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# Phenotypic analysis of a resting subpopulation of human peripheral blood NK cells: the FcRγIII (CD16) molecule and NK cell differentiation

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## SUMMARY

A subpopulation of human peripheral blood natural killer (NK) cells, defined by sedimentation at Percoll high buoyant densities ($P > 1{\cdot}0635$–$1{\cdot}0640$ g/ml) and unresponsiveness to interleukin-2 (IL-2), contained two distinct populations based on the intensity of CD16 (FcRγIII) expression, namely $CD16^{dim}$ and $CD16^{bright}$. This resting subpopulation of NK cells differed from the total population of peripheral blood NK cells, by containing a larger proportion of $CD16^{dim}$ cells, by the total absence of $CD56^{bright}$ $CD16^{-}$ cells, and by an inability to respond to high concentrations (500 U/ml) of rIL-2 despite the expression of an intermediate affinity (p70) IL-2R. Both $CD16^{dim}$ and $CD16^{bright}$ NK cells expressing high affinity IL-2R were initially generated following co-culture of resting NK cells with gamma-irradiated MM-170 cells and IL-2, but $CD16^{bright}$ NK cells became the dominant cell type later in culture. The CD16 molecule was not involved in the differentiation of resting NK cells since solid-phase-bound anti-CD16 monoclonal antibody neither enhanced nor inhibited NK cell generation. These studies demonstrate that the resting subpopulation of peripheral blood NK cells expresses a unique CD16 profile, that CD16 expression increases during NK cell generation, and that CD16 is not involved in the differentiation process.


## INTRODUCTION

Peripheral blood natural killer (NK) cells are a unique subset of cells which by phenotypic and functional criteria are distinct from T lymphocytes.[1] Phenotypically, NK cells are $CD3^{-}$, $CD56^{+}$, $CD16^{+}$ and express only a germline configuration of the T-cell receptor genes. Functionally, NK cells mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity, and through ligand interaction with the CD16 (FcRγIII) molecule mediate antibody-dependent cytotoxicity and secrete certain cytokines.

The majority of NK cells are large granular lymphocytes which sediment at Percoll low buoyant densities.[2] A minority of NK cells are small lymphocytes which sediment at Percoll high buoyant densities with resting T lymphocytes.[3,4] Separation of resting lymphocytes (R-Ly) on Percoll density gradients is facilitated by an IL-2 preculture step. The NK cells in R-Ly require a two-signal process to initiate cell differentiation and proliferation.[4,5] This process is blocked by cyclosporine, and is therefore distinct from the IL-2-dependent activation observed with the total population of NK cells.[5,6] The identity of NK cell surface molecules involved in the differentiation process is not known. We recently reported that CD16 expression was initially low on culture-generated NK cells.[7] This study presents data showing that the resting subpopulation of NK cells expresses a unique CD16 profile, documents an increase in CD16 expression during NK cell generation, and shows that CD16 is not involved in the differentiation process.

Abbreviations: FMF, flow microfluorimetry; IL-2-CM, interleukin-2-conditioned medium; IL-2R, IL-2 receptor; mAb, monoclonal antibody; NK, natural killer; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PI, propidium iodide; rIL-2, recombinant IL-2; RLy, resting lymphocytes; SPB, solid-phase bound.

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## MATERIALS AND METHODS

*Cells and cytokines*

Interleukin-2 (IL-2)-conditioned medium (CM) was a mitogen-free tonsil lymphocyte-CM.[8] Recombinant IL-2 (rIL-2) was obtained from Hoffman La Roche (Nutley, NJ). IL-2 activity was measured using the IL-2-dependent murine cell line CTLL by procedures previously described.[9]

The cell lines used were the human erythroleukaemic cell line K562, the DBA/2 murine mastocytoma P815 and the human malignant melanoma cell line MM-170. Maintenance of these cell lines was as previously described.[4,5]

Peripheral blood mononuclear cells (PBMC) were isolated from normal donors (ACT Red Cross Blood Transfusion Service) by sedimentation of peripheral blood over Ficoll–Paque (Pharmacia, Uppsala, Sweden). Resting lymphocytes (R-Ly) were isolated from PBMC by a previously described

procedure.[3] Briefly, PBMC were cultured at $10^6$/ml with 5 U/ml IL-2-CM for 3 days and the R-Ly isolated by sedimentation at high Percoll (Pharmacia) densities ($P > 1{\cdot}0635$–$1{\cdot}0640$ g/ml; osmolarity 304mOsm). The precise density varied from donor to donor and cells were defined as resting if an aliquot of $10^5$ cells cultured for a further 7 days in IL-2-CM had low proliferation (< 1000 c.p.m.) and low cytotoxic activity on K562 ($< 15 \times 10^2$ CU). R-Ly were stored in liquid $N_2$ and thawed as required. Resting NK cells were obtained by depleting R-Ly of $CD3^+$ cells by incubation with OKT3 mAb for 30 min on ice, followed by two treatments with rabbit complement (Cedarlane, Ontario, Canada), for 1 hr and for 30 min at 37°, with one wash in between treatments. A total population of peripheral blood NK cells was obtained from plastic-adherent cell-depleted PBMC (1 hr, 37°) by similarly depleting $CD3^+$ cells. The $CD3^-$ R-Ly and $CD3^-$ PBMC were used without purification for cell cultures and analysis by flow microfluorimetry (FMF).

