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Immunology

Fc_γ receptor type III (CD16) is included in the ζ NK receptor complex expressed by human natural killer cells

(large granular lymphocytes/immunoglobulin Fc receptors/cytolysis)

PAUL ANDERSON*†, MICHAEL CALIGIURI*, CARMELINE O'BRIEN*, THOMAS MANLEY*, JEROME RITZ*, AND STUART F. SCHLOSSMAN*

*Division of Tumor Immunology, Dana–Farber Cancer Institute, and †Department of Rheumatology and Immunology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

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ABSTRACT **We recently reported that CD3⁻ natural killer (NK) cells express the ζ chain of the T-cell receptor complex (ζ NK) in association with higher molecular weight structures whose expression differs between individual NK cell clones. Because NK cell cytolytic activity is known to be triggered by perturbation of the type III Fc_γ receptor (CD16), we sought to determine whether this activating molecule is included in the ζ NK molecular complex. Biochemical evidence for a physical association between CD16 and ζ NK was obtained by comparing immunoprecipitates formed using monoclonal antibodies reactive with each of these molecules by SDS/polyacrylamide gel electrophoresis, immunoblotting, and peptide mapping. In both clonal and polyclonal populations of CD3⁻ NK cells, CD16 and ζ NK specifically associated with one another. Functional evidence for a specific association between CD16 and ζ NK in intact cells was obtained by demonstrating a coordinate down-modulation of both of these molecules induced by either phorbol 12-myristate 13-acetate or monoclonal antibodies reactive with CD16. Our results suggest that Fc_γ receptor type III (CD16) is included in the ζ NK complex and that this complex is likely to play an important role in NK cell activation.**

Natural killer (NK) cells are large granular lymphocytes capable of killing tumor cells and certain virally infected cells in a manner not restricted by the major histocompatibility complex (MHC) (1, 2). This cytolytic effector function does not require prior sensitization or antigen presentation by accessory cells, properties that allow NK cells to effect an innate host defense prior to the elicitation of an antigen-specific immune response. The molecular interactions responsible for target cell recognition by NK cells are poorly understood. NK cells do not express either of the T-cell antigen receptor (TCR) heterodimers ($\alpha\beta$ or $\gamma\delta$) or their noncovalently associated CD3 subunits (γ, δ, and ε, refs. 3–7). We have recently shown (8) that NK cells do express the ζ chain of the TCR complex. In NK cells, ζ is found in equal amounts as a disulfide-linked homodimer and as a disulfide-linked heterodimer composed of ζ and a 12-kDa protein (p12) (8). In contrast to T cells, which express ζ in association with CD3–TCR, NK cells express ζ in association with two clonally distributed structures (p60–70 and p80–90) whose identities are not known (8). Given the central importance of the ζ chain as a transducing structure involved in T-cell activation (9–12), it seems likely that the NK cell ζ-containing complex may be functionally analogous to the T-lymphocyte antigen-recognition complex. The molecular characterization of the ζ-associated structures found on NK cells is therefore likely to provide important insights into the mechanism by which NK cells recognize their tumor targets. Here we provide biochemical and functional evidence that one of the components of the ζ NK complex is Fc_γ receptor type III (Fc_γRIII, CD16).

MATERIALS AND METHODS

Cell Culture. Polyclonal populations of peripheral blood NK cells were prepared as described (8). These cells were phenotypically <1% $CD3^+$, 75–95% $CD56^+$, and 65–80% $CD16^+$. The $CD3^-$ NK clone designated CNK7 was isolated and grown as described (13).

Antibodies. Monoclonal antibodies reactive with CD3 (RW24B6, IgG2b), CD4 (5H5, IgG1; 5A10, IgG1), class I MHC products (W6/32, IgG2a), CD56 (NKH1, IgG1), CD16 (3G8, IgG1), and ζ NK (TIA-2, IgG1) were used in this study. Their production and characterization have been described (14–18).

