CD56^{high}CD16$^-$CD62L$^-$ NK Cells Accumulate in Allergic Contact Dermatitis and Contribute to the Expression of Allergic Responses

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Allergic contact dermatitis is a common disease caused by an exaggerated T cell-mediated immune response to skin-applied haptens. We show in this study that NK cells affect skin immune responses to haptens by releasing type 1 cytokines and inducing keratinocytes apoptosis. Immunohistochemical stainings demonstrated that NK lymphocytes constitute ~10% of the inflammatory infiltrate mostly distributed in the superficial dermis and in the epidermis at the site of intense spongiotic changes. More than 90% of NK cells isolated from allergic contact dermatitis skin showed a CD3$^-$CD56^{high}CD16$^-$ phenotype by FACS analysis. In addition, they uniformly expressed NKG2A, intermediate to high levels of perforin, and the activating receptors, NKG2D, NKp44, and NKp46, but lacked NKp30 and killer Ig-related receptors. Skin NK lymphocytes displayed a CXCR3$^+$CCR6$^+$CCR5$^+$ chemokine receptor asset for homing into inflamed skin, but not CD62L and CCR7 for lymph node homing. When NK cells from nickel-allergic donors were exposed in vitro to the metal, they failed to proliferate, to upregulate CD69, and to release IFN-γ, thus indicating that NK lymphocytes do not exhibit memory-like properties to haptens. However, IL-2 released by hapten-driven T lymphocytes rapidly induced the release of IFN-γ by NK cells and promoted the NK-mediated apoptosis of autologous keratinocytes in a hapten-independent manner. Our findings underline the importance of the interaction between innate and adaptive immune mechanisms for amplification of skin allergic responses to haptens and full expression of allergic contact dermatitis

Allergic contact dermatitis (ACD) and its murine counterpart contact hypersensitivity (CHS) are delayed type immune responses directed toward skin applied small chemicals, so-called haptens (1, 2). In sensitized individuals, the immune reaction is initiated by skin-infiltrating hapten-reactive CD8$^+$ T lymphocytes, which are very efficient in inducing keratinocyte apoptosis, resulting in e-cadherin cleavage and spongiosis (3–6). Effector CD4$^+$ T lymphocytes, which outnumber CD8$^+$ T cells in ACD skin, also contribute to disease expression, being the major source of proinflammatory cytokines that promote the activation of resident cells (7, 8). Keratinocytes are not only the primary target cells for cytotoxic skin lymphocytes, but they actively regulate the magnitude of the immune response by releasing a variety of chemotactic factors which affect the quality of the lymphocytic infiltrate. In particular, T cell-derived IFN-γ and TNF-α induce

keratinocyte synthesis and release CXCL9, CXCL10, CCL2, CCL5, and CCL20, which are critical for the accumulation of type 1 CD8$^-$ and CD4$^-$ T lymphocytes, and thus for the amplification of the ACD reaction (7, 9). Although ACD is considered a Th1-dominated disease, other cytokines, such as IL-4 and IL-17. are abundantly expressed in acute ACD skin, and contribute to disease expression by enhancing the release of CXCR3 agonists induced by IFN-γ (10), and by increasing ICAM-1 expression by keratinocyte, respectively (11).

Although CD3$^+$ T lymphocytes are the most prominent cellular type recruited in ACD skin, the inflammatory infiltrate also comprises of myeloid and plasmacytoid dendritic cells, as well as NK cells and NK-T lymphocytes (11–14).

NK cells are innate immune lymphocytes that lack rearranged TCR for Ag recognition. NK effector functions rely on the homeostasis between germline-encoded activating and inhibitory receptors (15). Missing recognition of MHC class I, or MHC class I-related molecules on transformed or viral-infected cells rapidly induces NK cell activation, cytokine release, perforin and granzyme exocytosis, and finally lysis of target cells (16). Two major subsets of human NK cells have been distinguished on the basis of their functional properties, migratory capacity. and expression of surface markers (17). The majority of circulating NK cells are CD56^{low}CD16$^+$, contain high levels of preformed perforin and possess strong cytotoxic capacity. In contrast, CD56^{high}CD16$^-$ NK cells constitute 10–15% of circulating NK lymphocytes, but prevail in secondary lymphoid organs (18). CD56^{high}CD16$^-$ NK cells store lower levels of perforin, but have prominent immunomodulatory functions, as they release high amounts of cytokines (19, 20). Tissue distribution of these two major NK cell subsets depends on distinct expression of chemokine receptors. Cytotoxic CD56^{low}CD16$^+$ NK lymphocytes are mainly recruited in pathogen-invaded inflamed tissues because of expression of CXCR1 and CX3CR1 (21), whereas CD56^{high}CD16$^-$ NK cells express CD62L and CCR7 that allow migration from the bloodstream into lymph nodes (22–24). In addition, a subset of CD56^{high}

CD16$^-$ NK lymphocytes invades certain peripheral tissues, such as inflamed skin and uterus, in a CXCL10/CXCR3-dependent mechanism (25–27).

Evidence has been provided that NK cells accumulate in many immunomediated skin diseases, such as psoriasis, lichen planus, and ACD, but their contribution to skin inflammation has been poorly investigated (27, 28). A direct role of NK cells in murine CHS has been recently evidenced by O'Leary and Coll (29). Using Rag$^{-/-}$ mice, which lack T cells but not NK lymphocytes, they demonstrated that CHS to 2,4-dinitrofluorobenzene could be adoptively transferred with NK cells from 2,4-dinitrofluorobenzene-sensitized, but not oxazolone-sensitized or naive mice. However, the mechanisms of hapten recognition by murine NK cells and the NK cell subset specifically expanded on hapten sensitization remain undisclosed.

In this study, we identify a distinct CD56^{high}CD16$^-$ CD62L$^-$ NK cell population that homes into ACD skin by virtue of expressing of the chemokine receptors CXCR3, CCR5, and CCR6 and that contributes to amplifying the allergic reaction by releasing IFN-γ and inducing keratinocyte apoptosis in a hapten nonspecific manner.

Materials and Methods

Patients included in the study

Patients with allergic contact dermatitis to nickel (five), thiouram (one), diaminopropylamine (one), cobalt (one), and healthy volunteers (four) were included in this study. Diagnosis was confirmed or excluded by the clinical history and positivity or negativity to a patch test series (International Contact Dermatitis Research Group Series) applied on the back under occlusion. Before blood samples and punch biopsies from lesional ACD skin or from 24- to 72-h positive patch tests for nickel were obtained, each patient gave written informed consent. The study was performed according to the Declaration of Helsinki with regard to scientific use, and approved by the ethical committee of the Istituto Dermopatico Dell'Immacolata, Rome, Italy.

