.6–140 kU per l, reference ranges 1.6–122 kU per l), and Phadiatop negative, i.e., did not show IgE reactivity to 11 common airborne allergens (Pharmacia Diagnostics, Sweden). Monocytes were isolated and MDDCs generated as previously described (Romani *et al*, 1994; Buentke *et al*, 2001). Briefly, the CD14⁺ cells were diluted to 4×10^5 cells per ml in complete culture medium, RPMIc [RPMI 1640 medium supplemented with 25 μg per ml gentamicin, 2 mM L-glutamine, 100 IU per ml penicillin, 100 μg per ml streptomycin (Gibco BRL, Life Technologies, U.K.), and 10% (vol/vol) fetal bovine serum (FBS; Hyclone, Logan, UT), denoted "RPMI 1640 with 10% FBS", supplemented with 50 μM 2- β -mercaptoethanol (Sigma), 550 U per ml

granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering Plough, Kenilworth, NJ), and 800 U per ml of recombinant human interleukin-4 (rIL-4; Nordic BioSite, Sweden), and cultured in 25 or 75 cm² culture flasks (Costar, Cambridge, MA). To generate immature MDDCs the CD14⁺ cells were cultured in a humidified incubator with 6% CO₂ in air for 6 d with refeeding on day 3. To mature the MDDCs, RPMIc supplemented with cytokines (550 U per ml GM-CSF and 800 U per ml rIL-4) and 100 ng per ml of lipopolysaccharide (LPS) (L8274, *Escherichia coli* serotype 026-B6, Sigma) was added to the cells on day 6 of culture for an additional 46 h. The cells were harvested by gentle resuspension. This study was approved by the local ethics committee.

Interaction of MDDCs with *Malassezia* After 6 d of culture, the MDDCs appeared as loosely adherent cell aggregates with typical dendritic morphology. At this time point, the immature MDDCs were harvested and cocultured with *Malassezia*, at a 1:5 ratio, in 25 cm² culture flasks (Costar) at 37°C , in RPMIc supplemented with cytokines (550 U per ml GM-CSF and 800 U per ml rIL-4) or in complete culture medium alone, at a cell density of 4×10^5 MDDCs per ml, for approximately 46 h. *Malassezia* was also incubated in RPMIc culture medium alone for 46 h. Upon harvest, the cells were centrifuged, and cell viability was assessed by trypan blue exclusion. Some MDDCs, cocultured with or without *Malassezia* were air-dried onto three-well (\rightarrow internal 14 mm) microscope glass-slides ($\approx 2 \times 10^4$ cells per well, Novakemi, Sweden), and stored at -80°C until use. In some experiments, the culture supernatants were sterile filtered (0.2 μm) and used to stimulate immature DCs or NK cells.

Characterization of the MDDCs At day 8 of culture, the MDDCs' surface phenotype was assessed by flow cytometry. The following FITC- or phycoerythrin- (PE) conjugated mouse MoAbs were used: anti-CD1a PE (T6-RD1, Coulter, Beckman-Coulter, Sweden), anti-CD14 FITC (Leu-M3), anti-CD40 FITC (5C3), anti-CD54 PE (Leu-54), anti-CD80 FITC (L307.4), anti-CD83 FITC (HB15e), anti-CD86 FITC (2331 FUN-1), anti-HLA-DR FITC (L243), and anti-HLA-A, B, C FITC (G46-2.6) from Pharmingen/Becton Dickinson. Isotype-matched antibodies, mouse IgG₁ FITC- or PE-conjugated (X40), and mouse IgG_{2a} FITC-conjugated (X39) from Becton Dickinson were used as negative controls. Approximately 5×10^4 cells were incubated for 30 min on ice with MoAbs. Gates were set according to a characteristic forward-scatter pattern (Romani *et al*, 1996). The flow cytometer was calibrated according to the manufacturer's instructions before each acquisition, and a minimum of 10^4 cells was acquired on a FACSCalibur flow cytometer and analyzed using CellQuest software (Becton Dickinson). A typical immature DC phenotype was present in the MDDCs cultured only in medium (Table I) (Romani *et al*, 1994; 1996), and the LPS-matured MDDCs showed a characteristic mature DC phenotype (Table I) (Banchereau *et al*, 2000). Owing to autofluorescence of the yeast, flow cytometry could not be used to assess the phenotype of MDDCs cultured with *Malassezia*. Instead the expression of CD83, the DC maturation marker (Zhou and Tedder, 1996), was studied by immunocytochemistry. Frozen and acetone-fixed MDDCs were stained with anti-CD83 (HB15e, Pharmingen) MoAb, using Vectastain ABC-Elite (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol and as previously described (Buentke *et al*, 2001). The cells were evaluated with a Leitz microscope. A CD83⁺ cell was defined as one with a definite brownish-red staining and a visible nucleus. A minimum of 500 MDDCs were counted, and data were compared using the nonparametric Wilcoxon matched pairs test. The percentage of MDDCs cultured with *Malassezia*-expressing CD83 was significantly higher ($p < 0.05$) than for MDDCs cultured in medium, i.e., 77% (median, range 46%–87%, $n = 7$) and 7.2% (1.4%–16%, $n = 7$), respectively. The median purity of the MDDC cultures, as determined by cell size and cell granularity using flow cytometry, was 95% (median, range 78%–98%, $n = 12$). The median viability of MDDCs incubated with *Malassezia* for 46 h was 92% (range 84%–100%, $n = 12$), with LPS 93% (range 88%–97%, $n = 5$), and with medium only 96% (range 92%–98%, $n = 12$), as determined by trypan blue exclusion.