Flow Cytometry. Experiments designed to measure the surface modulation of Fc_γRIII (CD16) were performed in the following way. Polyclonal populations of peripheral blood $CD3^-$ NK cells were cultured in 24-well plastic dishes containing 10^6 cells per ml in RPMI-1640 supplemented with 10% fetal bovine serum. Individual wells were treated either with medium alone, with medium containing phorbol 12-myristate 13-acetate (PMA, 10 ng/ml), or with saturating concentrations of monoclonal antibodies (1:500 dilutions from ascites) for the indicated incubation periods. Cells were then washed three times with ice-cold minimal essential medium (GIBCO) containing 2% newborn calf serum, resuspended at 10^7 cells per ml in this same solution, and further incubated with a saturating concentration of a monoclonal antibody reactive with CD16 (3G8) for an additional 30 min on ice. Cells were then washed three times with ice-cold minimal essential medium containing 2% newborn calf serum, resuspended at 10^7 cells per ml in this same medium, and further incubated with fluorescein-conjugated goat anti-mouse immunoglobulin (Tago) for 30 min on ice. After three further washes, cells were analyzed with an Epics V flow cytometer (Coulter).

Immunoblotting. Immunoprecipitates were electrophoresed in 10% polyacrylamide gels, transferred to nitrocellulose, and probed with monoclonal antibodies reactive with ζ NK (TIA-2), as described (17).

Immunoprecipitations. Monoclonal antibodies were affinity-purified on protein A-Sepharose, rebound to protein A-Sepharose via their Fc domains, and then covalently coupled to protein A with dimethyl pimelimidate (19, 20). Antibody-coupled beads (25 μl of packed beads) were used to

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Abbreviations: NK, natural killer; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell antigen receptor; ADCC, antibody-dependent cell-mediated cytolysis; MHC, major histocompatibility complex; Fc_γRIII, Fc_γ receptor type III.

immunoprecipitate the indicated molecules from digitonin lysates of radioiodinated NK cells prepared as described (8, 17). Immunoprecipitates were analyzed in two-dimensional nonreducing/reducing diagonal gels (17).

V8 Protease Mapping. Autoradiograms prepared from unfixed, dried two-dimensional diagonal gels were used to identify the 16-kDa off-diagonal spot corresponding to ζ NK and the 60- to 70-kDa above-diagonal spot corresponding to CD16. These spots were cut out of the dried gel, hydrated, and loaded onto a SDS/20% polyacrylamide gel. The hydrated gel slices were overlaid with a solution containing 1 μg of *Staphylococcus aureus* V8 protease, as described by Cleveland (21). After electrophoresis, gels were fixed, dried, and set up for autoradiography.

RESULTS

Biochemical Evidence for a CD16–ζ NK Complex. Monoclonal antibodies reactive with CD16 are capable of inducing cytolytic activity in NK cells (22–25). Furthermore, the migration pattern of one of the ζ-associated molecules expressed in NK cells (p60–70) has been shown to be similar to that of CD16 (8, 18). For these reasons, we used monoclonal antibodies reactive with ζ NK, CD16, or CD4 to immunoprecipitate lysates of peripheral blood CD3⁻ NK cells, as shown in Fig. 1. Each of the antibodies used was of the IgG1 isotype. To prevent nonspecific associations with Fc domains, antibodies were purified, bound to protein A-Sepharose via their Fc domains, and crosslinked to protein A with dimethyl pimelimidate (19, 20). When analyzed on two-dimensional nonreducing/reducing diagonal gels, ζ NK migrated as two 16-kDa off-diagonal spots, both of which were found to have identical V8 protease digestion patterns (data not shown). This result suggests that these spots correspond to a ζ–ζ homodimer and a ζ–p12 heterodimer (Fig. 1 *Center*, arrow). These immunoprecipitates also contained a 60- to 70-kDa species migrating as a spot above the diagonal (arrowhead), as previously described (8). Immunoprecipitates prepared using monoclonal antibodies reactive with CD16 contained a 60- to 70-kDa protein migrating above the diagonal (Fig. 1 *Right*, arrowhead), corresponding to the transmembrane form of CD16 expressed by NK cells. These immunoprecipitates also included a 16-kDa off-diagonal spot migrating in an identical position as the ζ NK homodimer (Fig. 1 *Right*, arrow). Immunoprecipitates prepared using monoclonal antibodies reactive with CD4, a T-cell surface molecule not expressed in NK cells, did not include this 16-kDa off-diagonal spot (Fig. 1 *Left*). These results suggest that CD16 is capable of specifically associating with ζ NK.