Culture medium, reagents, Abs, and chemokines

Lymphocytes were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Lonza, Basel, Swisserland) (complete RPMI) plus 5% human serum (HS) (Sigma-Aldrich, St. Louis, MO) or 10% FBS (Hyclone, Logan, UT). Keratinocytes were grown in keratinocyte-modified medium (KGM) (Lonza), or in supplemented Ham F12 and DMEM (Biochrom KG, Berlin, Germany) medium, as previously described (7). For immunohistochemical analysis, mouse pure mAb anti-human CD16 (2H7, IgG2a) (Vector Laboratories, Burlingame, CA), CD56 (CD564, IgG2b), perforin (5B13, IgG1) (both from Novocastra, Newcastle, U.K.), and polyclonal rabbit anti-human CD3 (DAKO, Glostrun, Denmark) were used as primary Abs. Mouse FITC-PE- allophycocyanin-PB-, or PerCP-conjugated mAb anti-human CD3 (SK7, IgG1), CD16 (3G8, IgG1), CD56 (B159, IgG1), CD69 (L78, IgG1), CD62L (Dreg-56, IgG1), CD158a (HP3E4, IgM), CD158b (CH-L, IgG2b), NKG2A (HP3D9, IgG1), NKB1 (DX9, IgG1), CCR6 (11A9, IgG1), Perforin (G9, IgG2b), IFN-γ (B27, IgG1), TNF-α (Mab11 IgG1), IL-4 (8D4-08, IgG1), and rat anti-human cutaneous lymphocyte-associated Ag (HECA-452, IgM) were from BD Bioscience (San Diego, CA). Mouse PE-conjugated mAb anti-human CCR1 (53504,IgG2b), CCR2 (48607, IgG2b), CCR4 (205410, IgG2b), CCR5 (CTC5, IgG1), CCR7 (150503, IgG2a), CXCR1 (42705, IgG2a), CXCR3 (49801, IgG1), IL-22 (142928, IgG1), rat CCR8 (191704, IgG2b), and rat CCR10 (314305, IgG2a) were from R&D Systems (Abingdon, U.K.). Mouse PE-conjugated mAb anti-human CX3CR1 (2A9–1, IgG2b) was obtained from MBL (Woburn, MA). Allophycocyanin-anti–human IL17A (eBio64-DEC17,IgG1) was from e-Bioscience (San Diego, CA), whereas anti-human NKG2D (ON72, IgG1), NKp30 (Z25, IgG1), NKp44 (Z231, IgG1), and NKp46 (BAB281, IgG1) were from Beckman Coulter (Fullerton, CA). Mouse or rat IgG/IgM isotype controls were purchased from BD Bioscience. For cell culture recombinant human IL-2 (rhIL-2) from Novartis (Origgio, Italy), recombinant human IFN-γ from R&D Systems, PMA, and ionomycin from Sigma-Aldrich were used. For migration assays, the following chemokines were used: CCL1, CCL2, CCL5, CCL17, CCL20, and CXCL10 from R&D Systems, whereas CCL3, CCL4, CXCL8, and CX3CL1 were purchased from Peprotech (London, U.K.).

Immunohistochemistry

Skin biopsies were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were dewaxed and rehydrated. After quenching endogenous peroxidase, achieving Ag retrieval, and blocking nonspecific binding sites, sections were incubated with primary mAbs for 1 h at room temperature in a humid atmosphere. Staining kits were purchased from ScyTek (ScyTek Laboratories, West Logan, UT). Single stainings were developed using 3-amino-9-ethylcarbazole (DAKO) as chromogen, followed by counterstaining with hematoxylin. In double immunostaining, the second primary mAb was added and the avidin-biotin-alkaline phosphatase system and the chromogen alkaline phosphatase substrate kit III (Vector Laboratories) were used to develop the second reaction. As a negative control, primary Abs were omitted or replaced with irrelevant isotype-matched Abs. Quantification of CD56 or CD3 positive cells was performed on five randomly selected photographic fields/slide at 10× magnification by two independent analyzers. Three slides were examined for a total of 30 observations for each time point. The values are the mean ± SD of total observations.

Isolation of skin-infiltrating lymphocytes

Six-millimeter punch skin biopsies were minced with a scalpel and placed in culture in complete RPMI with 5% autologous plasma and 100 U/ml rhIL-2. After 3–6 d, infiltrating lymphocytes emigrated from tissue samples were collected for phenotypic characterization and functional assays.

Migration assay

The chemotactic property of each chemokine was evaluated by measuring cell migration through a 5-μm pore polycarbonate filter in 24-well transwell chambers (Corning Costar, Cambridge, MA). Briefly, cells recovered from skin samples were incubated for 20 min on ice with FITC-conjugated anti-CD56 and PE-conjugated anti-CD3 mAbs, washed once with sterile PBS plus 1% HS, suspended in complete RPMI plus 0,5% BSA (Sigma-Aldrich) at 1×10^6 cells/ml and then added 100 μl to the top chamber. Various concentrations of different chemokines (0.6 ml) were added to the lower chamber of the transwell. After 2 h incubation at 37°C with 5% CO$_2$, cells that had transmigrated into the lower chamber were recovered and analyzed with a FACSCalibur by acquisition for 70 s at a flow rate of 60 μl/min. Results are shown as net migration, which represents the number of cells migrated to the lower chamber in the presence of the chemokine subtracted from the cells migrated in response to the medium alone.

Flow cytometry analysis

For surface marker staining, cell populations were washed with PBS 1% HS/ 0.01% NaN$_3$ and stained with FITC-, PE-, allophycocyanin- PerCP-, or PB-conjugated mAb. Staining with matched isotype control Ig was included. For intracytoplasmic detection of cytokines, lymphocytes were incubated with PMA (10 ng/ml), ionomycine (1 μg/ml), and monensin for 6 h at 37°C and 5% CO$_2$. After 2 h, brefeldin (BD Bioscience) was added for the last 4 h. After a first staining with Abs toward surface markers, cells were collected, fixed, and permeabilized with Cytofix/Cytoperm (BD Bioscience) and stained for 20 min with the indicated anti-cytokine mAb in the presence of Perm/Wash solution (BD Bioscience). Acquisition and analysis were performed using a FACS ARIA equipped with FACSDiva software (BD Biosciences).

Peripheral blood-derived NK lymphocyte purification and cultures

PBMCs derived from healthy and allergic individuals were separated by density gradient centrifugation over Lymphoprep (Nycomed-Pharmacia, Oslo, Norway). To obtain monocytes, PBMCs, after extensive washing, were separated by immunomagnetic selection into CD14$^+$ and CD14$^-$ populations by using magnetic beads according to the manufacturer's protocol (Miltenyi Biotec, Gladbach, Germany). The CD14$^-$ fraction was further purified into CD3$^-$CD56$^+$ fraction using MACS NK isolation kit (Miltenyi Biotec). A further immunomagnetic selection was performed by using CD16 microbeads (Miltenyi Biotec) to obtain NK CD16$^-$ subpopulation. The resulting NK cells (>96% CD56$^+$) were suspended in complete RPMI supplemented with 10% FBS.