*mAb and fluorochrome-conjugated reagents*

OKT3 (CD3, IgG2a) was a culture supernatant prepared from the cell line CRL 8001 (American Type Culture Collection, Rockville, MD). Coulter T3 (CD3, IgG1) and Coulter B4 (CD19, IgG1) were obtained from Coulter Electronics (Luton, Beds, U.K.). The B73.1 mAb (CD16, IgG1)[10] was a culture supernatant or an ascites prepared from the hybridoma cell line (kindly provided by Dr G. Trinchieri, Wistar Institute, Philadelphia, PA). Leu-11b (CD16, IgM) was purchased from Becton-Dickinson (Mountain View, CA). Leu-19 (CD56, IgG1) (Dr L. Lanier, Becton-Dickinson), NKHIA (CD56, IgM) (Dr J. Ritz, Dana-Faber Cancer Institute, Boston, MA), FcRGran1 (CD16, IgG2a) (Dr T. Huizinga, Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and CLB-T11.2/1 (CD2, IgG1) (Dr R. Van Lier, Netherlands Red Cross Blood Transfusion Service) were supplied by the organisers of the 4th International Workshop on Human Leucocyte Differentiation Antigens, as indicated. Mik-$\beta$1 mAb (IgG2a) recognizing the p70 IL2-R was a kind gift from Dr M. Tsudo (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan).[11] Anti-Tac (CD25, mixed Ig isotypes) recognizing the p55 IL2-R was obtained from Dr T. Waldmann (NIH, Bethesda, MD). TCRdeltal was obtained from Dr M. Brenner (Dana-Faber Cancer Institute). Anti-IL-2R (CD25), directly conjugated with phycoerythrin, was purchased from Becton-Dickinson. The biotin-conjugated IgGs were obtained from Amersham (Amersham, Bucks U.K.), and the fluorescein-conjugated IgGs and the streptavidin-phycoerythrin were purchased from Serotec (Oxford, Oxon, U.K.).

*FMF analysis*

Cells were treated with predetermined optimum concentrations of mAb, washed and incubated with biotin-conjugated anti-mouse Ig of the appropriate subclass, followed by streptavidin-phycoerythrin. For two-colour FMF analysis the cells were then incubated with the required second mAb followed by fluorescein-conjugated anti-mouse Ig of the appropriate subclass. Incubations were in 96 well V-shaped plates using between 2 and $10 \times 10^4$ cells. All incubations were for 30 min on ice, and cells were washed three times in between the different incubations. Cells were either fixed with paraformaldehyde (1%) and analysed within 2 days or were stained with propidium iodide (PI) (10 $\mu$g/ml) to label the dead cells and analysed immediately. A Becton-Dickinson FACScan was used in this analysis.

*Generation of NK cells in culture*

The culture medium was Eagles' medium (410–1500; Gibco, Grand Island, NY) containing 100 $\mu$g/ml penicillin, 100 $\mu$g/ml streptomycin, 60 $\mu$g/ml gentamycin, 24 mM $NaHCO_3$, 0·1 mM 2-mercaptoethanol with a 10% addition of heat-inactivated foetal calf serum (HIFCS). Cultures were maintained in a gas phase of 10% $CO_2$, 7% $O_2$, 83% $N_2$ at a temperature of 38°.

NK cells were generated in culture as described previously.[5] $10^4$ $CD3^-$ R-Ly were cultured in 0·2 ml volumes in flat wells (Linbro 76-032-05, Flow Laboratories Inc, McLean, VA) with $10^{3.5}$ $\gamma$-irradiated (5000 rads, $Co^{60}$ source) MM-170 cells and 5 U/ml IL-2-CM. For analysis of the frequency of responding cells, between 1 and 32 $CD3^-$ R-Ly per well were cultured under the same conditions. Between 24 and 48 wells were set up at each cell concentration. Cultures were fed with fresh medium containing IL-2-CM on Days 8 and 14 and the experiment was terminated on Day 21. For some experiments $10^4$ $CD3^-$ PBMC or $CD3^-$ R-Ly were cultured in round wells (Linbro 76-013-05) with 500 U/ml rIL-2.

Culture wells containing solid-phase-bound (SPB) antibody were prepared as follows. Tissue culture wells were incubated overnight at 4° with 100 $\mu$l of 10 $\mu$g/ml affinity-isolated sheep anti-mouse Ig (Silenus-AMD-ICI, Melbourne, Australia) in 0·1 M $NaHCO_3$ buffer, pH 9·6. After washing three times with phosphate-buffered saline (PBS) and once with PBS containing 1% HIFCS, the plates were incubated at room temperature for at least 1 hr with 100 $\mu$l medium, or 1/10 dilution of B73.1 culture supernatant, or 1/1000 dilution of OKT3 culture supernatant. These mAb concentrations were 10-fold greater than saturating for cell binding estimated by FMF analysis.