To confirm that the 32-kDa homodimer included in immunoprecipitates prepared using anti-CD16 was ζ NK, immunoprecipitates prepared from peripheral blood CD3⁻ NK cells were fractionated in a SDS/10% polyacrylamide gel, transferred to nitrocellulose, and probed with antibodies reactive with ζ NK (TIA-2). Immunoprecipitates prepared using TIA-2 included a 32-kDa ζ-reactive protein (Fig. 2, lanes a and e). Immunoprecipitates prepared using antibodies reactive with either CD4 (lane b) or class I MHC products (lane c) did not include any ζ-reactive material, whereas immunoprecipitates prepared using antibodies reactive with CD16 (lane d) included a 32-kDa ζ-reactive band. Although the antibody reactive with class I MHC products is an IgG2a antibody, it is an appropriate control to rule out binding via CD16, since CD16 has been shown to bind IgG2a isotypes as well as, or better than, IgG1 isotypes (26). These results provide further evidence that CD16 is included in the ζ NK complex expressed in NK cells.

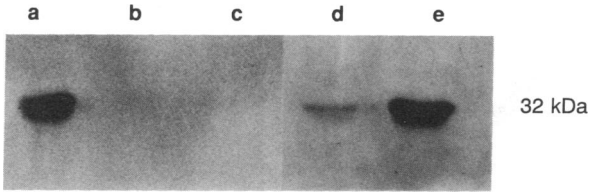

FIG. 2. Immunoblot analysis of immunoprecipitates prepared from CD3⁻ NK cells. Digitonin lysates prepared from peripheral blood CD3⁻ NK cells. (5×10^6 cells per sample) were immunoprecipitated using monoclonal antibodies reactive with ζ NK (TIA-2, lanes a and e), CD4 (5H5, lane b), class I MHC products (W6/32, lane c), and CD16 (3G8, lane d). After SDS/polyacrylamide gel electrophoresis and transfer to nitrocellulose, blots were developed using monoclonal antibodies reactive with ζ NK. This figure is a composite prepared from two separate immunoblots.

Biochemical Analysis of the CD16–ζ NK Complex in Cloned CD3⁻ NK Cells. We similarly immunoprecipitated digitonin lysates prepared from a CD3⁻, CD16⁺ NK clone (CNK7) with antibodies reactive with either ζ NK or CD16 (Fig. 3). CD16 migrates as a 60- to 70-kDa smear above the diagonal, a migration pattern closely resembling the ζ NK-associated p60–70 (arrowheads). Both immunoprecipitates also included a 16-kDa below-diagonal spot corresponding to the 32-kDa ζ NK homodimer (arrows). The structural relatedness of each of these molecules was examined by peptide mapping using V8 protease (Fig. 4). The 16-kDa off-diagonal spots were excised from the unfixed, dried gels used to make the autoradiograms shown in Fig. 3. The V8 protease digestion patterns obtained from these spots were identical (Fig. 4). Similarly, the 60- to 70-kDa spots included in immunoprecipitates prepared using anti-ζ NK and anti-CD16 were compared by V8 protease mapping (Fig. 4). The corresponding structures from each immunoprecipitate produced similar