Generation of short-term activated polyclonal NK cells Peripheral blood mononuclear cells, depleted of CD14⁺ cells, were frozen in RPMI 1640 (Gibco BRL, Life Technologies) supplemented with 50% heat-inactivated FBS and 10% dimethyl sulfoxide, and stored at -150°C . To generate autologous short-term activated polyclonal NK cells, peripheral blood mononuclear cells were thawed and diluted to 2×10^6 cells per ml in RPMI 1640 with 10% FBS supplemented with 1000 U per ml of rIL-2 (Pepro Tech EC, U.K.), and then cultured in 25 cm² culture flasks at 37°C for 48 h. K562 cells, a human erythroleukemia line (a kind gift

Table I. Phenotype of the generated MDDCs

Phenotypic Marker	Immature MDDCs, Day 8 (n = 6-12) ^a		LPS-matured MDDC ^b , Day 8 (n = 3-5)	
	% ^c	MFI ^d	% ^c	MFI ^d
CD1a	97 (88-100)	486 (104-847)	91 (85-98)	267 (56-332)
CD14	1.0 (0.0-4.8)	25 (17-179)	0.6 (0.2-0.9)	32 (17-42)
CD40	92 (82-96)	44 (26-83)	100 (94-100)	118 (39-136)
CD54	60 (12-94)	39 (24-64)	94 (92-96)	36 (24-75)
CD80	61 (36-95)	31 (12-59)	99 (88-100)	107 (35-198)
CD83	2.5 (0.3-7.6)	27 (13-40)	67 (57-83)	24 (14-36)
CD86	28 (13-59)	52 (21-89)	100 (99-100)	348 (130-440)
HLA-DR	100 (98-100)	349 (207-829)	100 (99-100)	614 (452-1644)
MHCI	100 (99-100)	197 (108-337)	100 (100-100)	763 (534-1022)

^aNumber of experiments using cells from different healthy blood donors.

^bMDDCs matured with LPS for 46 h.

^cPositively stained MDDCs as analyzed by flow cytometry. Results are given as median % (range). At least 10⁴ cells per sample were analyzed.

^dMean fluorescence intensity values presented as median (range).

from Professor Giorgio Trinchieri, Schering-Plough Research Institute, Laboratory for Immunological Research, Dardilly, France), were cultured in RPMI 1640 with 10% FBS.

Time-lapse study MDDCs were seeded on glass coverslips in a 24-well plate (Costar) at a cell density of 2×10^5 per ml in RPMI supplemented with GM-CSF and rIL-4 (550 U per ml GM-CSF and 800 U per ml rIL-4), and incubated at 37°C overnight to allow adhesion. The coverslips were transferred to an inert aluminum chamber containing RPMI medium, and NK cells and *Malassezia* were added at a 1:5 ratio before the chamber was sealed with silicon immediately before the onset of the time-lapse photography as previously described (Buentke *et al*, 2001).

NK-cell-mediated cytotoxicity The susceptibility of MDDCs to autologous short-term activated polyclonal NK-cell-induced cell death was measured in a standard 4 h ⁵¹Cr-release assay, at 37°C, using Na₂⁵¹CrO₄-labeled target cells in triplicate at various effector:target ratios. 4×10^5 MDDCs and K562 cells were labeled with 40 μl Na₂⁵¹CrO₄ (Amersham, Sweden) for 1 h at 37°C, washed, and resuspended in complete culture medium. The amount of specific ⁵¹Cr released was expressed as a percentage and calculated as: % release = [(experimental release - spontaneous release)/(maximum release - spontaneous release)] × 100. The spontaneous release was usually less than 50% of the maximum. K562 cells, which do not express major histocompatibility complex (MHC) class I molecules, were used as control targets to assess the cytotoxic capacity of NK cells.

Effect of soluble factors from MDDC-*Malassezia* cocultures on the interaction of NK cells and MDDCs In some experiments NK cells and MDDCs were incubated in a 1:2 dilution of culture supernatant from MDDCs, cultured with or without *Malassezia*, during the 4 h ⁵¹Cr-release assay. To assess the effect of soluble factors on the phenotype of the MDDCs, 4×10^5 immature MDDCs per ml were resuspended in RPMI and incubated in 5 ml tubes (Falcon, Becton Dickinson) for 4 h in a 1:2 dilution with sterile filtered supernatants from MDDCs cultured either in medium or with *Malassezia*, and with supernatants from *Malassezia* alone. The cells were harvested, labeled with MoAbs for 30 min at 4°C, and analyzed by flow cytometry. Data were compared using the nonparametric Wilcoxon matched pairs test. NK cells were also preincubated for 4 h at 37°C, in a 1:2 dilution of sterile filtered culture supernatant from autologous MDDC cultures, with or without *Malassezia*, and with supernatant from *Malassezia* cultured alone. The NK cells were then incubated with Na₂⁵¹CrO₄-labeled K562 cells, and their cytotoxic capacity was measured in the standard 4 h ⁵¹Cr-release assay.

RESULTS

NK cells are in close proximity to CD1a⁺ DCs in *Malassezia* APT-positive skin from AIDS patients Immunohistochemical staining revealed few scattered CD56⁺ cells in the dermis, close to the epidermis, in skin biopsy specimens from healthy individuals

(**Fig 1A**) and in nonlesional skin from AIDS patients (**Fig 1B**). In lesional and *Malassezia* APT-positive skin from the AIDS patients, CD56⁺ cells were also found in the epidermis (**Fig 1C**) and numerous CD56⁺ cells were observed in the dermal cell infiltrates (**Fig 1D, E**). To exclude the possibility that these CD56⁺ cells could be NK T cells, double immunofluorescence staining with anti-CD3 and anti-CD56 antibodies was performed. In healthy skin, nonlesional, lesional, and *Malassezia* APT-positive skin at 24 h and 72 h, all the CD56⁺ cells detected were CD3⁻ and therefore most likely NK cells (**Fig 1F**). We next asked the question whether interaction between NK cells and DCs might take place in the skin during the inflammatory disorder AIDS associated with *Malassezia*. To address this question we performed double immunofluorescence staining using antibodies to CD56 and CD1a. In the dermis, close to the epidermis, we were able to detect CD56⁺ NK cells in close contact with CD1a⁺ DCs in *Malassezia* APT-positive skin from AIDS patients (**Fig 2**).

