Regular paper

Chemerin and the recruitment of NK cells to diseased skin[★]

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Natural killer (NK) cells play a major role in the initial control of many viral pathogens and in the rejection of tumors. Consistent with their roles as immune sentinels, NK cells are found in inflamed skin, including lichen planus, psoriasis and atopic dermatitis (AD) lesions. In oral lichen planus lesions, the recruitment as well as intradermal colocalization of NK cells and pDC (plasmacytoid dendritic cells) appear to be mediated by chemerin, a recently identified protein ligand for chemokine-like receptor 1 (CMKLR1), a chemoattractant receptor expressed by both cell types. Dendritic cells can regulate NK cell activity, and NK cells can regulate DC-mediated responses. Since chemerin was recently implicated in recruitment of pDC to psoriatic skin, in this work we determined whether chemerin facilitates interactions between NK and pDC in psoriatic plaques through controlling influx of NK cells to diseased skin. We demonstrate that circulating NK cells from normal donors as well as psoriasis and AD patients respond similarly in functional migration assays to chemerin. However, differences in the distribution of NK cells and pDC in skin lesions suggest that recruitment of both NK cells and pDC is unlikely to be controlled solely by chemerin.

Keywords: chemerin, NK cells, skin diseases, viral pathogens

INTRODUCTION

Chemerin was initially named *tazarotene-induced gene 2 (tig-2)*, a designation that reflects its transcriptional upregulation in lesional psoriatic skin by the anti-psoriatic synthetic retinoid tazarotene (Nagpal *et al.*, 1997). More recently, through G protein-associated receptor screening assays, chemerin was identified as a protein ligand for the orphan seven-pass transmembrane receptor chemokine-like receptor 1 (CMKLR1) (Wittamer *et al.*, 2003; Zabel *et al.*, 2005b). Several specific functions have been ascribed to chemerin so far, including regulation of specific immune cell migration (Wittamer *et al.*, 2003; Zabel *et al.*, 2005b), anti-inflammatory effects on macrophages (Cash *et al.*, 2008), and regula-

tion of adipogenesis (Goralski *et al.*, 2007). Human chemerin is synthesized as an aprox. 18 kDa precursor which undergoes protease-mediated C-terminal truncation to generate a potent chemoattractant for CMKLR1-expressing cells. Enzymes that contribute to this conversion include host serine proteases of the coagulation, fibrinolytic and inflammatory cascades (Du *et al.*, 2009; Wittamer *et al.*, 2005; Zabel *et al.*, 2005a), circulating carboxypeptidases (Du *et al.*, 2009), as well as staphopain B, a cysteine protease secreted by *Staphylococcus aureus* (Kulig *et al.*, 2007).

CMKLR1 exhibits a selective expression pattern among immune cells. The best documented is the presence of this receptor on immature plasmacytoid dendritic cells (pDC) and resident macrophages (Zabel *et al.*, 2006a; Zabel *et al.*, 2005b). In addition,

recent data indicate that a subset of natural killer (NK) cells express functional CMKLR1 (Parolini *et al.,* 2007). NK cells represent an important component of the immune system through their ability to both lyse target cells and to secrete a variety of immuno-regulatory cytokines. Although NK cells are mainly recognized as cells of the innate immune system capable of killing virally-infected cells or tumor cells (Moretta *et al.,* 2008), recent data indicate that NK cells also posses adaptive immune features (Cooper *et al.,* 2009; Sun *et al.,* 2009). Interaction between NK cells and dendritic cells (DC) is of particular interest, due to mutual modulation of cell function. For example, NK cells play an important role in controlling DC differentiation or in killing immature DC (Moretta *et al.,* 2008). On the other hand, interferon alpha-producing pDC induce NK cytolytic activity (Colonna *et al.,* 2004). The molecules responsible for "bringing these cells together" at sites of inflammation remain poorly defined. In this context it is of interest that NK cells have been shown to colocalize with pDC in oral lichen planus lesions in a chemer-in-dependent manner (Parolini *et al.,* 2007). Chemer-in-mediated recruitment of pDC and NK cells might therefore represent a general mechanism to enable pDC and NK cell cross-regulation in diseased skin.