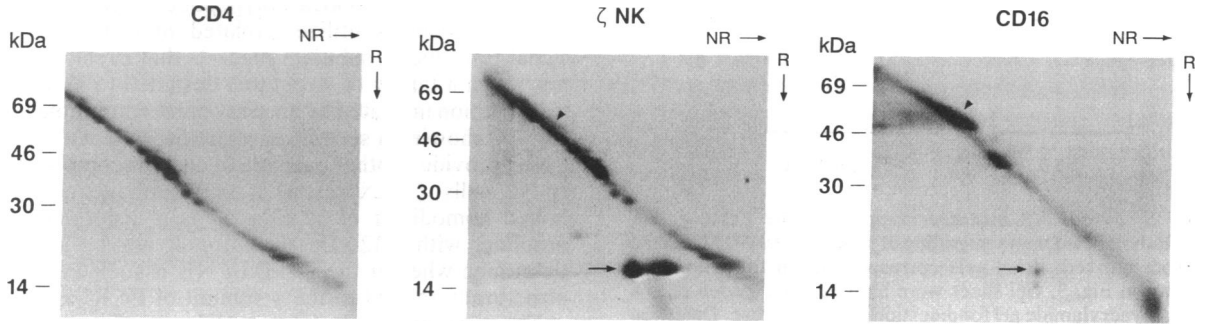

FIG. 1. Immunoprecipitation analysis of peripheral blood CD3⁻ NK cells (2×10^7 cells per sample) by nonreducing/reducing diagonal gel electrophoresis. Digitonin lysates prepared from radioiodinated peripheral blood CD3⁻ NK cells were immunoprecipitated using monoclonal antibodies reactive with the indicated lymphocyte surface molecules. (*Left*) CD4 (antibody 5H5). (*Center*) ζ NK (TIA-2). (*Right*) CD16 (3G8). The first dimension, run under nonreducing (NR) conditions, is depicted in the horizontal direction, whereas the second dimension, run under reducing (R) conditions, is depicted in the vertical direction. The relative migration of molecular size markers is indicated.

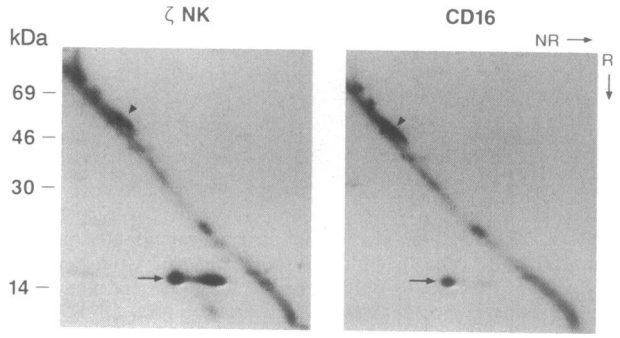

FIG. 3. Immunoprecipitation analysis of cloned CD3⁻ NK cells (CNK7) by nonreducing (NR)/reducing (R) diagonal gel electrophoresis. (*Left*) Immunoprecipitates prepared using monoclonal antibodies reactive with ζ NK (TIA-2). Arrow indicates the ζ homodimer. Arrowhead indicates the 60- to 70-kDa ζ-associated protein. (*Right*) Immunoprecipitates prepared using monoclonal antibodies reactive with CD16 (3G8). Arrowhead indicates the position of CD16. Arrow indicates the 16-kDa CD16-associated protein.

peptide maps, strongly suggesting that the molecular species contained in the individual immunoprecipitates were identical.