*Cytotoxicity assays*

Cytotoxic activity of cells was measured in a 4-hr $^{51}Cr$-release assay as described previously[4] using K562 targets and P815 targets coated with rabbit anti-P815 IgG. The anti-P815 antibody was prepared in rabbits following once weekly s.c. injections of $10^7$ P815 in Freund's complete adjuvant (1:1, v/v) for a period of 10–12 weeks. Blood was collected, serum prepared, and IgG precipitated with $(NH_4)_2SO_4$ using the 40–45% cut.[12] After dialysis against PBS, the antibody was clarified by centrifugation and stored in aliquots at −20°. P815 cells were incubated for 15 min at room temperature with the antibody (20 $\mu$l/$0{\cdot}5 \times 10^6$ cells in 0·5 ml) following $^{51}Cr$ labelling. Five-thousand $^{51}Cr$-labelled target cells were incubated with serial dilutions of effector cells in 0·2-ml volumes in round wells (Linbro 76-013-05). Cytotoxic activity was calculated from the amount of $^{51}Cr$ released, as described by Davidson,[13] and is expressed as cytotoxic units (CU). One $CU = -Y_0 \ln(1-y)$, where $Y_0$ is the number of target cells and y is the proportion of target cells lysed. For comparison with other methods for expressing cytotoxic activity, $100 \times 10^2$ CU corresponds to 40% specific lysis at an effector to target cell ratio of 0·5:1, and $1 \times 10^2$ CU corresponds to 20% lysis at an effector to target cell ratio of 25:1.

*Cell proliferation*

Cell proliferation was measured as [$^3$H]methyl thymidine (Amersham) incorporation over a 5-hr incubation period using 1 $\mu$Ci (1Ci = 37GBq)/0·2 ml culture volume, as described previously.[3]

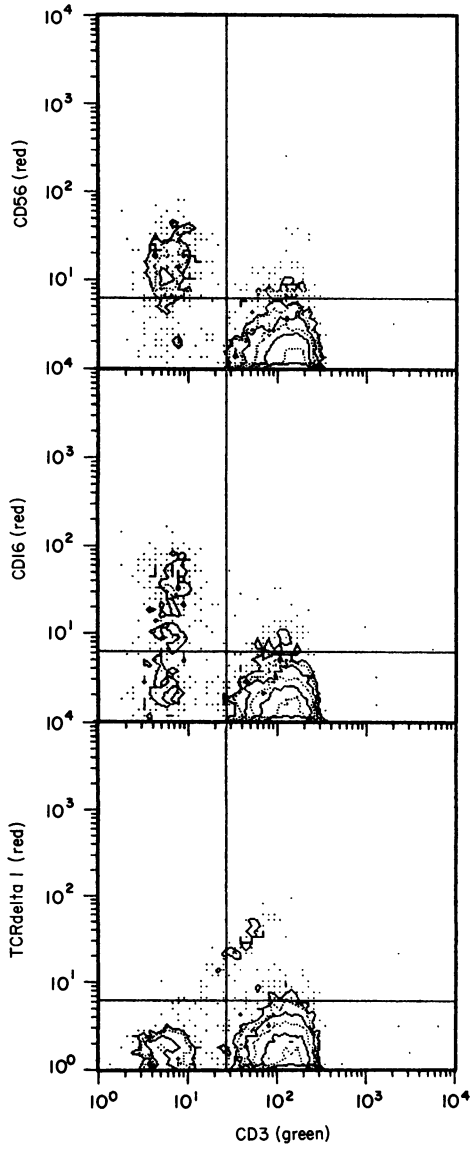


**Figure 1.** Two-colour fluorescence analysis of R-Ly (donor 82298) showing CD56 (Leu-19), CD16 (B73.1) and TcRdeltal stained with biotinylated anti-mouse IgG1 and streptavidin-PE, and CD3 (OKT3) stained with fluorescein-conjugated anti-mouse IgG2a.

## RESULTS

### Properties of resting NK cells

The resting NK cells used in this study are present in a selected resting lymphocyte (R-Ly) subpopulation of PBMC.[3] R-Ly respond minimally to IL-2-CM and sediment at Percoll densities greater than 1·0635–1·0640 g/ml. R-Ly are a source of resting T cells,[3] resting gamma/delta T cells[5] and resting NK cells.[4-6] The yield of R-Ly is donor dependent, averaging 14% of PBMC.[3] A representative phenotypic profile of R-Ly is illustrated in Fig. 1 for donor 82298, and shows the presence of T cells ($CD3^+$), including gamma/delta T cells ($TCRdeltal^+$), and the presence of NK cells ($CD3^-$ $CD56^+$; $CD3^-$ $CD16^+$). Analysis of R-Ly from donors 69096, 82298 and 72984 in three different experiments for each donor showed that 10·6±0·8%, 8·5±0·8% and 12·5±0·6% of R-Ly were $CD3^-$. For all donors the $CD3^-$ subpopulation could be accounted for by $CD19^+$ (B) cells and $CD56^+$ (NK) cells. The percentage of $CD3^-$ $CD56^+$ cells in R-Ly for the different donors was 4·1±0·4%, 6·9±0·9% and 3·2±0·4%, respectively. The number of $CD3^-$ R-Ly which showed strong expression of CD16 was lower than the number expressing CD56, and was 2·2±0·5%, 4·0±0·8% and 2·2±0·2% for the different donors.