Recently, we and others have demonstrated that chemerin is present in psoriatic skin lesions, and likely plays a key role in regulating skin infiltration by pDC in psoriasis (Albanesi *et al.,* 2009; Skrzeczyn-ska-Moncznik *et al.,* 2009). Although pDC are absent in normal skin, lesional skin from patients suffering from psoriasis vulgaris is characterized by substantial numbers of pDC. Infiltrating NK cells have also been described in psoriatic skin, although accumulation of NK cells was reported to be mediated by CXCL10, a ligand for CXCR3, and CCL5, a ligand for CCR5 (Ottaviani *et al.,* 2006).

In this work we demonstrate that circulating NK cell subsets derived from psoriatic patients migrate to chemerin, suggesting that this chemoattractant contributes to NK cell migration to diseased skin. However, since NK cell infiltration does not always correlate with the presence of pDC in diseased skin, other chemoattractants may provide additive or alternative signals in recruiting these cells to affected skin.

MATERIALS AND METHODS

Chemoattractants. Recombinant human CXCL12 (SDF-1α) and recombinant human CXCL10 (IP-10) were purchased from R&D Systems. Sta-phopainB (SspB) activated chemerin was prepared as described previously (Kulig *et al.,* 2007). Briefly, recombinant human pro-chemerin, expressed in in-

sect cells was treated with *S. aureus*-derived cysteine protease SspB at a 100:1 pro-chemerin:active SspB molar ratio at 37°C in a buffer containing 50 mM Hepes, 100 mM NaCl, pH 7.2. The 16 155.4 Da SspB-mediated cleavage product, further referred to as chemerin/SspB was purified by HPLC and used for cell migration experiments. Since SspB treatment of pro-chemerin generates a potent chemoattractant (Kulig *et al.,* 2007), chemerin/SspB was used in all experiments as a bioactive form of this molecule.

Patients. All human studies were performed in accordance with guidelines established by the Jagiellonian University Institutional Bioethics Committee under approved protocols. Declaration of Helsinki protocols were followed. A total of 16 psoriasis patients (age 36.7±15.1 years; F: 7, M: 9), 14 AD patients (age 28.9±6.9 years; F: 6, M: 8) and 12 healthy individuals (age 33.3±10 years; F: 5, M: 7) were enrolled into these studies. The severity of the psoriasis skin lesions was assessed according to the Psoriasis Area Severity Index score (PASI) (minimum point 0, maximum point 72) and ranged from 9.6 to 34.6 (mean±S.D., 21.4±8.1). The AD patients were diagnosed according to the criteria defined by Hanifin and Rajka (1993). Disease severity in AD was measured according to the Severity Scoring of Atopic Dermatitis (SCORAD) (minimum point 0, maximum point 100) and ranged from 12 to 82 (mean±S.D., 42.1±19.3). Patients on UV therapy, systemic or local corticosteroid treatment, or with viral infections were excluded from the studies. Healthy control subjects had no clinical signs of dermatologic or allergic diseases.

Flow cytometry analysis. Human blood was collected and peripheral blood mononuclear cells (PBMC) were harvested following LSM1077 (PAA Laboratories) gradient separation. Cells were blocked with 50–80% autologous plasma and then stained for flow cytometry using mAbs to CD56, CD16 and CD3 (reagents from BD Pharmingen eBioscience, Beckman Coulter and Dako). Stained cells were analyzed on an LSRII flow cytometer (Becton Dickinson).