Comodulation of CD16 and ζ NK. It was possible that the physical association between CD16 and ζ NK was formed after cells were solubilized in digitonin lysis buffer. The physiologic relevance of this association required the demonstration of a functional link between these molecules on viable NK cells. If CD16 and ζ NK are associated in cells, then stimuli that down-modulate the expression of one member of the complex should also reduce the expression of the other member of the complex. In a similar manner, the physical association between CD3 and the αβ antigen-recognition unit on T cells was first suggested by the comodulation of these molecules by monoclonal antibodies directed against CD3 (14). As previously reported by Perussia *et al.* (27), monoclonal antibodies reactive with CD16 (3G8) were

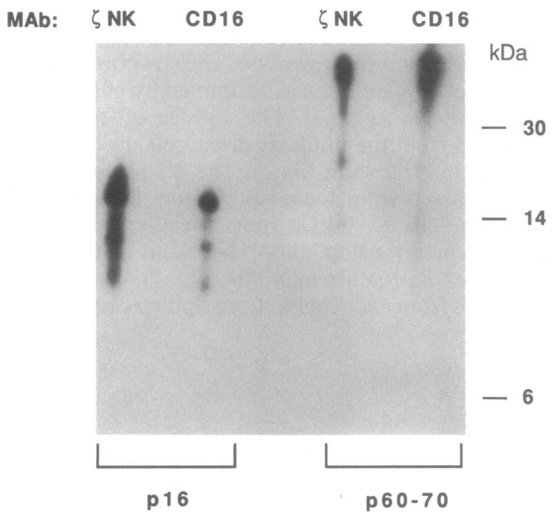

FIG. 4. *S. aureus* V8 protease mapping of the CD16–ζ NK complex. Radiolabeled spots migrating at 16 kDa or 60–70 kDa were excised from unfixed, dried gels corresponding to the autoradiograms shown in Fig. 3. Gel slices were hydrated and loaded onto a SDS/20% polyacrylamide gel for digestion by V8 protease. Digestion products were separated electrophoretically, after which the gel was dried and exposed for autoradiography. The specificities of the monoclonal antibodies (MAb) used to immunoprecipitate the individual spots are indicated above the lanes. The apparent molecular masses of the immunoprecipitated species are indicated below the lanes. The relative migration of molecular size markers is indicated.

capable of down-modulating the surface expression of CD16 on peripheral blood CD3⁻ NK cells (Fig. 5*A*). Monoclonal antibodies reactive with CD3, a structure not expressed in NK cells, had no effect on the surface expression of CD16. PMA was also able to reduce the surface expression of CD16, an effect previously reported by Trinchieri *et al.* (28). To determine whether the expression of ζ NK was comodulated with CD16, we measured its expression in immunoblots (Fig. 5*B*). Over the 2-day incubation period, the expression of ζ NK was unchanged in NK cells cultured in medium alone. Whereas monoclonal antibodies reactive with CD3 have been shown to reduce the expression of ζ in T cells (17), NK cells were unaffected by this treatment. Both PMA and monoclonal antibodies reactive with CD16, on the other hand, down-modulated the expression of ζ NK in these cells. These results, showing a coordinate down modulation of CD16 and ζ NK, suggest that these molecules are functionally linked in intact cells.

DISCUSSION

The molecular nature of the receptor complex used by NK cells for target cell recognition is poorly understood. The recent demonstration that NK cells express ζ TCR as part of a novel molecular complex (8) has provided a direction for research aimed at identifying the elusive NK receptor complex. In the present report, we provide biochemical evidence for a physical association between $Fc_\gamma RIII$ (CD16) and ζ NK in clonal and polyclonal populations of CD3⁻ NK cells. Furthermore, comodulation experiments using monoclonal antibodies reactive with CD16 provide functional evidence for a CD16–ζ NK complex in viable NK cells.

NK cells are capable of mediating cytolytic effector function in one of two ways. They are able to recognize tumor cells and certain virally infected target cells in a non-MHC-restricted manner without prior sensitization. Alternatively, in the presence of antibodies reactive with target cell surface molecules, NK cells can effect an antibody-dependent cell-mediated cytotoxicity (ADCC). Antibody-dependent cytolysis is mediated through Fc receptors ($Fc_\gamma RIII$) expressed on the surface of NK cells (27–29). Down-modulation of $Fc_\gamma RIII$ by either phorbol esters (28) or immune complexes (27) results in the abrogation of ADCC. Our results, showing a specific association between $Fc_\gamma RIII$ and ζ NK, suggest that this receptor complex is responsible for the antibody-dependent activation of cytolytic effector function in NK cells.