The phenotype of $CD3^-$ R-Ly was analysed in more detail following depletion of $CD3^+$ cells from R-Ly by OKT3 and double complement treatment. Double rather than single complement treatment was necessary to ensure depletion of gamma/delta T-cell precursors,[5] which expressed low levels of CD3 (Fig. 1). The yield of cells following OKT3 and double complement treatment of R-Ly was usually between 1% and 1·7%, corresponding to a recovery of approximately 25% of the $CD3^-$ $CD56^+$ cells. $CD3^-$ R-Ly were analysed by two-colour immunofluorescence using PI to gate out the dead cells. T cells were totally depleted following treatment with OKT3 and complement, and, unexpectedly, B cells ($CD19^+$) were also depleted by this procedure (data not shown). The results obtained from one of two experiments with donor 69096 are presented in Fig. 2a, and these results are representative of those obtained for other donors. Almost all of the $CD3^-$ R-Ly were $CD56^+$, with two distinct populations of $CD16^+$ cells, $CD16^{dim}$ and $CD16^{bright}$. The results for CD56 were obtained using Leu-19, and the results for CD16 using FcRGran1, although identical results were obtained using the mAb NKH1A (CD56), B73.1 (CD16) and Leu-11b (CD16). The proportion of $CD16^{dim}$: $CD16^{bright}$ cells in $CD3^-$ R-Ly varied from donor to donor and was 27:73, 59:41 and 8:92 for donors 69096, 82298 and 72984.

For comparison with the results obtained for the resting subpopulation of NK cells using $CD3^-$ R-Ly, we also examined the phenotype of the total population of NK cells using $CD3^-$ PBMC. This total population of NK cells would include the resting subpopulation of NK cells. The yield of cells following OKT3 and double complement treatment of non-adherent PBMC was between 3% and 5%. As with $CD3^-$ R-Ly, B cells were also absent from $CD3^-$ PBMC (data not shown). The results presented in Fig. 2b are for donor 69096 and were obtained in the same experiment as those in Fig. 2a. $CD3^-$ PBMC differed from $CD3^-$ R-Ly in containing a greater proportion of large cells. Thus the percentage of cells exceeding the arbitrary forward scatter value of 110 was 27% for $CD3^-$ PBMC compared to 3% for $CD3^-$ R-Ly. This result is consistent with the fact that $CD3^-$ PBMC would contain the larger NK cells sedimenting at Percoll low buoyant densities.[14] $CD3^-$ PBMC contained a distinct population of cells which were $CD56^{bright}$ $CD16^-$. By setting electronic gates at different forwards scatter values it was possible to reprocess the data in Fig. 2b to show that the $CD56^{bright}$ $CD16^-$ cells were the largest cells in the $CD3^-$ PBMC population. For donors 69096, 82298 and 72984, 14%, 10% and 6% of $CD3^-$ PBMC had the phenotype $CD56^{bright}$ $CD16^-$. The $CD56^{bright}$ $CD16^-$ cells were never seen in $CD3^-$ R-Ly (Fig. 2a). $CD3^-$ PBMC also differed from $CD3^-$ R-Ly in their relatively higher expression of CD16 (mean fluorescence intensity of 200 compared to 90) and relatively lower expression of CD56 (mean fluorescence intensity 80 compared to 120). $CD16^{dim}$ cells were present in $CD3^-$ PBMC, but they were a smaller and less distinct population than