NK cell chemotaxis assay. Blood was collected from psoriasis and AD patients as well as normal individuals, and PBMC were harvested as described above. The chemotaxis assay was performed using 5-μm pore Transwell inserts. PBMC (0.4–0.5×10⁶) in 100 μl were added to the top wells, and various chemoattractants were added to the bottom wells in a 600 μl volume. Migration was assayed for 2 h at 37°C, then the inserts were removed, and the cells that had migrated through the filter to the lower chamber were harvested, stained with anti-CD16-FITC, anti-CD56PE, and analyzed by flow cytometry. The number of gated NK cell subsets defined as CD56^{hi} CD16- or CD56^{low} CD16^{+} subsets counted in 60 s was used as the migration output.

Immunocytochemistry. Frozen 8-μm sections were prepared from skin biopsies. Sections were fixed in acetone, blocked with mouse IgG and stained with the following directly conjugated antibodies; APC-anti-CD3 (BD Pharmingen) and PE-anti-CD56 (Biolegend) or PE-anti-BDCA2 (Miltenyi Biotech).

Statistical analysis. For statistical evaluation, two-tailed Student's *t*-test was performed.

RESULTS

Psoriasis and atopic dermatitis (AD) represent chronically relapsing skin diseases with skin-infiltrating leukocytes playing a crucial role in the initiation and maintenance of both disorders (Leung *et al.*, 2004; Nestle *et al.*, 2005). However, whereas autoimmune psoriasis is characterized by substantial numbers of skin-infiltrating pDC that significantly contribute to the pathogenesis of the disease (Nestle *et al.*, 2005), few pDC are present in skin lesions of AD patients (Wollenberg *et al.*, 2002). Notably, the percentage of circulating pDC is lower in psoriasis patients compared to healthy individuals, whereas a higher frequency of pDC is noted in AD patients, suggesting that pDC leaving the circulation could account for the lower percentage of these cells among circulating leukocytes in patients suffering from psoriasis (Skrzeczynska-Moncznik *et al.*, 2009). To examine whether NK cells follow a similar frequency pattern, we analyzed NK cell subsets derived from the blood of psoriasis and AD patients as well as normal donors by flow cytometry. Two subsets of circulating NK cells have been reported; CD56^{low} CD16$^+$ NK effector cells, and CD56^{hi} CD16$^-$ NK cells, characterized by limited cytotoxic function but a pronounced immunoregulatory phenotype (Ottaviani *et al.*, 2006; Parolini *et al.*, 2007). As shown in Fig. 1, similar percentages of CD56^{hi} CD16$^-$ NK cells were found in normal individuals as well as psoriasis and AD patients. In contrast, the percentage of circulating CD56^{low} CD16$^+$ NK cells was lower in AD, but not psoriasis patients, compared to healthy individuals. The reduced frequency of NK cells in patients suffering from AD is in agreement with a previous report (Katsuta *et al.*, 2006). Taken together, these data suggest that the recruitment of pDC and NK cell is regulated by different mechanisms.

To assess functional migratory responses of NK cells, we performed *in vitro* transwell chemotaxis assays on freshly isolated PBMC from normal, psoriasis, and AD patients, and stained for NK cell subsets after migration. The migratory response of NK cells to CXCL12, CXCL10 and chemerin was analyzed. All three chemoattractants have been shown before to support pDC migration to diseased skin
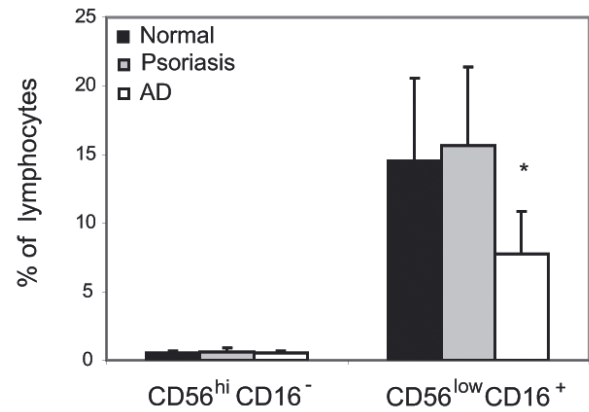

Figure 1. Circulating NK cells are present at a lower frequency in AD patients compared with psoriasis patients and normal donors.
PBMC were isolated from the blood of indicated donors and stained for NK cells using anti- CD56 and CD16 mAbs. The cells were then analyzed by flow cytometry. Data are shown as percentage of NK cell subsets among the cells falling in a lymphocyte light scatter gate (mean ±S.D. for the indicated number of donors; normal n=6; psoriasis n=10; AD n=8). Statistically significant difference between normal and AD NK cell levels is indicated by asterisk (*P<0.05; Student's *t*-test).