Recently, Kinet and colleagues have described a ζ-related molecule (designated γ) that appears to be a subunit of the immunoglobulin Fc receptors expressed on murine mast cells ($Fc_\varepsilon RI$, ref. 30) and monocytes ($Fc_\gamma RIIa$, ref. 31). The γ subunit of $Fc_\varepsilon RI$ and $Fc_\gamma RIIa$ is a disulfide-linked homodimer composed of two 10-kDa polypeptides. The demonstration that Fc receptors utilize ζ-related molecules as putative signal-transducing subunits suggests that ζ related molecules may form a family of structures designed to assist in signal transduction mediated by an array of receptor molecules. Our results, showing a specific association between $Fc_\gamma RIII$ and ζ NK, provide another example of an Fc receptor–ζ complex. In NK cells, ζ is expressed in equal amounts as a disulfide-linked homodimer of 32 kDa or as a disulfide-linked heterodimer with a 12-kDa structure. It will be interesting to determine whether the 12-kDa ζ NK-associated molecule is structurally related to the γ subunit of $Fc_\varepsilon RI$ and $Fc_\gamma RIIa$.

The role played by the ζ NK complex in non-MHC-restricted killing is less clear. Whereas non-MHC-restricted killing is abrogated by immune complexes, it is enhanced by phorbol esters (28). Non-MHC-restricted killing can also be enhanced by perturbation of either CD16 or CD2 (22–25, 32). Moreover, co-aggregation of CD2 and CD16 synergistically