NK-cell-mediated lysis of autologous MDDCs is lower after preincubation with *Malassezia* By using time-lapse photography, the interactions between NK cells and MDDCs in the presence of *Malassezia* were followed for up to 6 h (**Fig 3**). One consequence of the interaction between NK cells and DCs is the NK-cell-mediated induction of cell death in DCs (Wilson *et al*, 1999). We observed that the interaction of several NK cells with an MDDC resulted in death of the MDDC after approximately 2-3 h, as apparent by the cell's rounding, swelling, loss of membrane integrity, and release of small vesicles (**Fig 3F-I**). The MDDC's dendrite comes into contact with NK cell no. 1 (**Fig 3B**) in a manner like that shown in the skin, pictured in **Fig 2**. The effect of *Malassezia* on the interaction between NK cells and DCs was further studied using an *in vitro* assay for NK-cell-induced cytotoxicity. MDDCs, preincubated with the yeast *Malassezia* for 46 h at a 1:5 ratio, were relatively less susceptible to NK-cell-induced cell death compared to the control MDDCs incubated in culture medium for the same time period (**Fig 4**). LPS-matured MDDCs were less susceptible to NK-cell-induced cell death than MDDCs incubated in medium and MDDCs cocultured with *Malassezia* (**Fig 4**). A shorter coculture period (20 h) for MDDCs and *Malassezia* did not result in reduced susceptibility to NK-cell-mediated lysis (data not shown).

Soluble factors from cocultures of MDDCs and *Malassezia* affect the cytotoxic capacity of NK cells The reduced susceptibility of *Malassezia*-pretreated MDDCs to NK-cell-mediated cytotoxicity might be a consequence of induction of a more mature phenotype in the MDDCs, or an effect of soluble

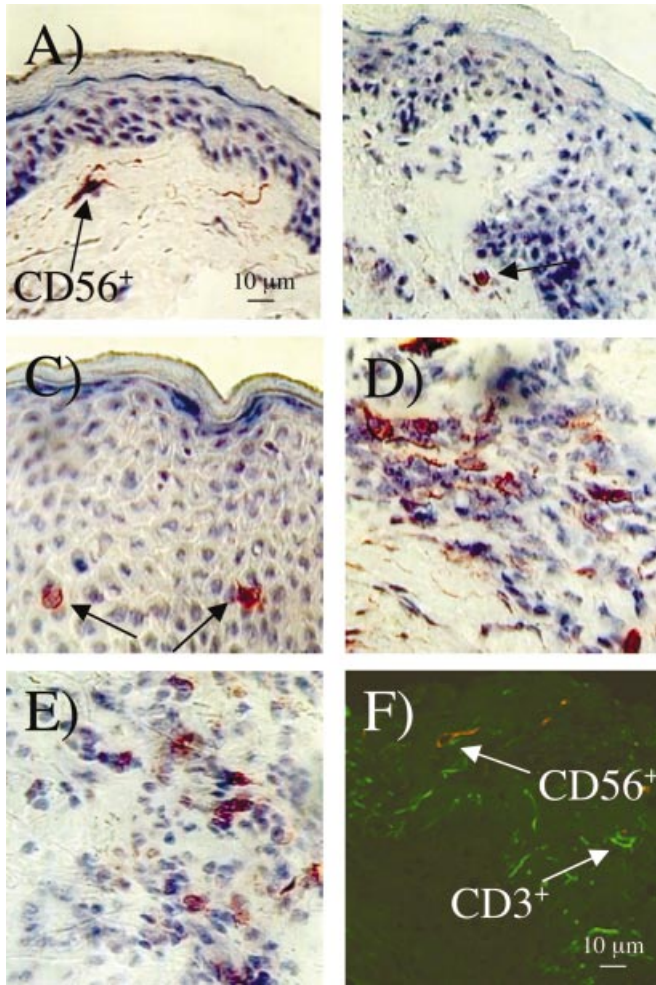


Figure 1. Different localization of NK cells in nonlesional compared to lesional and *Malassezia* APT-positive skin from AIDS patients. Using immunohistochemistry, CD56⁺ cells were found in the dermis close to the epidermis in healthy individuals (A), and in nonlesional skin from AIDS patients (B). In lesional (C, D) and *Malassezia* APT-positive skin at 72 h (E) from AIDS patients, CD56⁺ cells were found in the epidermis (C) and dermal cell infiltrates (D, E). Epidermis is upwards in the pictures. Double immunofluorescence staining, and confocal laser scanning microscopy, showed that the CD56⁺ cells were CD3⁻ (lesional skin, F). Scale bar: 10 μ m.

mediators produced by the MDDCs when activated with *Malassezia*. To investigate whether soluble factors produced by MDDCs preincubated with *Malassezia* affected NK-cell-induced death in immature MDDCs, sterile filtered culture supernatant was added during the 4 h ⁵¹Cr-release assay. This rendered the MDDCs less susceptible to NK-cell-induced cell death (Fig 5).