(Vanbervliet *et al.*, 2003; Albanesi *et al.*, 2009; Skrzeczynska-Moncznik *et al.*, 2009). Whereas the homeostatic chemokine CXCL12 and the inducible pro-inflammatory chemokine CXCL10 are present in both psoriatic and AD skin, active chemerin appears to be primarily associated with psoriasis (Albanesi *et al.*, 2009; Skrzeczynska-Moncznik *et al.*, 2009). In addition to pDC, NK cells have been reported to express CMKLR1 (Vermi *et al.*, 2005; Zabel *et al.*, 2005b; 2006b; Parolini *et al.*, 2007), suggesting that, similarly to pDC, they might be recruited *via* chemerin activation in skin disorders. We first determined whether circulating NK cells derived from psoriasis patients migrated more efficiently to chemerin compared with normal controls. Of the two NK cell subsets, only the CD56^{low} CD16$^+$ NK cells migrated to chemerin (Fig. 2), in agreement with previous studies demonstrating selective expression of functional CMKLR1 by CD56^{low} CD16$^+$ NK cells (Parolini *et al.*, 2007). However, there were no significant differences in CD56^{low} CD16$^+$ NK cell migration to chemerin among the patient groups (Fig. 2A). CD56^{low} CD16$^+$ NK cells from all groups also responded with similar migration efficiency to CXCL12 (Fig. 2A). The CD56^{hi} CD16$^-$ NK cell subset, although unresponsive to chemerin, migrated efficiently to CXCL12 (Fig. 2B). CXCL10 was a more potent chemoattractant for CD56^{hi} CD16$^-$ NK cells than for CD56^{low} CD16$^+$ NK cells, perhaps reflecting preferential expression of CXCR3 on the CD16$^-$ subset (Ottaviani *et al.*, 2006). A combination of CXCL12 and CXCL10
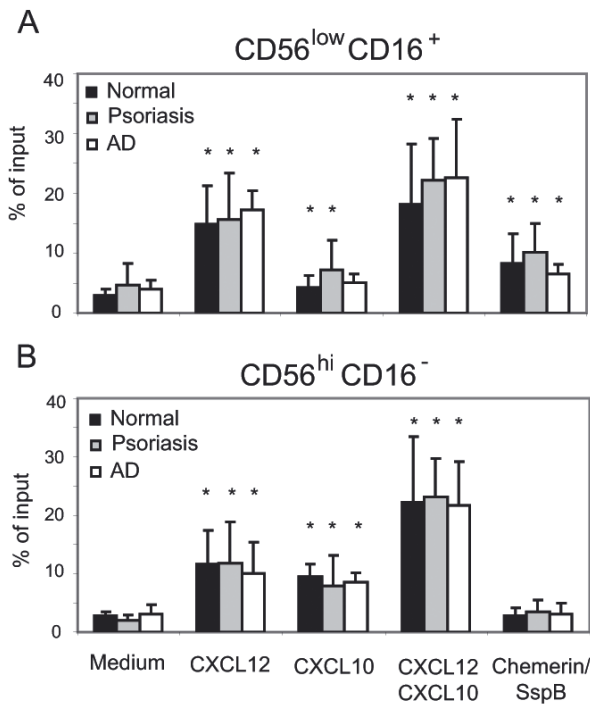
Figure 2. CD56^{low} CD16^{+} blood NK cells from normal donors as well as psoriasis and AD patients respond to active chemerin.