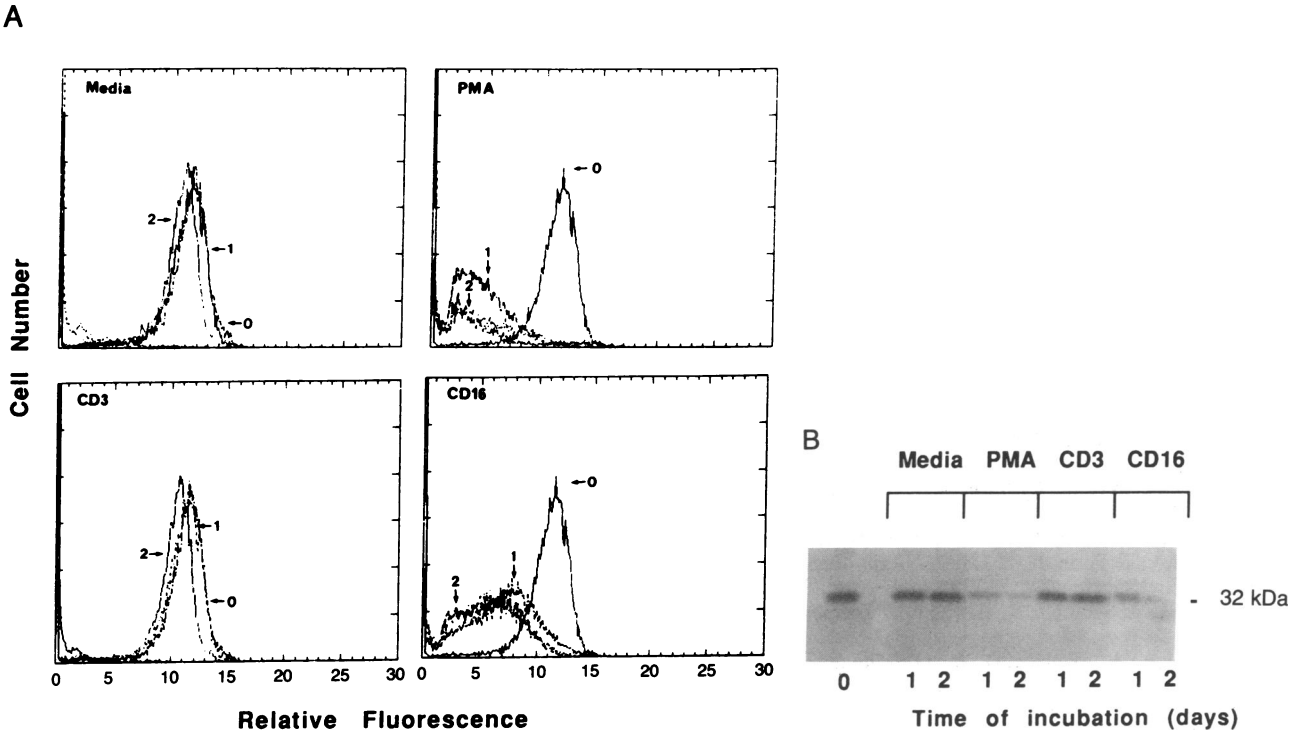

FIG. 5. CD16 and ζ NK are down-modulated in a coordinate fashion. (*A*) Flow cytometric analysis of CD16 expression following culture for 0, 1, or 2 days in the presence of medium alone, PMA (10 ng/ml), anti-CD3 (RW24B6, 1:500 dilution from ascites), or anti-CD16 (3G8, 1:500 dilution from ascites). Individual histograms were derived from analysis of 10,000 individual cells. Time of incubation in days (0, 1, or 2) is indicated on each profile. (*B*) Immunoblot analysis using a monoclonal antibody reactive with ζ NK (TIA-2). Cell lysates were prepared from peripheral blood $CD3^-$ NK cells (5×10^6 cells per sample) incubated for the indicated times with the indicated stimuli. Whole-cell lysates were fractionated in SDS/10% polyacrylamide gels, transfered to nitrocellulose, and probed with TIA-2 as described.

enhances non-MHC-restricted killing (33, 34). Some evidence suggests that CD2-mediated activation of NK cells requires the coexpression of CD16 (25). This observation raises the possibility that the ζ subunit is responsible for the functional link between CD2 and CD16 in NK cells. The relative expression of each of the ζ-associated molecules (CD16, p80–90, and possibly CD2) appears to differ on individual NK cell clones (8), suggesting that none of these molecules alone is sufficient for non-MHC recognition. It seems more likely that non-MHC-restricted killing results from the interplay of several NK cell surface molecules. NK cells express several recognition structures (e.g., CD2, CD16, LFA-1) capable of interacting with target cell ligands (e.g., LFA-3, immunoglobulin, ICAM-1). NK triggering may in turn require the aggregation of some or all of these structures, an event facilitated by the particulate nature of the target cell itself. It is interesting that many of these intercellular interactions are mediated by molecules possessing immunoglobulinlike domains (e.g., CD2, CD16, ICAM-1, LFA-3). Regulatory interactions between members of the immunoglobulin superfamily have been extensively documented (35). Although CD16, itself a member of the immunoglobulin superfamily, has been operationally defined as an Fc receptor, its affinity for immunoglobulin is extremely low (26, 36). One might speculate that its physiologic ligand is not the Fc domain of immunoglobulin, but rather another domain of an immunoglobulin superfamily member expressed on target cells. The aggregation of activating molecules expressed on NK cells (e.g., CD2, CD16, and possibly the ζ-associated p80–90) that results from specific interactions with target cell ligands may be sufficient to trigger NK cell killing. Differential expression of individual receptors and ligands on NK cells and target cells, respectively, might allow for differences in target cell recognition by individual NK clones. We would therefore predict that the ζ NK complex will play an important role in both antibody-dependent and antibody-independent, non-MHC-restricted killing mediated by NK cells.

Note Added in Proof: After this manuscript was submitted for review, Lanier *et al.* (37) reported similar results demonstrating the association of CD16 and ζ in human NK cells.

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