Next we investigated whether the soluble factors produced by MDDCs preincubated with *Malassezia* affected the phenotype of the immature MDDCs. Using flow cytometry, we found a significant increase of CD86⁺ MDDCs after addition of the MDDC-*Malassezia* supernatant as well as after addition of MDDC-LPS supernatant (data not shown). Heating of the supernatants to 56°C for 30 min, or addition of culture supernatants from yeast alone, did not affect the CD86 expression (data not shown). No differences were detected in the levels of CD1a, CD80, CD83, HLA-DR, or MHC class I expression after addition of MDDC-*Malassezia* supernatant (data not shown).

To further investigate the effect of soluble factors on the cytotoxic capacity of NK cells, NK cells were preincubated with culture supernatants. NK cells preincubated with MDDC-

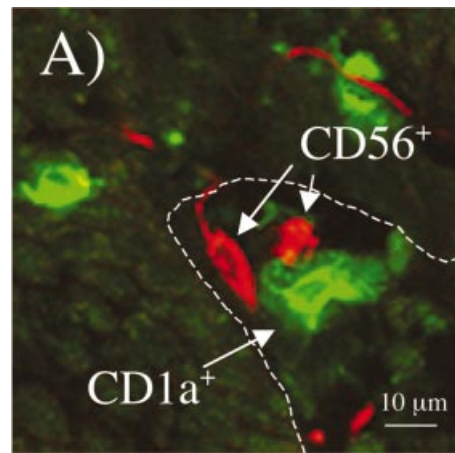


Figure 2. CD56⁺ NK cells and CD1a⁺ DCs in close contact in the skin. Double immunofluorescence staining was performed with MoAbs against CD56 and CD1a in skin biopsy specimens from AIDS patients with *Malassezia* APT-positive reactions (72 h). The dendrites from the CD1a⁺ DC can be seen in close proximity to the CD56⁺ NK cells. The dotted line indicates the border between epidermis and dermis, with dermis to the lower right in the picture. The specimen was analyzed using confocal laser scanning microscopy (Leica Microsystems). Scale bar: 10 μ m.

Malassezia coculture supernatant killed K562 MHC class I negative target cells to a lower degree than did NK cells incubated in medium alone (Fig 6). Preincubation in supernatants from *Malassezia* alone also resulted in less killing of the K562 cells, indicating that factors derived directly from the yeast might affect the activity of NK cells (Fig 6). Thus in addition to cell-cell contact, soluble factors might also play a role in the interaction between NK cells and DCs.

DISCUSSION

In this study we show that CD56⁺/CD3⁻ NK cells are located close to CD1a⁺ DCs in *Malassezia* APT-positive skin from AIDS patients and that uptake of the allergenic yeast *Malassezia* by immature MDDCs affects the subsequent interaction of MDDCs and NK cells.

Despite increasing interest in NK cell-DC interactions, little evidence indicates where this interaction takes place *in vivo*. It has been shown, however, that cell-cell contact is important in the cross-talk (Fernandez *et al*, 1999). Therefore we questioned whether NK cells were present in the skin of patients with the chronic inflammatory skin disease AIDS and, if so, might be distributed in a way that they could interact with DCs. As described here, we have demonstrated that NK cells and CD1a⁺ DCs can appear in close contact in *Malassezia* APT-positive skin, suggesting a role for NK cell-DC cross-talk. To our knowledge this is the first time that human NK cells and DCs have been shown to be in actual contact *in vivo*. NK cells in human skin have been associated with skin tumors, and occasional NK cells have been described in lesional skin from patients with AIDS (Zachary *et al*, 1985; Nakamura *et al*, 1995). The possible role of NK cells in AIDS has been studied with somewhat contradictory results. The main focus has been on the number of NK cells in peripheral blood and their level of activation. In AIDS patients, both a numerical decrease of NK cells and lowered NK cell activity compared to individuals without AIDS has been reported (Wehrmann *et al*, 1990; Hashiro and Okumura, 1998). Other groups found no difference in the number of NK cells (Bouloc *et al*, 2000), or higher NK cell activity than in healthy individuals (Strannegård and Strannegård, 1980). Such conflicting results may reflect the possibility that AIDS is a heterogeneous disease (Johansson *et al*, 2001), or that treatments, like topical steroid treatment, may have a systemic effect on NK