Keratinocyte culture

Keratinocyte cultures were prepared from skin blister roofs obtained from nickel-allergic patients. Blisters were induced by generating a vacuum on normal skin of the forearms, and the epidermal sheets obtained were treated with 0.05% trypsin (Invitrogen, San Giuliano Milanese, Italy) to obtain single-

cell suspensions. Primary cultures were prepared by seeding cell suspensions on a feeder layer of irradiated 3T3 mouse fibroblasts and were cultured according to an optimized Rheinwald and Green culture technique. Second- or third-passage keratinocytes were used in all experiments, with cells cultured in 6-well plates in serum-free medium (KGM) for at least 3 to 5 d before performing experiments. Keratinocytes were treated or not with recombinant human IFN-γ (200 U/ml) in the absence of hydrocortisone for the last 24 h before being used in coculture experiments.

Skin-infiltrating nickel-specific T cell clones

T cell clones derived from short-term nickel-specific CD4$^+$ T cell lines were obtained from nickel ACD skin, as previously described (30, 31). Skin-emigrated T cells were cloned by limiting dilution (0.5 cell/well in U-bottom 96-well plates) in complete RPMI plus 10% FBS and 3% HS in the presence of irradiated allogeneic feeder cells plus 1% PHA (Invitrogen) and 50 U rhIL-2/ml. Ag specificity in the presence of NiSO$_4$ (10 μg/ml) (Sigma-Aldrich) plus monocytes was assessed as [^{3}H]thymidine (Amersham, Little Chalfont, UK) incorporation and measured in a β counter (PerkinElmer, Waltham, MA).

Treatment of NK cells with T cell-derived supernatants

Resting CD56^{high}CD16$^-$ NK cells (1 × 10^5 cells/well) purified from peripheral blood of nickel-allergic donors were incubated with Th1, Th2, Th17 supernatants (1:2 of the final volume), or rhIL-2 (80 U/ml) as positive control in U-bottom 96-well plates. After 60 h, NK cell surface activation markers were analyzed using a FACSAria cytometer. To detect IFN-γ release by NK cells, T cell clones conditioning supernatants were removed from cultures, NK cells were washed twice with PBS and left in culture for the last 24 h in RPMI plus 10% FBS before collecting NK supernatants. Cytokine content was measured by ELISA (R&D Systems).

Proliferation assays

Proliferative capacity of NK cells was measured using the 5,CFSE (Molecular Probes, Eugene, OR) dilution assay. NK cells, purified from the PBMCs of healthy and nickel-allergic individuals, were intracellular fluorescent labeled with CFSE at a final concentration of 5 μM. Ag-specific assays were performed in flat-bottom 96-well plate (Corning Costar) by incubating NK cells (1.25 × 10^5 cells/well) plus autologous monocytes (2.5 × 10^4 cells/well) in the presence of NiSO$_4$ (20 μg/ml), 3,4-dibutoxy-3-cyclobutene-1,2-dione (SABDE) (Sigma-Aldrich) (25 μg/ml), or rhIL-2 (80 U/ml) as positive control. On day 7, cell-free supernatants were collected and the percent of CFSE^{low} CD69$^+$ was analyzed using a FACSAria cytometer.

Keratinocyte induction of apoptosis

Resting or IL-2-activated CD56^{high}CD16$^-$ NK cells (1 × 10^5 cells/well), purified from the peripheral blood of nickel-allergic donors, were cocultured for 6 h with resting or IFN-γ stimulated autologous keratinocytes (1 × 10^4 cells/well) in flat-bottom 96-well plates. Nickel (20 μg/ml) was added to selected keratinocyte cultures 30 min prior to NK cell exposure. Induction of keratinocyte apoptosis was determined using the caspases 3 and 7 FLICA kit (ImmunoChemistry Technologies, Bloomington, MN) according to the manufacturer's protocol, and evaluated by FACS.

ELISA

Supernatants of NK cells activated with NiSO$_4$, SABDE, or rhIL-2 in the presence of monocytes were collected at day 7. NK cells conditioned with TCC supernatants, were washed twice with PBS and left in culture for the last 24 h in RPMI plus 10% FBS before collecting NK supernatants. Cell-free supernatants were tested for IFN-γ content using the DuoSet ELISA system (R&D Systems) according to the manufacturer's instructions.

Supernatants of T cell clones were stimulated with autologous monocytes, and NiSO$_4$ (10 μg/ml) were collected after 48 h and examined for IFN-γ, TNF-α, IL-2, IL-4, IL-10, and IL-17 content by R&D Systems ELISA according to the manufacturer's instructions.

The pattern of cytokine release of Ni-specific T cell clones used as a source of conditioning supernatants is shown in Supplemental Table II.

Statistical analysis

Statistical analysis was calculated using Student t test.

Results

NK cells accumulate in ACD reactions

Hapten challenge (patch test) in sensitized individuals induces an eczematous skin reaction, which peaks at 48–72 h and simulates spontaneous ACD. Three nickel-allergic donors were punch biopsied at the site of nickel-positive patch tests at different time points (24–72 h), and the cellular infiltrate was analyzed by immunohistochemistry. Double staining with anti-CD3 and anti-CD56 Abs showed that ACD infiltrate comprises CD3$^-$CD56$^+$ NK cells (Fig. 1A, inset) that uniformly express perforin, as shown in CD56/perforin double-staining immunohistochemistry (Fig. 1B). CD3$^+$CD56$^+$ double-positive cells were rarely detected in ACD skin. Anti-CD16 staining revealed only a few positive cells in the mid dermis and inside dilated dermal vessels (Fig. 1C). CD56$^+$ NK lymphocytes, albeit absent from normal skin (not shown), were already detectable in the superficial dermis at a 24-h patch test, and progressively increased in number at later time points (Fig. 1D–F). At 48 and 72 h, CD56$^+$ NK cells were detected into the epidermis at the site of prominent spongiosis (Fig. 1E, 1F), with a marked attitude to colocalize with T cells (Fig. 1A, inset). NK lymphocytes infiltrating ACD skin progressively augment in number,
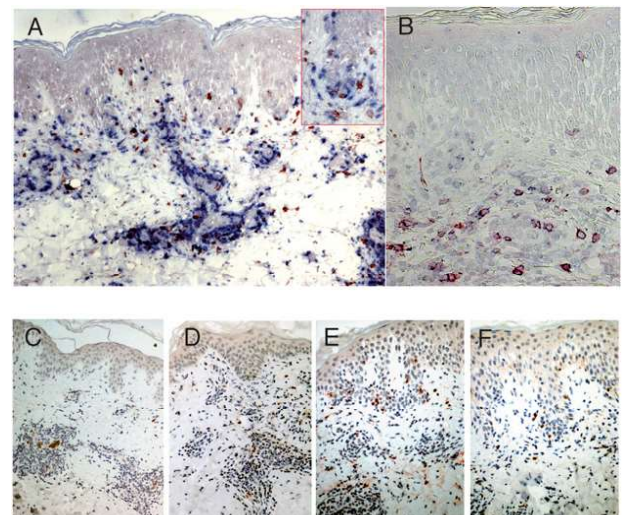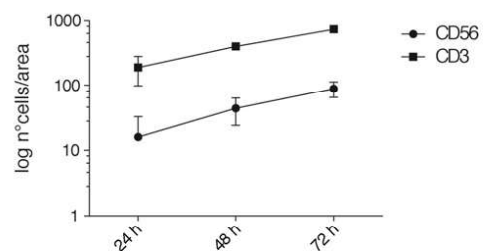