Total PBMC were tested in transwell chemotaxis, and the migrated cells were stained for CD56 and CD16 to define two circulating NK cell subsets, CD56^{low} CD16^{+} (**A**) and CD56^{hi} CD16^{-} (**B**). The following chemoattractants were used as indicated: CXCL12 (10 nM), CXCL10 (115 nM) and chemerin/SspB (50 pM). Migration to chemotaxis medium served as a negative control (Medium). NK cell subset migration is displayed as the mean of six independent donors in each group ±S.D. Statistically significant differences in the migration to the negative control (chemotaxis medium) *vs.* various chemoattractants in pairwise comparisons within patient groups was determined by Student's *t*-test (*P*<0.05) and indicated by *.

triggered the most robust chemotactic response in both NK cell subsets (Fig. 2). Taken together, these findings confirm a differential pattern of chemotactic responses by NK cell subsets but indicate similar chemotactic properties of NK cell subsets among the donor groups.

Previous immunohistochemical data suggest that NK cells are recruited to psoriasis and AD skin (Buentke *et al.*, 2002; Ottaviani *et al.*, 2006). In agreement with those studies, we found NK cells (CD56^{+}CD3^{-} cells of lymphocyte morphology) in the dermis of lesional skin from psoriasis and AD patients (Fig. 3A). NK cells were present throughout the T cell-rich infiltrate in the dermis, although small clusters of NK cells essentially devoid of T cells were also observed in inflamed AD skin (Fig. 3A). The same skin biopsies failed to stain for pDC (not shown), suggesting different recruitment pathways for NK cells and pDC. Since infiltration of psoriatic skin by pDC appears to be associated only with early phases of developing skin lesions (Albanesi *et al.*, 2009), we also analyzed biopsies of pDC-infiltrated psoriatic skin for NK cells. As demonstrated in Fig. 3B, pDC were found in the dermis and the dermal papillae, in the latter case without obvious co-localization of T cells. In contrast, NK cells were primarily associated with T cell infiltrates in the dermis. Moreover, the distribution of NK cells was similar in pDC-positive and pDC-negative skin biopsies (Fig. 3A and B), suggesting that NK cell trafficking to psoriatic skin is not paralleled by pDC recruitment.

DISCUSSION

Psoriasis is one of the most common autoimmune disorders in humans and recent findings
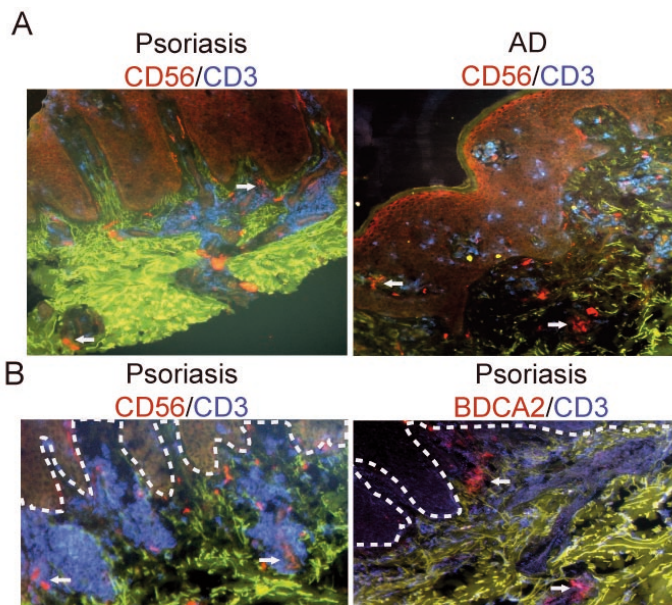

Figure 3. NK cell skin infiltration does not correlate with pDC influx.