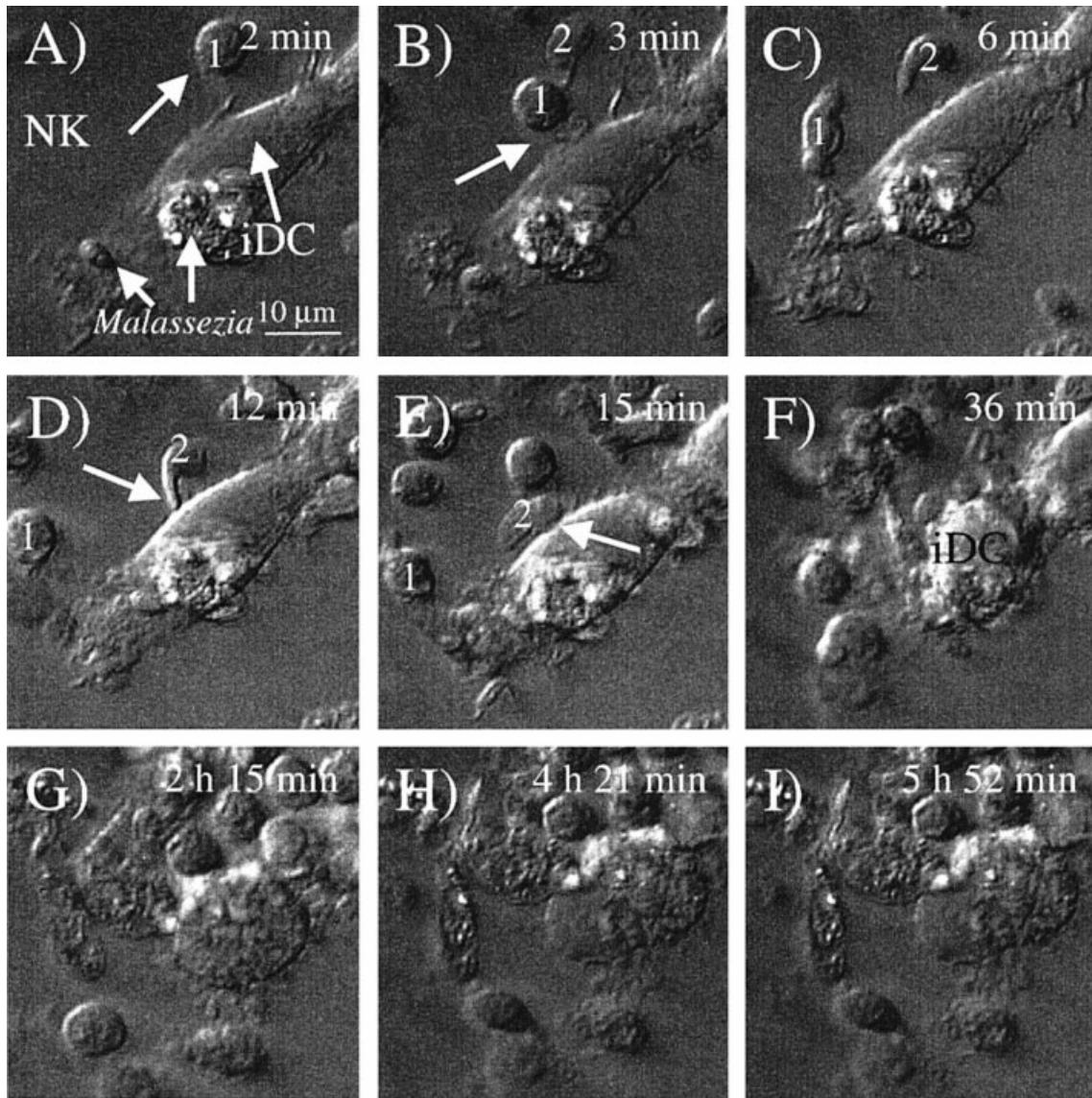


Figure 3. Time-lapse photography of short-term activated NK cells inducing cell death in an immature MDDC. Time-lapse photography was used to study the NK-cell-mediated lysis of an immature MDDC. One picture was taken every third second. The nine different time points, after coincubation of 2×10^5 immature MDDCs per ml with NK cells and *Malassezia* at a 1:5 ratio, are indicated in the upper right corner of each figure. The numbers 1 and 2 are used to indicate the movement of two different NK cells. Contact between NK cell no. 1 and the MDDC is seen in (B) and between NK cell no. 2 and the MDDC in (D), (E). Scale bar: 10 μ m. The photographs are from one experiment representative of two.

cells, thus influencing experimental outcomes (Lesko *et al*, 1989). Additionally, NK cells leaving the circulation could account for the lower number reported in most studies. Support for this seems to come from the numerous CD56⁺ cells we observed in dermal infiltrates in lesional and *Malassezia* APT-positive skin from AEDS patients (Fig 1D, E) and the report of increased levels of the NK cell chemoattractant MCP-1/CCL2 in patients with AEDS (Kaburagi *et al*, 2001). CD1a⁺ cells in lesional skin of AEDS patients can produce MDC/CCL22, another chemokine that attracts NK cells, as well as DCs and Th2 cells (Vulcano *et al*, 2001).

We showed that NK cells were differentially distributed in lesional and *Malassezia* APT-positive skin, with NK cells in the epidermis and dermal infiltrates, compared to nonlesional skin or skin from healthy individuals. Besides, an inverse relation between the NK cell activity and severity of eczema has been demonstrated (Chiarelli *et al*, 1987). This, together with our findings, points to a role for NK cells in AEDS. Moreover, NK cells have been implicated in the development of allergen-induced airway inflam-

mation in a mouse model, further supporting the role of NK cells in allergic diseases (Korsgren *et al*, 1999).

We then pursued our interest in studying the interaction of human NK cells and DCs preincubated with an allergen. That the opportunistic yeast *Malassezia* can act as an allergen and thereby contribute to the inflammatory reaction in AEDS has been shown in many studies (Faergemann, 1999; Scheynius *et al*, 2002), and we have previously demonstrated that *Malassezia* can be taken up by immature MDDCs, leading to their maturation and production of cytokines with a potential to skew the immune reaction towards a Th2-like response (Buentke *et al*, 2000; 2001). Using time-lapse photography, we visualized the interaction between MDDCs and several NK cells, in the presence of *Malassezia*. Similar events, like swelling and loss of membrane integrity in the dying cell, were recorded elsewhere by the same technique, although target cells other than MDDCs were used (Eriksson *et al*, 1999). Spaggiari *et al* showed the involvement of two activating receptors in the NK-cell-mediated induction of cell death in DCs, and several recent