FIGURE 1. CD3$^-$CD56$^+$CD16$^-$perforin$^+$ NK cells infiltrate acute ACD reactions. A 48-h positive nickel patch test was evaluated immunohistochemically for: A, anti-CD56 (3-amino-9-ethylcarbazole, red) and -CD3 (alkaline phosphatase substrate kit III, blue) (original magnification ×100; inset ×400); B, anti-CD56 (3-amino-9-ethylcarbazole, red) and -perforin (alkaline phosphatase substrate kit III, blue) (original magnification ×200); and C, anti-CD16 Abs. Time course of CD56 expression was performed on positive nickel patch tests 24 h (D), 48 h (E), and 72 h (F) after hapten challenge (single staining with 3-amino-9-ethylcarbazole, counterstained with hematoxylin [original magnification ×100]). G, Kinetics of CD3 or CD56 positive cells were calculated on randomly selected photographic fields taken from three ACD time course (five fields per slide). Measurements of cell number were carried out manually by two independent investigators. Values are expressed as number of cells per field (mean ± SD) of ~30 observations for each time point.

FIGURE 2. Characterization of NK cells infiltrating ACD. Lymphocytes infiltrating lesional ACD skin were isolated from the minced tissue and characterized by FACS. Results show one representative skin line of five lines investigated. Numbers indicate relative percentages in each quadrant.
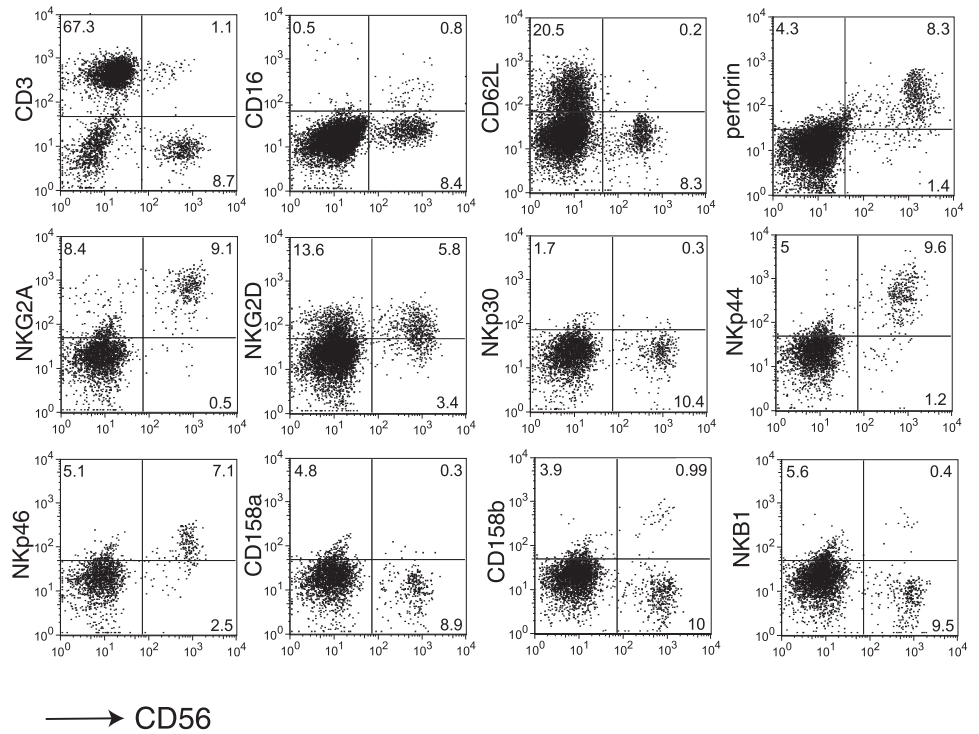
ranging from 6.5 ± 4.8% at 24 h to 10.9 ± 3.7% SD at 72 h of total number of infiltrating lymphocytes, as measured as CD3+ plus CD56+ cells (Fig. 1G).

NK cells from ACD skin belong to the CD56^{high}CD16^- subset

To further characterize skin-derived NK cells, lymphocytes were isolated from five ACD skin biopsies (obtained from five allergic donors: two nickel, one thiouram, one diaminopropylamine, and one cobalt) and investigated by flow cytometry. Percentage of NK cells, defined as CD3⁻CD56+ lymphocytes, ranged from 5.75 to 14.8% (mean 9.8 ± 4.1% SD) of the total lymphocytic infiltrate (Fig. 2). CD3 CD56 double-positive cells were rare (0.95 ± 0.4% SD of total

infiltrating cells). Interestingly, <10% of the total skin NK cell population expressed the marker CD16 (0.7 ± 0.5% of total infiltrating lymphocytes). Thus, the great majority of NK cells recruited in ACD belongs to the CD56^{high}CD16^- subset (9.1 ± 4.7% SD of total infiltrating lymphocytes, Supplemental Table I). In contrast to T lymphocytes, skin NK cells contained high amounts of perforin without prior activation. In addition, NK cells were homogeneously positive for NKG2A, and for the activating receptors NKG2D, NKp44, and NKp46, but they were negative for NKp30 and killer Ig-related receptors (KIRs) (CD158a, CD158b and NKB1). Interestingly, skin NK cells failed to express the molecule CD62L, which characterizes circulating CD56^{high}CD16^- NK
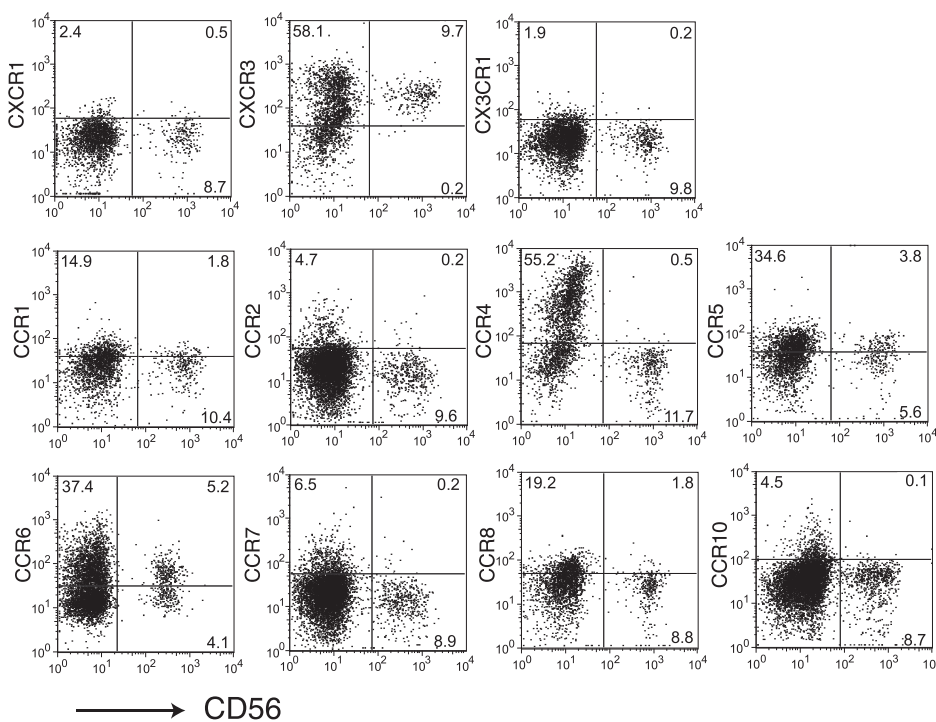