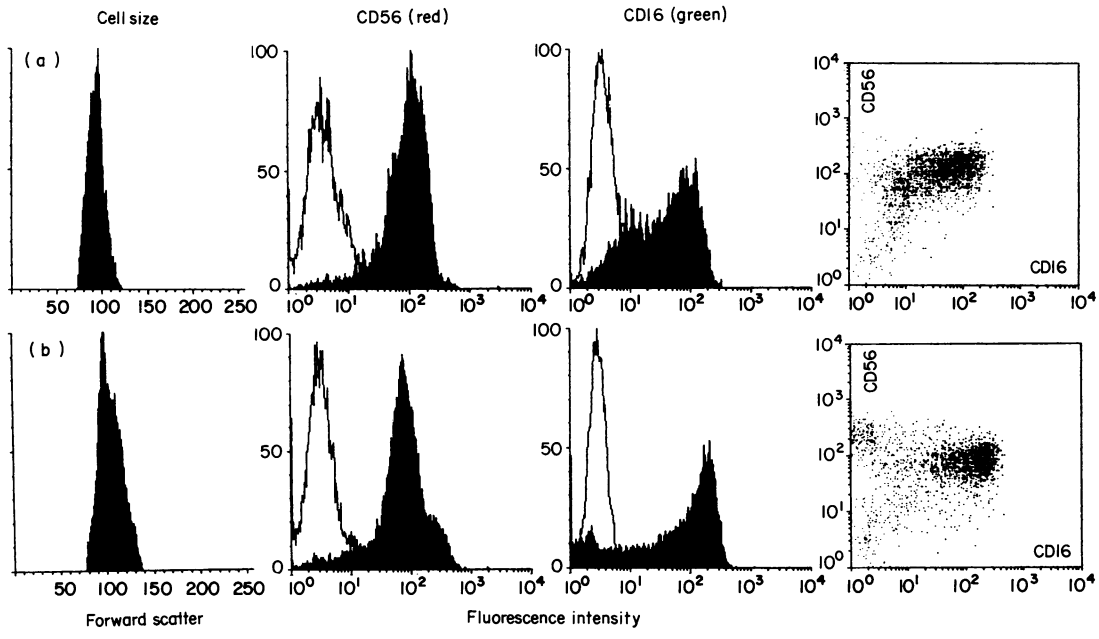


**Figure 2.** Analysis of CD3⁻ R-Ly (a) and CD3⁻ PBMC (b) from donor 69096 showing cell size and two-colour fluorescence analysis of CD56 (Leu-19) and CD16 (FcRGran1) expression. Data are displayed as filled single histograms with the medium control outlined, and as a two-dimensional dot plot.

**Table 1.** The effect of rIL-2 (500 U/ml) on the proliferation and cytotoxic activity of NK cells in R-Ly and PBMC

| | rIL-2 | | | MM-170$_\gamma$ + IL-2-CM | | |
|---|---|---|---|---|---|---|
| | | Cytotoxicity (CU × $10^{-2}$/culture) | | | Cytotoxicity (CU × $10^{-2}$/culture) | |
| NK cells | Proliferation (c.p.m. × $10^{-2}$/culture) | K562 | Ig-P815 | Proliferation (c.p.m. × $10^{-2}$/culture) | K562 | Ig-P815 |
| CD3⁻R-Ly | <1 | <2·4 | <2·2 | 84 | 103±9 | 92±10 |
| CD3⁻ PBMC | 32 | 198±50 | 118±40 | 145 | 501±88 | 326±28 |

$10^4$ NK cells (donor 91471) were cultured in at least triplicate with either rIL-2 (500 U/ml) or MM-170$_\gamma$ + IL-2-CM. Cultures were combined and assayed on Day 7.

in the corresponding CD3⁻ R-Ly. By setting electronic gates at different forward scatter values the data in Fig. 2b were re-analysed to show that the CD16$^{dim}$ cells were the smallest cells in the CD3⁻ PBMC population.

CD3⁻ R-Ly and CD3⁻ PBMC differed in their response to high concentrations of rIL-2. The results from one of three similar experiments with different donors is presented in Table 1. During 7 days culture with rIL-2 (500 U/ml) only cells in the CD3⁻ PBMC population proliferated and were cytotoxic on K562 and Ig-coated P815 target cells. Both populations responded in culture with MM-170 stimulator cells and IL-2-CM.

### NK cell differentiation from resting NK cells

Cultures containing $10^4$ CD3⁻ R-Ly were stimulated with $10^{3.5}$ γ-irradiated MM-170 cells and IL-2-CM, as previously described.[5] The first actively dividing cells were visible in the cultures on Days 5–6. By Days 7 or 8 and up to Days 12–14, the cultures required daily splitting and were maintained on IL-2-CM. After Day 14, when cell growth had virtually ceased, the cultures were fed every 2 days with fresh medium and IL-2-CM. This pattern of cell growth was reproducible for a number of donors. In a typical experiment (Fig. 3), the cell yields per culture were $1{\cdot}5 \times 10^4$, $2{\cdot}3 \times 10^4$ and $653 \times 10^4$ at Days 6, 7 and 14, respectively. Cells generated during culture were cytotoxic for K562 and Ig-P815 targets with activities between 1 and 2 CU/cell. The percentage of responding cells in CD3⁻ R-Ly was estimated, by limiting dilution analysis,[15] as 20% (69096), 6% (82298) and 12% (72984).