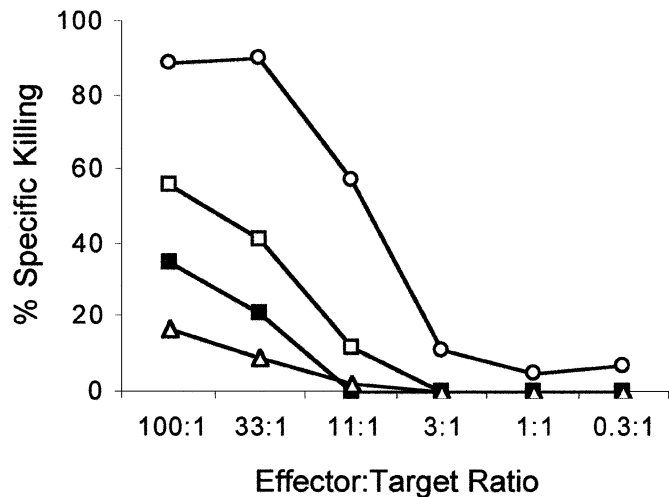


Figure 4. Immature MDDCs are less susceptible to autologous NK-cell-induced cell death if preincubated with the yeast *Malassezia*. The percentage lysis of K562 (circles), of MDDCs (4×10^3 per well) incubated in medium alone (squares), of MDDCs incubated for 46 h with the yeast *Malassezia* at a 1:5 ratio (filled squares), and of MDDCs cultured with LPS (100 ng per ml) for 46 h (triangles), by autologous short-term activated polyclonal NK cells, was detected in a 4 h standard ^{51}Cr -release assay. The results shown are from one of three representative experiments, using cells from different blood donors.

in vitro studies have demonstrated functional effects upon interaction between NK cells and DCs, implying an importance for their mutual regulation (Spaggiari *et al*, 2001; Ferlazzo *et al*, 2002; Gerosa *et al*, 2002; Piccioli *et al*, 2002).

Using a ^{51}Cr -release assay, we found that immature MDDCs cocultured with *Malassezia* for only 20 h were as susceptible to NK-cell-induced cell death as MDDCs incubated in medium. When immature MDDCs were cocultured with the yeast for 46 h, however, they became less susceptible to NK-cell-induced cell death, as were MDDCs matured with LPS. In one donor, an autologous NK cell clone was also produced and the results were the same as when using the short-term activated polyclonal NK cells (data not shown). Additionally, *Malassezia* induced maturation of MDDCs resulting in an increased number of CD80-, CD83-, and CD86-positive cells after coculture for approximately 46 h (Buentke *et al*, 2001). Evidently, MDDCs pretreated with *Malassezia* require time to fully mature for the reduced susceptibility to NK-cell-induced cell death to occur. DCs in different maturation stages have been reported to be differently susceptible to NK cell lysis. Human immature DCs were found to be more susceptible to autologous NK-cell-induced cell death than DCs matured by LPS, tumor necrosis factor α , monocyte-conditioned medium, or CD40L engagement (Carbone *et al*, 1999; Wilson *et al*, 1999). Viral infection also rendered the DCs less susceptible to NK-cell-induced cell death (Wilson *et al*, 1999), a conclusion that is in agreement with our observations. A balance of activating and inhibiting signals determines whether NK cells induce cell death in their target cells or not (Moretta *et al*, 2001). This indicates that maturation of DCs by microorganisms, like yeast or virus, or products from microorganisms, like LPS, influence the surface phenotype of DCs or the factors they secrete in such a way that the DCs become less susceptible to NK-cell-mediated lysis.

In addition to cell-cell contact, our data show that soluble factors may play a role in the NK cell-DC interaction. Supernatant from cocultures of MDDCs and *Malassezia* rendered untreated immature MDDCs less susceptible to NK-cell-induced cell death, possibly through soluble factors with an

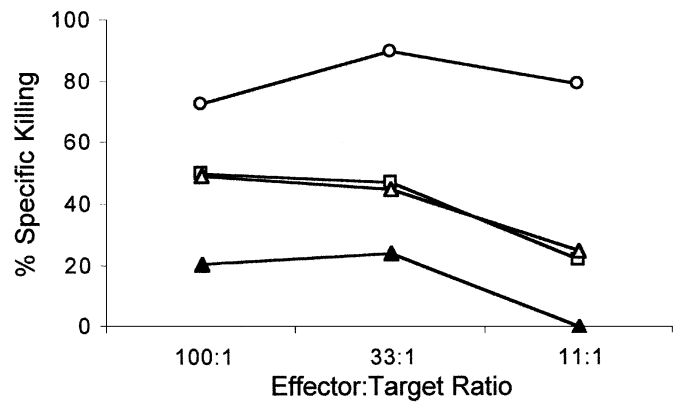


Figure 5. Soluble factors in supernatants from cocultures of MDDCs and *Malassezia* yeast cells lead to decreased induction of autologous NK-cell-mediated cell death in immature MDDCs. A 4 h standard ^{51}Cr -release assay was used to detect the percentage lysis by NK cells of K562 (circles), of MDDCs (4×10^3 per well) cultured in medium alone with no addition of culture supernatants (squares), of MDDCs with addition of culture supernatant from autologous MDDCs cultured in medium for 46 h (triangles), and of MDDCs with addition of culture supernatant from autologous MDDCs cocultured with the yeast *Malassezia* at a 1:5 ratio for 46 h (filled triangles). The results shown are from one of two representative experiments, using cells from different blood donors.