Frozen sections of skin biopsies from indicated patients were stained to detect T cells (CD3^{+}, blue), NK cells (CD56^{+}, red; CD3^{-}) or pDC (BDCA2^{+}, red). Autofluorescent components of extracellular matrix are shown in green. The slides were examined by fluorescence microscopy. Original magnification: ×10. Dotted lines in B indicate location of epidermis. Arrows indicate NK cells (upper panel and lower panel, left) and pDC (lower panel, right). Data shown in B represent serial sections. Data are representative of three different donors in each staining combination.

strongly implicate activated pDC in this pathologic condition (Nestle *et al.*, 2005; Lande *et al.*, 2007). Interestingly, both pDC and NK cells constitute the inflammatory infiltrate in psoriasis, suggesting possible crosstalk between these cells in the initiation and/or maintenance of psoriatic inflammation. The mutual interaction of pDC with NK cells has been reported to profoundly influence activation status of both types of cells, leading on one hand to the augmentation of NK cell cytolytic activity by pDC, and on the other hand, to the induction of pDC maturation by activated NK cells (Gerosa *et al.*, 2005). Therefore, reciprocal contact between pDC and NK cells would be predicted to be of important consequence for the development of psoriatic lesions and a better understanding of the rules that govern the pDC-NK relationship will help develop new approaches for the diagnosis and/or treatment of psoriasis.

Since circulating pDC and NK cells both express CXCR3 and CMKLR1, we reasoned that their respective ligands, CXCL10 and chemerin, might contribute to either pDC or NK cell influx to psoriatic skin, enabling local interactions between these cells in psoriatic plaques. Chemerin appears to cooperate with CXCL10 in directing pDC to skin in psoriasis (Skrzeczynska-Moncznik *et al.*, 2009). Notably, while CD56^{hi} CD16$^-$ NK cell infiltrates have been reported in psoriatic skin, CD16$^+$ NK cells have not been detected (Ottaviani *et al.*, 2006). Since circulating CD56^{hi} CD16$^-$ NK cells from psoriasis patients do not migrate to chemerin (while CD16$^+$ NK cells do) (Fig. 2), this implies that chemerin-independent mechanisms likely guide the recruitment of CD56^{hi} CD16$^-$ NK cells to psoriatic skin, and that the regulation of CD16$^+$ NK cell recruitment is more complex than simply by chemerin: CMKLR1 interactions. Other chemokines might provide alternative or additional signals to regulate NK cell infiltration of diseased skin, including CCL5, CCL4 or CXCL8 (Leung *et al.*, 2004; Ottaviani *et al.*, 2006; Parolini *et al.*, 2007).

Chemerin has been shown to play a role in the co-localization of NK cells and pDC in oral lichen planus lesions (Parolini *et al.*, 2007), suggesting that the accumulation of both NK cells and pDC observed in other skin diseases might depend on this chemoattractant. However, the results of this work together with previous reports (Parolini *et al.*, 2007; Albanesi *et al.*, 2009; Skrzeczynska-Moncznik *et al.*, 2009) suggest that, despite being chemerin-responsive, pDC and NK cells follow distinct trafficking patterns in skin disorders. First, the differences in the frequency of circulating pDC and NK do not similarly reflect disease-associated variations in pDC and NK cell migration to local sites. Furthermore, NK cells infiltrate either psoriasis or AD skin, even though AD skin appears to be largely chemerin-

negative (Skrzeczynska-Moncznik *et al.*, 2009) and pDC are thought to be generally absent in AD skin lesions (Wollenberg *et al.*, 2002). Moreover, psoriatic skin infiltration by NK cells does not correlate with pDC recruitment into skin, since NK cells mixed with T cells can be found in psoriatic skin despite the absence of pDC. Finally, pDC-positive biopsies of lesional psoriatic plaques that are likely to contain chemotactively active chemerin (Albanesi *et al.*, 2009; Skrzeczynska-Moncznik *et al.*, 2009) do not show obvious co-localization of NK cells and pDC.

Taken together these data suggest at least partially distinctive homing properties of pDC and NK cells to diseased skin and implicate other chemoattractants that may play additive effects in recruiting these leukocytes to inflamed skin.

Acknowledgements

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