FIGURE 3. Skin NK cells express the chemokine receptors CXCR3, CCR5, and CCR6. Total lymphocytes emigrated from ACD skin biopsies were collected and analyzed for their chemokine receptor expression by FACS. Results show the data obtained for one representative skin line of five lines investigated.
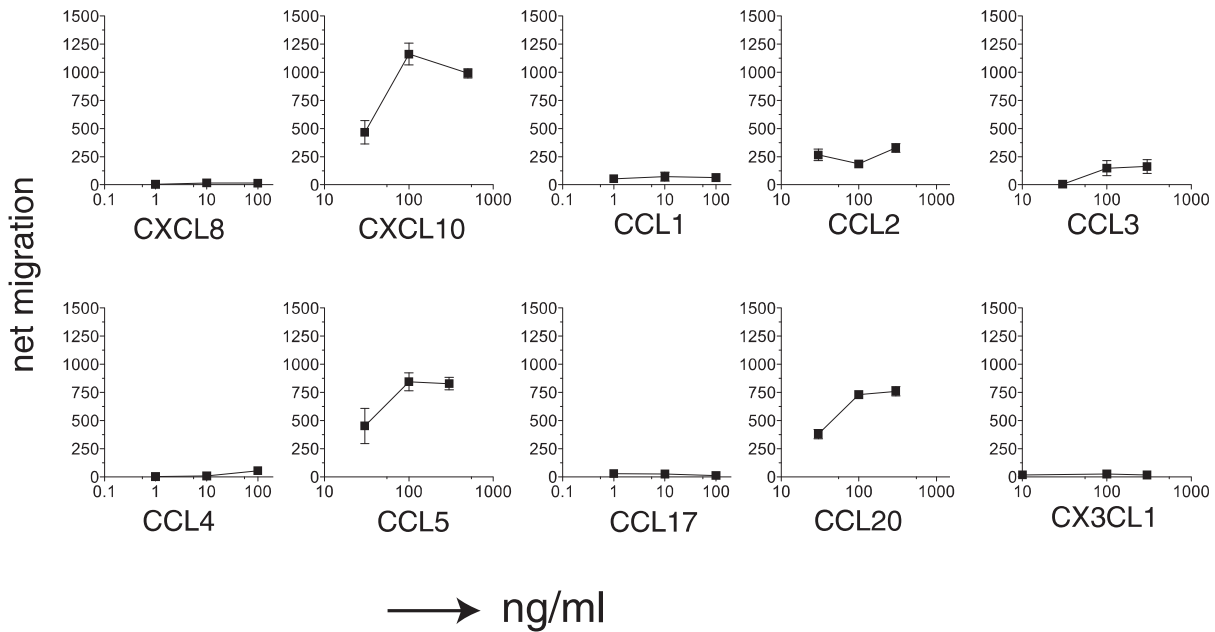
FIGURE 4. Skin NK cells migrate in vitro to CXCL10, CCL20, and CCL5. Skin lymphocytes were stained with FITC-conjugated anti-CD56 and PE-conjugated anti-CD3 and analyzed for their migratory capacity to selected chemokines using a transwell system. Data are shown as net migration (number of cells migrated to the chemokine minus the number of cells migrated in the presence of medium alone) ± SD of CD3⁻CD56⁺ cells emigrated in the lower chamber. Experiment was performed in duplicate. Data shown as representative experiment of three performed.

lymphocytes that home to secondary lymphoid organs, and the cutaneous lymphocyte-associated Ag (data not shown).

Skin CD56^{high}CD16⁻ NK cells express CXCR3, CCR6, and CCR5 chemokine receptors and preferentially migrate to CXCL10, CCL20, and CCL5 in vitro

Entry of lymphocytes at the site of hapten challenge is a crucial event for development of ACD reactions. Selective migration of circulating lymphoid cells in tissue depends on their chemokine receptor asset and is strictly regulated by sequential and co-ordinated release of chemokines from resident and migrated cells. As detected by FACS analysis, skin CD56^{high}CD16⁻ NK cells were homogeneously positive for the chemokine receptor CXCR3. In addition, they express intermediate levels of CCR6 and CCR5, and very low levels of CCR1 (Fig. 3). Other chemokine receptors involved in skin recruitment, including CXCR1, CCR2, CCR4, CCR8, and CCR10, were not expressed by skin NK cells. CX3CR1, which allows responsiveness to fractalkine (CX3CL1), has been shown to be exclusively expressed on CD56⁺CD16⁺, but not on CD56^{high}CD16⁻ skin NK cells. (Fig. 3) (21). CCR7, which identifies lymph node homing of CD56^{high}CD16⁻ NK cells, was also negative on ACD-derived NK cells. Direct comparison between skin and blood-derived NK cells reveals a substantial enrichment of CXCR3, CCR5, and CCR6 positive cells in tissue-derived lymphocytes, and of CX3CR1 and CCR8 in circulating NK cells, which mostly belong to the CD56^{low}CD16⁺ fraction (Supplemental Fig. 1A, 1B). Migratory behavior of skin-derived NK cells to chemokines was studied in vitro using a transwell chemotaxis assay (Fig. 4). Congruent with their chemokine receptor asset, skin CD56⁺CD3⁻ NK cells migrated preferentially to CXCL10, and to a lesser extent to CCL20 and CCL5. No significant migration toward CXCL8, CCL1, CCL2, CCL3, CCL4, CCL17, and CX3CL1 was observed. Taken together, skin ACD NK cells show a CD56^{high}CD16⁻ phenotype, do not express the lymph node homing receptors CD62L and CCR7, but uniformly display CXCR3, and consecutively migrate toward CXCL10 that is produced in high amounts during ACD.