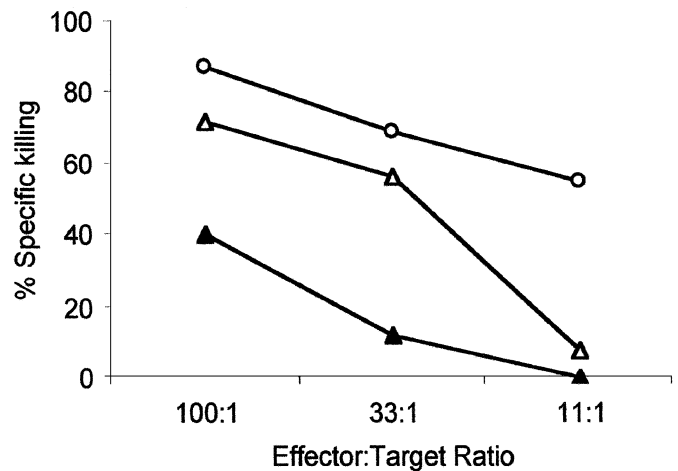


Figure 6. The activity of autologous NK cells is inhibited by soluble factors in supernatants from cocultures of MDDCs and *M. sympodialis*. Short-term activated polyclonal NK cells were preincubated with culture supernatant from MDDCs cultured with or without *Malassezia* or from *Malassezia* alone, for 4 h, before the cytotoxic capacity of the NK cells were assessed on the MHC class I negative target cell line K562. Shown is the percentage lysis of K562 by NK cells preincubated in medium (circles), of K562 by NK cells preincubated in MDDC-*Malassezia* coculture supernatant (1:5 ratio for 46 h, filled triangles), and of K562 by NK cells preincubated in *Malassezia* culture supernatant (46 h, triangles). These results are from one of four representative experiments, using cells from different blood donors in a 4 h standard ^{51}Cr -release assay. Using supernatants from MDDCs incubated with medium resulted in a background induction of NK-cell-mediated cell death of median 93% of the K562 cells at the 100:1 ratio (range 91%–95%, $n = 2$).

inhibitory effect on the NK cell. Cytokines produced by DCs, such as IL-10 and IL-12, have been shown to modulate NK cell activity (Goodier and Londei, 2000; Grufman and Kärre, 2000). We reported previously, however, that DC interaction

with *Malassezia* induced the production of IL-1 β , tumor necrosis factor α , and IL-18, but little or no IL-10 or IL-12p70 (Buentke *et al*, 2001). Soluble factors in the coculture supernatant did result in an increased number of CD86⁺ MDDCs, which might indicate that the soluble factors only indirectly affected the NK cell–DC interaction. Upregulation of MHC class I has been suggested to be of importance in the interaction between mature DCs and NK cells (Ferlazzo *et al*, 2001), but we could not detect any increase in MHC class I mean fluorescence intensity values for MDDCs stimulated with coculture supernatant. Our results also indicate that factors from *Malassezia* itself might have an inhibitory effect on the NK cell cytotoxicity. Possibly the yeast cells produce some substance similar to the reported viral IL-10 (Minter *et al*, 2001). *In vitro* studies have shown that the ratio between NK cells and DCs is important for the outcome of the cell interaction. At high ratios NK-cell-induced cell death is the preferential outcome, whereas mutual activation occurs at lower ratios (Piccioli *et al*, 2002; own unpublished results). We have shown NK cell and DC contact in *Malassezia* APT-positive skin, and that *Malassezia* can influence the interaction of NK cells and DCs *in vitro*, leading to lower NK-cell-induced cell death. What the preferential effect of *Malassezia* on the cross-talk *in vivo* is needs to be further investigated. Our findings that *Malassezia* directly or indirectly affects the interaction of NK cells and CD1a⁺ DCs, in concert with previous reports on the involvement of *Malassezia* in AEDS (Faergemann, 1999; Scheynius *et al*, 2002), suggest that the allergenic yeast may interfere and/or affect the cross-talk between NK cells and DCs in this disease.

In conclusion, our data indicate that CD56⁺ CD3⁻ NK cells and CD1a⁺ DCs can interact in allergen-provoked APT-positive skin of AEDS patients. The differential distribution of NK cells in nonlesional compared to lesional and *Malassezia* APT-positive skin from AEDS patients suggests that NK cells may play a role in regulating DCs in AEDS. These data also imply that the allergenic yeast *Malassezia* affects the interaction of NK cells and DCs, and that soluble factors from the DCs or the yeast may be of importance in the NK cell–DC cross-talk in addition to cell–cell contact.

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REFERENCES

Alm JS, Swartz J, Lilja G, Scheynius A, Pershagen G: Atopy in children of families with an anthroposophic lifestyle. *Lancet* 353:1485–1488, 1999

Banchereau J, Briere F, Caux C, *et al*: Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767–811, 2000

Boulloc A, Charue D, Nikolova M, Bensussan A: No modulation of circulating natural killer cell and natural killer receptor bearing memory T cell subsets in patients with atopic dermatitis. *J Invest Dermatol* 115:1160–1162, 2000

Buentke E, Zargari A, Heffler LC, Avila-Carino J, Savolainen J, Scheynius A: Uptake of the yeast *Malassezia furfur* and its allergenic components by human immature CD1a⁺ dendritic cells. *Clin Exp Allergy* 30:1759–1770, 2000

Buentke E, Heffler LC, Wallin RP, Lofman C, Ljunggren HG, Scheynius A: The allergenic yeast *Malassezia furfur* induces maturation of human dendritic cells. *Clin Exp Allergy* 31:1583–1593, 2001

Carbone E, Terrazzano G, Ruggiero G, *et al*: Recognition of autologous dendritic cells by human NK cells. *Eur J Immunol* 29:4022–4029, 1999

Chambers BJ, Salcedo M, Ljunggren HG: Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). *Immunity* 5:311–317, 1996

Chiarelli F, Canfora F, Verrotti A, Amerio P, Morgese G: Humoral and cellular immunity in children with active and quiescent atopic dermatitis. *Br J Dermatol* 116:651–660, 1987

Cooper KD: Atopic dermatitis: recent trends in pathogenesis and therapy. *J Invest Dermatol* 102:128–137, 1994

Eriksson M, Leitz G, Fallman E, Axner O, Ryan JC, Nakamura MC, Sentman CL:

Inhibitory receptors alter natural killer cell interactions with target cells yet allow simultaneous killing of susceptible targets. *J Exp Med* 190:1005–1012, 1999

Faergemann J: *Pityrosporum* species as a cause of allergy and infection. *Allergy* 54:413–419, 1999