The data presented in Fig. 3 show cell size, the expression of the p55 (Tac) IL-2R, the p70 IL-2R, CD56 and CD16 during NK cell generation from CD3⁻ R-Ly of donor 69096. Similar results were obtained with at least four different donors and in repeat experiments with the same donor. On Day 6, 75% of the cells were very large and expressed the p55 (anti-Tac) IL-2R chain. The small cells (data inset, Fig. 3), like the CD3⁻ R-Ly

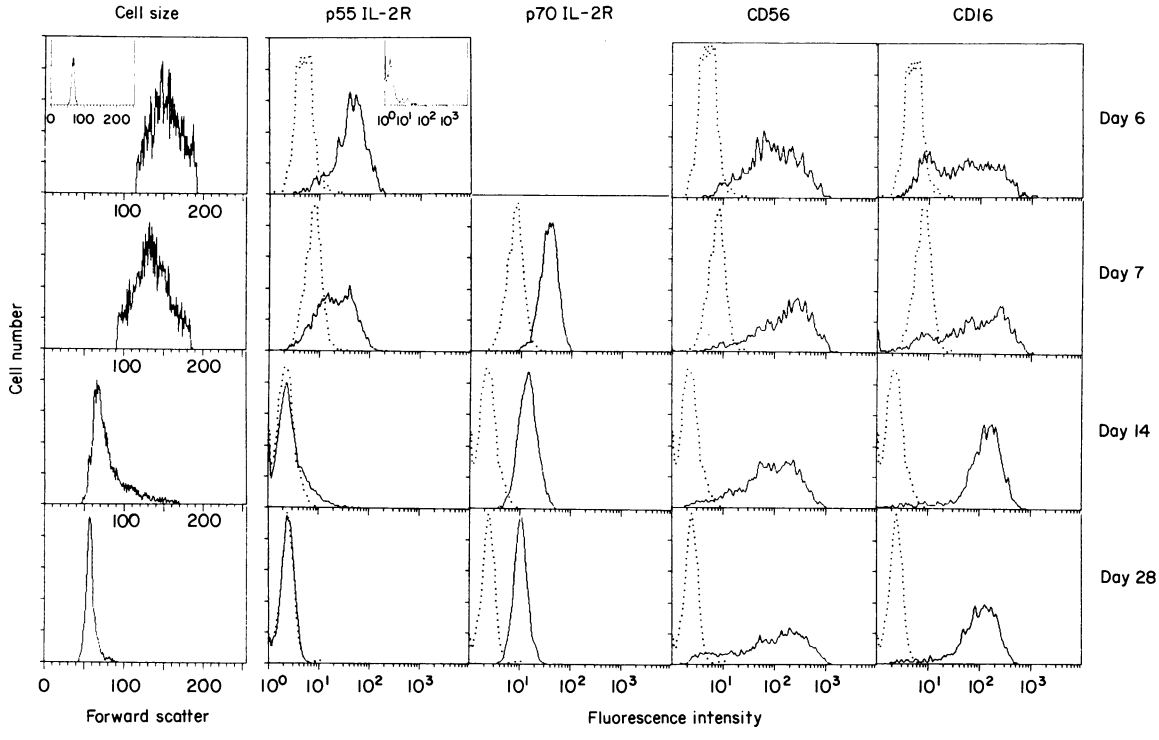


**Figure 3.** Analysis of NK cells generated from CD3$^-$ R-Ly by stimulation with $\gamma$-irradiated MM-170 cells and IL-2-CM (donor 69096). Analysis shows cell size (forward scatter), expression of the IL-2R chains p55 (anti-Tac) and p70 (Mik-$\beta$1), and expression of CD56 (Leu-19) and CD16 (B73.1) using biotinylated anti-mouse subclass-specific Ab and streptavidin-PE. Samples were analysed on the days indicated using the same instrument settings. These instrument settings differed from those used in Fig. 2. Inset data (Day 6) show the absence of p55 IL-2R on small cells.

**Table 2.** Effect of CD16 interaction on NK cell generation

| $10^4$ CD3$^-$ R-Ly (no. 82298) cultured with | Cell* yield/culture $\times 10^{-4}$ | Proliferation c.p.m./culture $\times 10^{-2}$ | Cytotoxicity CU $\times 10^2$/culture | |
|---|---|---|---|---|
| | | | K562 | Ig-P815 |
| IL-2-CM | 0·31 | <1 | <1 | ND |
| MM-170+IL-2-CM | 1·73 | 110 | 175±9 | 468±56 |
| IL-2-CM on SPB† | 0·25 | <1 | <1 | ND |
| IL-2-CM on SPB-OKT3 | 0·03 | <1 | <1 | ND |
| IL-2-CM on SPB-B73.1 | 0·11 | <1 | <1 | ND |
| MM-170+IL-2-CM on SPB | 3·03 | 189 | 195±42 | 300±56 |
| MM-170+IL-2-CM on SPB-OKT3 | 2·63 | 181 | 167±25 | 276±31 |
| MM-170+IL-2-CM on SPB-B73.1 | 2·60 | 171 | 79±5 | 76±8 |

* Cultures from six wells were combined and assayed on Day 8.

† SPB (solid-phase bound) refers to culture wells coated with sheep anti-mouse Ig and mAb as indicated.

ND, not determined.