The CD56^{high}CD16⁻ CD62L⁻ NK cell subpopulation is detectable in circulation

To disclose whether the described NK subpopulation that preferentially migrates into inflamed skin is also detectable in the circulation, we studied peripheral blood-derived NK cells from both nickel-allergic (four) and healthy (four) nonallergic donors. FACS analysis showed that within CD3⁻CD56^{high}CD16⁻ cells two clear-cut subpopulations can be distinguished on the basis of CD62L expression. Between 25% and 60% of the CD56^{high}CD16⁻ NK cells were CD62L⁻, with a high interindividual variability. Interestingly, the expression of the chemokine receptor CXCR3 was
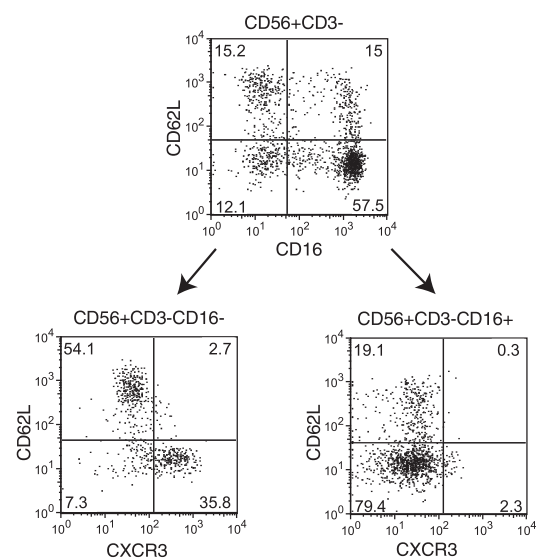

FIGURE 5. CXCR3 expression is restricted to a minor subpopulation of circulating CD3⁻CD56^{high}CD16⁻CD62L⁻ NK cells. Five color FACS analysis was performed on PBLs obtained from four nickel-allergic and four healthy subjects. Panels show the result from one representative experiment.
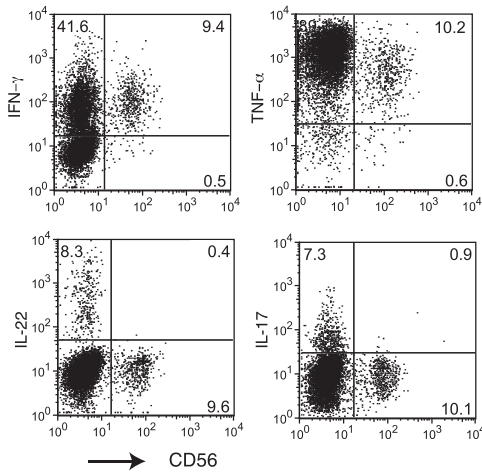
FIGURE 6. Skin NK cells release IFN-γ and TNF-α, but not IL-17 and IL-22. Cytokine release of skin lymphocytes from ACD skin after PMA/ionomycine stimulation was detected by intracellular staining with specific Abs and analyzed by FACS. One experiment of five is shown.

restricted to the $CD56^{high}CD16^-CD62L^-$ NK cell subset (Fig. 5). Thus, the $CD3^-CD56^{high}CD16^-CD62L^-CXCR3^+$ population can be identified as a NK cell subset uniquely equipped to traffic to hapten-challenged sites.

$CD56^{high}CD16^-$ NK cells derived from ACD skin produce IFN-γ and TNF-α, but not IL-22 and IL-17

Type 1 cytokines are critical for the development of ACD, because they are potent activators of resident skin cells. FACS analysis of skin ACD lines demonstrated that $CD56^{high}CD16^-$ NK cells produce high amounts of both IFN-γ and TNF-α, but not IL-22, IL-17, and IL-4 (Fig. 6, and data not shown). Thus, NK cells significantly contribute to the maintenance of a type 1 microenvironment and to the amplification of the immune reaction.

Nickel fails to induce activation, proliferation, and cytokine release of peripheral blood NK cells in sensitized individuals

To disclose whether NK cells from nickel-allergic donors may exhibit memory-like properties and could be specifically activated in vitro by the metal, we compared peripheral blood $CD3^-CD56^+$ cells from four allergic and four healthy volunteers in terms of nickel-specific proliferative response, upregulation of activation markers, and cytokine release. CFSE–labeled NK cells from both allergic and nonallergic individuals failed to proliferate and to upregulate the activation marker CD69 when cultured with nickel or with the unrelated hapten SABDE in the presence of autologous monocytes (Fig. 7A). In parallel, NK cells cultured in the same experimental setting did not release IFN-γ on nickel exposure (Fig. 7B). In contrast, NK cells exposed to IL-2 showed a high rate of proliferation, significant upregulation of CD69 and substantial IFN-γ release, without significant

FIGURE 7. NK from sensitized donors are not activated by nickel in vitro. NK cells were purified from peripheral blood of nickel-allergic or healthy donors, stained with CFSE and incubated with monocytes plus nickel or the unrelated hapten SABDE. At day 7, cells were investigated for proliferation, expression of activation markers (A) by FACS and IFN-γ release and (B) by ELISA. IL-2 treated cultures were used as a positive control. Shown are the results of one representative experiment of four distinct experiments performed.
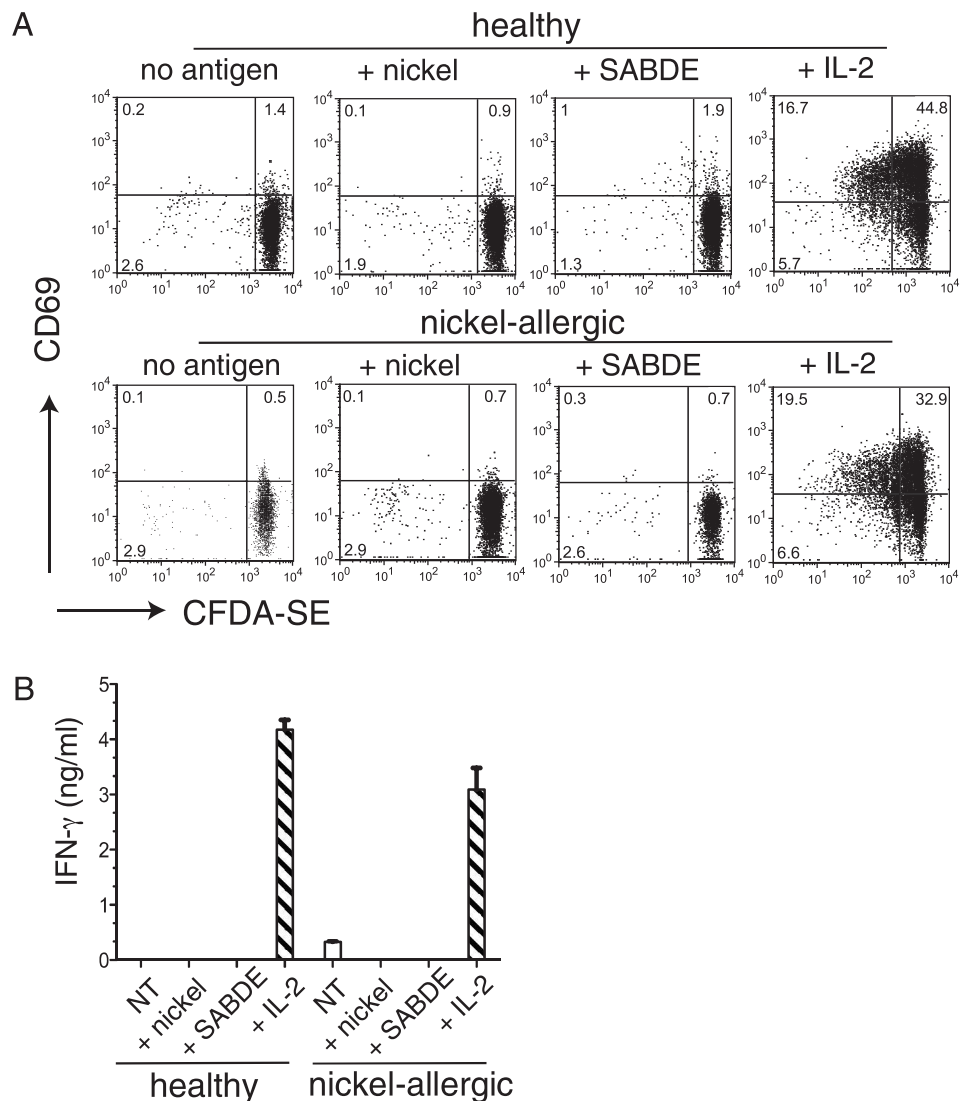
differences between the allergic and nonallergic groups. These findings support the notion that NK cells from allergic patients do not display memory-like properties to the causative hapten.