Ferlazzo G, Semino C, Melioli G: HLA class I molecule expression is up-regulated during maturation of dendritic cells, protecting them from natural killer cell-mediated lysis. *Immunol Lett* 76:37–41, 2001

Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C: Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 195:343–351, 2002

Fernandez NC, Lozier A, Flament C, *et al*: Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses *in vivo*. *Nat Med* 5:405–411, 1999

Geldhof AB, Moser M, Lespagnard L, Thielemans K, De Baetselier P: Interleukin-12-activated natural killer cells recognize B7 costimulatory molecules on tumor cells and autologous dendritic cells. *Blood* 91:196–206, 1998

Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G: Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* 195:327–333, 2002

Goodier MR, Londei M: Lipopolysaccharide stimulates the proliferation of human CD56⁺ CD3⁻ NK cells: a regulatory role of monocytes and IL-10. *J Immunol* 165:139–147, 2000

Granucci F, Vizzardelli C, Pavelka N, *et al*: Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nat Immunol* 2:882–888, 2001

Gruftman P, Kärre K: Innate and adaptive immunity to tumors: IL-12 is required for optimal responses. *Eur J Immunol* 30:1088–1093, 2000

Hashiro M, Okumura M: The relationship between the psychological and immunological state in patients with atopic dermatitis. *J Dermatol Sci* 16:231–235, 1998

ISAAC: Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 351:1225–1232, 1998

Johansson C, Eshaghi H, Tengvall Linder M, Jacobson E, Scheynius A: Positive atopy patch test reaction to *Malassezia furfur* in atopic dermatitis correlates with a Th2-like PBMC response. *J Invest Dermatol* 118:1044–1051, 2002

Johansson SGO, Hourihane JO, Bousquet J, *et al*: A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy* 56:813–824, 2001

Johnson GD, Davidson RS, McNamee KC, Russell G, Goodwin D, Holborow EJ: Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J Immunol Meth* 55:231–242, 1982

Kaburagi Y, Shimada Y, Nagaoka T, Hasegawa M, Takehara K, Sato S: Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-1 α , MIP-1 β , and eotaxin) in patients with atopic dermatitis. *Arch Dermatol Res* 293:350–355, 2001

Korsgren M, Persson CG, Sunder F, *et al*: Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice. *J Exp Med* 189:553–562, 1999

Lesko MJ, Lever RS, Mackie RM, Parrott DM: The effect of topical steroid application on natural killer cell activity. *Clin Exp Allergy* 19:633–636, 1989

Leung DY: Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 105:860–876, 2000

Mayer P, Gross A: IgE antibodies to *Malassezia furfur*, *M. sympodialis* and *Pityrosporum orbiculare* in patients with atopic dermatitis, seborrheic eczema or pityriasis versicolor, and identification of respective allergens. *Acta Derm Venereol* 80:357–361, 2000

Minter RM, Ferry MA, Rectenwald JE, *et al*: Extended lung expression and increased tissue localization of viral IL-10 with adenoviral gene therapy. *Proc Natl Acad Sci USA* 98:277–282, 2001

Moretta L, Bottino C, Vitale M, *et al*: Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu Rev Immunol* 19:197–223, 2001

Nakamura S, Suchi T, Koshikawa T, *et al*: Clinicopathologic study of CD56 (NCAM) -positive angiocentric lymphoma occurring in sites other than the upper and lower respiratory tract. *Am J Surg Pathol* 19:284–296, 1995

Piccioli D, Sbrana S, Melandri E, Valiante NM: Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* 195:335–341, 2002

Romani N, Gruner S, Brang D, *et al*: Proliferating dendritic cell progenitors in human blood. *J Exp Med* 180:83–93, 1994

Romani N, Reider D, Heuer M, *et al*: Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J Immunol Meth* 196:137–151, 1996

Sallusto F, Lanzavecchia A: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179:1109–1118, 1994

Scheynius A, Johansson C, Buentke E, Zargari A, Tengvall Linder M: Atopic eczema/dermatitis syndrome and *Malassezia*. *Int Arch Dermatol* 127:161–169, 2002

Spaggiari GM, Carosio R, Pende D, *et al*: NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46. *Eur J Immunol* 31:1656–1665, 2001

Strannegård IL, Strannegård Ö: Natural killer cells and interferon production in atopic dermatitis. *Acta Dermato-Venerologica Suppl* 92:48, 1980

Tengvall Linder M, Johansson C, Scheynius A, Wahlgren C: Positive atopy patch test

- reactions to *Pityrosporum orbiculare* in atopic dermatitis patients. *Clin Exp Allergy* 30:122–131, 2000
- Vulcano M, Albanesi C, Stoppacciaro A, et al: Dendritic cells as a major source of macrophage-derived chemokine/CCL22 *in vitro* and *in vivo*. *Eur J Immunol* 31:812–822, 2001
- Wehrmann W, Reinhold U, Kukul S, Franke N, Uerlich M, Kreysel HW: Selective alterations in natural killer cell subsets in patients with atopic dermatitis. *Int Arch Allergy Appl Immunol* 92:318–322, 1990
- Werfel T, Kapp A: Environmental and other major provocation factors in atopic dermatitis. *Allergy* 53:731–739, 1998
- Wilson JL, Heffler LC, Charo J, Scheynius A, Bejarano MT, Ljunggren HG: Targeting of human dendritic cells by autologous NK cells. *J Immunol* 163:6365–6370, 1999
- Zachary CB, Allen MH, MacDonald DM: *In situ* quantification of T-lymphocyte subsets and Langerhans cells in the inflammatory infiltrate of atopic eczema. *Br J Dermatol* 112:149–156, 1985
- Zhou LJ, Tedder TF: CD14⁺ blood monocytes can differentiate into functionally mature CD83⁺ dendritic cells. *Proc Natl Acad Sci USA* 93:2588–2592, 1996