(data not shown), did not express the p55 IL-2R. By Day 7, 90% of the cells were large with p55 IL-2R expression markedly reduced. Cell size decreased as the cells entered the non-proliferative phase (Day 14), and this coincided with low levels of the p55 IL-2R. By Day 28 all cells were small and no p55 IL-2R was detectable. In contrast to the transient expression of the p55 IL-2R, the p70 IL-2R chain was present on the CD3$^-$ R-Ly (data not shown) and on all cells throughout culture. All culture-generated NK cells were CD3$^-$ and for most donors all cells were CD2$^+$ (data not shown). Virtually all cells expressed the NK cell marker CD56. At least two subpopulations expressing a CD16 phenotype were present early in culture,

namely CD16$^{dim}$ and CD16$^{bright}$, with the proportion of CD16$^{bright}$ cells increasing with time in culture. A similar range of CD56 expression was seen on both CD16$^{dim}$ and CD16$^{bright}$ cells.

### The role of CD16 in NK cell differentiation

Since varying levels of CD16 were expressed on CD3$^-$ R-Ly, we asked whether CD16 was involved in the process of NK cell differentiation triggered by MM-170 stimulator cells. CD3$^-$ R-Ly were cultured on untreated culture wells or on wells pretreated with sheep anti-mouse Ig and subsequently with either medium or with B73.1 or OKT3, allowing presentation of mAb in a solid-phase-bound (SPB) form. Cultures contained IL-2-CM with or without $\gamma$-irradiated MM-170 stimulator cells. The results of one of three experiments are presented in Table 2. The data presented show that differentiation and proliferation of NK cells from CD3$^-$ R-Ly only occurred in cultures containing MM-170 and IL-2-CM. The presence of SPB-B73.1 did not interfere with NK cell generation, and SPB-B73.1 with IL-2-CM was an insufficient stimulus in this process. Similar results were obtained using anti-CD16 mAb in soluble rather than SPB form. The increased cell yield and proliferation in culture wells containing SPB-B73.1 was not specific for the anti-CD16 mAb but also occurred with the control SPB and SPB-OKT3 culture wells. The presence of SPB-B73.1 inhibited the cytotoxic activity of the culture-generated NK cells. Compared to NK cells generated in control (SPB and SPB-OKT3) wells, NK cells generated in SPB-B73.1 wells were less cytotoxic per cell. Cytotoxic activity per cell was reduced from 0·64 to 0·30 CU (K562 targets), and from 1·0 to 0·29 CU (Ig-P815 targets) in culture wells containing SPB-B73.1. As a positive control in these experiments, culture-generated NK cells from Day 17 of culture were stimulated with SPB-B73.1 and the culture supernatants analysed for interferon-gamma (IFN-$\gamma$) production (Commonwealth Serum Laboratories, Melbourne, Australia). Activated NK cells produced 3800 pg IFN-$\gamma$/5 $\times$ 10$^4$ cells over a 20-hr period when stimulated with SPB-B73.1 compared to 360 pg/5 $\times$ 10$^4$ cells in control wells.

## DISCUSSION

The present studies define the CD16 phenotype of a resting subpopulation of human peripheral blood NK cells. Resting NK cells sediment at Percoll high buoyant densities with resting T cells,[3,4] and, as shown in these studies, are small cells unresponsive to high concentrations of IL-2. In contrast, the majority of NK cells are large cells which sediment at Percoll low buoyant densities[2] and proliferate with high concentrations of IL-2.[14] The resting subpopulation of NK cells contained two distinct populations based on the intensity of CD16 expression, namely CD16$^{dim}$ and CD16$^{bright}$. Both populations expressed similar levels of CD56. The proportion of CD16$^{dim}$ and CD16$^{bright}$ cells varied from donor to donor. In comparison, the total population of NK cells, which includes the resting NK cells, contained three populations, CD16$^{dim}$, CD16$^{bright}$ and CD16$^-$. The CD16$^{dim}$ phenotype may be unique to the resting subpopulation of NK cells for the following reasons. Firstly, the CD16$^{dim}$ cells were a greater proportion of the resting subpopulation of NK cells compared to the total population of NK cells for any particular donor. Secondly, CD16$^{dim}$ cells were identified as small cells in the total population of NK cells. It should be noted that the minor population of CD16$^{dim}$ cells described by Nagler *et al.*[14] differed from the CD16$^{dim}$ resting NK cells since they were CD56$^{bright}$ and proliferated with low concentrations of IL-2. CD16$^{bright}$ cells were present in both the resting subpopulation and the total population of NK cells, and these cells were the major type identified in Percoll low buoyant density NK cells by Nagler *et al.*[14] The CD16$^{bright}$ cells in the resting subpopulation of NK cells differed from those in the total population of NK cells by an increased expression of CD56 and a decreased expression of CD16. The resting subpopulation of NK cells was devoid of the CD16$^-$ cells which were present in the total population of NK cells as large cells expressing high levels of CD56. CD16$^-$ CD56$^{bright}$ cells were recently described by Nagler *et al.*,[14,16] and by Caliguiri *et al.*[17] as a small population of NK cells expressing high affinity IL-2R and proliferating with low concentrations of IL-2. This analysis demonstrates a unique CD16 profile for a resting subpopulation of peripheral blood NK cells.