Cytokines secreted by skin-infiltrating Th1 and Th17, but not Th2 lymphocytes, induce IFN-γ release and upregulation of activation markers by CD56^{high}CD16$^-$ NK cells

Given the incapacity of haptens to directly activate NK lymphocytes, we wondered whether cytokines released by hapten-activated T lymphocytes could trigger the effector function of NK cells. To this aim, nickel-specific Th1, Th17, and Th2 clones (Supplemental Table II) were stimulated in the presence of 20 μg/ml of nickel plus APC, and 48 h culture supernatants added 1:1 to CD56^{high}CD16$^-$ NK cell cultures. Exogenous rhIL-2 (80 U/ml) was used as positive control. NK cells exposed to Th1-derived and, to a lesser extent, Th17-derived cytokines, but not Th2 supernatant, upregulated the activation markers CD69 and NKp44 (Fig. 8A). Moreover, Th1 and Th17 supernatants significantly induce IFN-γ production from NK cells (Fig. 8B). Thus, during ACD responses, hapten-activated T lymphocytes release a variety of inflammatory cytokines, which critically amplify the immune reaction by arming NK cell effector functions.

Activated CD56^{high}CD16$^-$ NK cells from nickel-allergic donors kill autologous keratinocytes in a nickel-independent manner

Immunohistochemistry of ACD lesions demonstrated the presence of CD56$^+$ NK cells in close contact with keratinocytes at the site of intense spongiosis. Because this observation strongly suggests a direct role of NK cells in the induction of keratinocyte apoptosis, we studied the capacity of NK cells to kill autologous keratinocytes in in vitro cytotoxicity assays. Resting CD56^{high}CD16$^-$ NK cells from nickel-allergic subjects failed to induce apoptosis in either untreated or IFN-γ–activated keratinocytes, as determined by quantification of the apoptosis markers caspase 3 and 7 in keratinocytes. As expected, nickel-exposure of target keratinocytes did not enhance NK cell-mediated cytotoxicity. However, IL-2–activated CD56^{high}CD16$^-$ NK cells became fully capable of killing both resting and IFN-γ–treated autologous keratinocytes (Fig. 9). This finding confirms that NK cells infiltrating ACD skin can be activated by IL-2 that is released by T lymphocytes in the inflammatory microenvironment and can thus directly contribute to the expression of the immune reaction by inducing keratinocyte apoptosis in an Ag nonspecific manner.

FIGURE 8. NK cells exposed to skin Th1- and Th17-derived supernatants upregulate NKp44 and CD69 and release IFN-γ. Resting CD56^{high}CD16$^-$ NK cells purified from peripheral blood of allergic donors were incubated with Th1, Th2, or Th17 supernatants. *A*, Expression of NK activation markers NKp44 and CD69 was investigated after 60 h of treatment. *B*, To detect cytokine secretion, NK cells were pulsed 60 h with T cell-derived supernatants, washed extensively, and left an additional 24 h in culture before collection of cell culture supernatants. IFN-γ content was measured by ELISA. IL-2–treated cultures were used as a positive control. One experiment of two is shown.
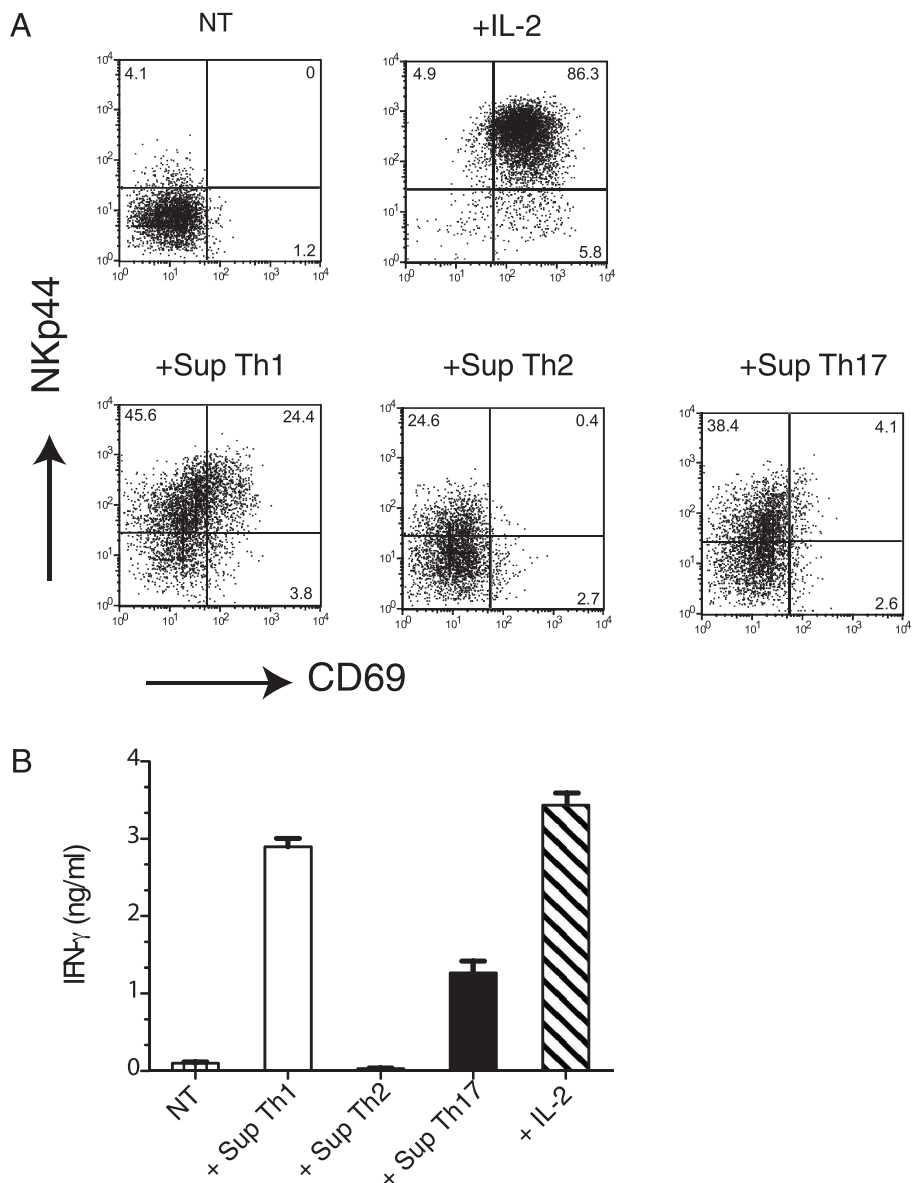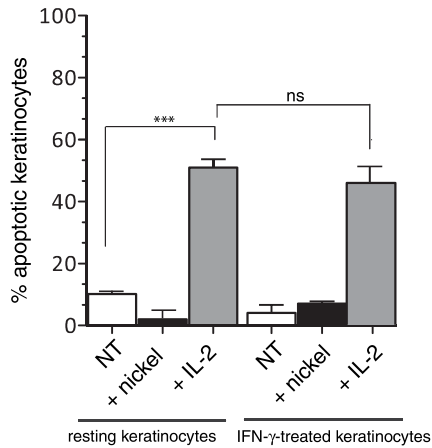
FIGURE 9. IL-2–activated CD56^{high}CD16$^-$ NK cells induce keratinocyte apoptosis. CD56^{high}CD16$^-$ NK cells were purified from peripheral blood of nickel-allergic individuals, exposed to 80 U/ml IL-2 or left untreated, and cocultured 6 h with resting or IFN-γ–treated keratinocytes. A total of 20 μg/ml NiSO$_4$ was added to keratinocytes 30 min prior to the NK cell coculture. Induction of apoptosis was determined by FACS as percentage of caspase 3 and 7 positive keratinocytes (***$p < 0.0001$ by paired Student t test; NS, $p < 0.3$) One experiment of four performed is shown.

Discussion

The role of NK cells in inflammatory skin diseases remained as yet obscure. In this study, we identify a distinct CD56^{high}CD16$^-$ CD62L$^-$ NK cell subpopulation that is specifically recruited in inflamed skin through a CXCL10-dependent mechanism and that directly aggravate ACD reactions by releasing IFN-γ and TNF-α, and by inducing keratinocyte apoptosis.

NK cells are roughly divided into two major subsets with defined functional properties. The majority of circulating NK lymphocytes display a CD56^{low}CD16$^+$ phenotype, express KIRs and high levels of perforin, and exert protective functions by killing virus-infected and transformed cells (18). In contrast, CD56^{high}CD16$^-$ NK lymphocytes, which express the secondary lymphoid organ receptors CCR7 and CD62L, are enriched in lymph nodes, where they modulate dendritic cell functions and T cell priming by releasing type 1 cytokines (32). In this study, we identify a minor population of circulating CD56^{high}CD16$^-$ NK cells that do not express CD62L and CCR7, but high levels of CXCR3, which allows their recruitment into inflamed peripheral tissue, in particular the skin. Indeed, NK cells derived from ACD reactions show a CD56^{high}CD16$^-$CD62L$^-$ phenotype and uniformly express the chemokine receptor CXCR3. Like other CD56^{high} CD16$^-$ NK cells, they highly express C-type lectin, NKG2A, intermediate to high levels of perforin, but not the KIRs CD158a, CD158b, and NKB1. Moreover, skin NK cells are positive for NKp44, NKp46, and NKG2D. According to their expression of CXCR3, CCR6, and CCR5, recruitment of NK cells into ACD skin is mostly regulated by CXL10, and to a lesser extent by CCL20 and CCL5, which are abundantly released by keratinocytes and infiltrating cells during ACD reaction.

Consistent with their homing ability into inflamed skin, NK cells critically mediate the pathogenesis of eczematous skin diseases. A major pathogenic event in eczema is the induction of apoptosis in keratinocytes, as it leads to rapid cleavage of adhesion molecules such as e-cadherins, loss of keratinocyte adhesion and, finally, spongiosis (4, 5). Keratinocyte apoptosis is induced mainly by CD8$^+$ cytotoxic T cells. However, on exposure to IFN-γ and TNF-α, keratinocytes express ICAM-1 and MHC class II molecules, and consecutively become susceptible to additional CD4$^+$ T cell-mediated killing (4, 7). In this scenario, activated NK cells are crit-

ically involved in the pathogenesis of ACD in two ways: first, they are a source of proinflammatory cytokines, thus promoting a CD4$^+$ T cell-mediated killing of keratinocytes, and second, they themselves induce keratinocyte apoptosis. We demonstrate skin-homing CD56^{high}CD16$^-$ NK cells contribute to the maintenance of a type 1-dominated microenvironment and to the activation of resident cells by releasing IFN-γ and TNF-α. However, they do not produce IL-17 and IL-22. Thus, these NK cells are not related to the recently identified mucosa-associated NK subset named NK-22, which secretes high amounts of IL-22, expresses NKp44, and is associated with immune responses to extracellular pathogens (33). This obvious difference indicates that distinct NK cell subpopulations fulfill specific functions in peripheral tissue, with the identified CD56^{high}CD16$^-$CD62L$^-$ NK cells being critical for inflammatory skin reactions that target host cells rather than extracellular pathogens.

It has been suggested that hapten recognition by NK cells is responsible of development of CHS in T cell- and B cell-depleted mice (29). However, we could not confirm a specific recognition of nickel or the acquisition of specific memory to haptens by the human NK cell compartment. Rather, NK cells are activated by cytokines locally released by infiltrating Th1 and Th17 cells.

Moreover, NK-mediated killing is independent on hapten recognition on target cells, and mostly controlled by T cell-derived IL-2. To definitely exclude that NK cells specifically recognize chemical molecules, we investigated their response profile to nickel. We found nickel does not promote any activation of NK cells regarding proliferation, cytokine release, and upregulation of activation markers. Thus, in our hands, skin-derived NK cells do not recognize haptens. Evidence exists, however, for Ag-driven selective amplification of NK cells in the context of infection. In particular, mice infected with CMV expand Ly49H$^+$ NK cells, which respond more rapidly at subsequent Ag challenges (34). In line with our observations, this phenomenon can be explained by the fact that Ly49H is a specific receptor for the virally encoded m157 protein expressed on the membrane of CMV-infected cells rather than by specific memory responses.

In summary, our data indicate that NK cells, although being extremely potent in killing transformed or viral-infected autologous cells, do not induce apoptosis of hapten-coupled keratinocytes. However, once activated by infiltrating Th1 and Th17 lymphocytes, they become potent effector cells in the pathogenesis of ACD by releasing proinflammatory cytokines and by induction of keratinocyte apoptosis. Although in this scenario, NK cells appear as a T cell-dependent magnification tool for ACD expression, we could not exclude a role of NK lymphocytes in an earlier phase of the immune reaction, as a consequence of T cell-independent activation pathways.

Although NK cells represent only 10% of the infiltrating lymphocytes and are outnumbered by IFN-γ–producing T cells, their contribution to the maintenance of a Th1-dominated microenvironment could be relevant. In fact, whereas only a fraction of infiltrating lymphocytes is specific for the causative hapten, NK cells uniformly release IFN-γ when exposed to T cell-derived cytokines, IL-2 in particular.

In conclusion, our data reinforce the notion that full expression of allergic contact dermatitis requires a coordinated interaction between adaptive and innate immune mechanisms.

Acknowledgments

Disclosures
The authors have no financial conflicts of interests.

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