Differentiation of resting NK cells triggered by co-culture with MM-170 cells and IL-2-CM resulted initially in the generation of both CD16$^{dim}$ and CD16$^{bright}$ NK cells. The simplest interpretation of these data is that these cells differentiate from CD16$^{dim}$ and CD16$^{bright}$ resting NK cells, respectively. However, we could not obtain data to unequivocally demonstrate this. An analysis of the frequency of responding cells compared to the proportion of CD16$^{dim}$ cells made it unlikely that CD16$^{dim}$ cells were the sole precursors of the culture-generated NK cells. Thus, for the three donors analysed, the proportion of resting cells responding was 20%, 6% and 12%, whereas the proportion of CD16$^{dim}$ cells in the resting subpopulation of NK cells was 27%, 59% and 8%, respectively. It was not practical to sort the resting subpopulation of NK cells into CD16$^{dim}$ and CD16$^{bright}$ cells to assess whether both populations were activated, since after OKT3 and double complement treatment the R-Ly contained approximately 99% dead cells, and the number of live cells obtained was never greater than 2 $\times$ 10$^5$. The CD16$^{bright}$ cells became the dominant cell later in culture. A simple explanation for this result is that CD16$^{bright}$ cells grow more rapidly than CD16$^{dim}$ cells. An alternative explanation is based on the observation that CD16 is rapidly shed from the surface of activated NK cells.[18] Accordingly, the dominance of CD16$^{bright}$ cells could reflect a net increase in the rate of synthesis compared to the rate of shedding of cell-surface CD16. The high level of CD16 expression was a stable feature of culture-generated NK cells maintained for several weeks. During this time NK cells became smaller, were no longer responsive to IL-2-CM, and had therefore reverted to a resting state. From these observations we predict that the CD16$^{bright}$ subpopulation of resting NK cells consists of cells which have been previously activated *in vivo* and have reverted to a resting state.

The subpopulations of peripheral blood NK cells distinguished by their different activation requirements may represent distinct NK cell lineages. The CD56$^{bright}$ CD16$^-$ cells are the most activated NK cell subpopulation since they constitutively express high affinity IL-2R and proliferate with low IL-2 concentrations.[14,16,17] These NK cells may be those active in the early response to viral infections. A second subpopulation, which expresses the phenotype CD56$^{dim}$ CD16$^{bright}$, expresses an intermediate affinity IL-2R and responds to high concentrations of IL-2.[14] The third NK cell

subpopulation is the resting subpopulation of NK cells described in our studies, which contains a mixed population of $CD16^{dim}$ and $CD16^{bright}$ cells, the latter cells possibly reflecting a previous state of activation. These resting NK cells do not respond to low or high concentrations of IL-2 unless there is an additional signal provided by stimulator cells. The activated cells generated from this resting subpopulation of NK cells never express a $CD56^{bright}$ $CD16^-$ phenotype, and only transiently express high affinity IL-2R. Thus it is unlikely that the activated NK cells isolated from peripheral blood are related in a differentiation sequence to the resting subpopulation of NK cells described in these studies.

Our results show that the differentiation of resting NK cells does not involve the CD16 molecule. Thus CD16 interaction using SPB-linked anti-CD16 mAb did not induce proliferation of resting NK cells in the presence of IL-2, and neither enhanced nor inhibited the differentiation process triggered by the stimulator cells and IL-2. However, CD16 interaction did reduce the cytotoxic activity of the culture-generated cells, and did stimulate them to produce IFN-$\gamma$. These results are consistent with the studies of others showing that the effector functions of NK cells are influenced by CD16 interaction,[19,20] but that such interaction does not induce NK cell proliferation.[21,22] Thus it can be concluded that CD16 functions only as an effector stage molecule for NK cells.

The process of NK cell differentiation described in these studies is similar to that known for T-cell differentiation.[23] Thus, like resting T cells,[24] resting NK cells express the p70 IL-2R but do not respond to high concentrations of rIL-2. As with resting T cells, co-culture with the required stimulator cells and IL-2 initiates cell differentiation with an increase in cell size and the expression of the p55 IL-2R. Expression of the p55 IL-2R is transient and corresponds to the period of cell growth. Following cessation of growth, NK cells revert to a smaller size. Culture-generated NK cells, unlike activated T cells, do not produce IL-2.[25] Thus the differentiation of resting NK cells *in vivo* would be dependent upon activated T cells to provide the required stimulating signals,[4,5] including IL-2.

Generation of NK cells in culture from the total population of peripheral blood NK cells has been reported by others.[21,26] The role of tumour cell lines in these co-culture procedures is not fully defined. In one study, NK cell proliferation induced in cultures containing B-lymphoblastoid cells was dependent upon $CD4^+$ T cells, possibly activated against the tumour cells.[1] In another study, the presence of tumour cell lines increased the degree of proliferation and the number of NK cells maintained in clonal growth.[27] Harris *et al.*[21] suggested that target cell interaction may provide a stimulating signal for NK cell differentiation. However, it is unlikely that the resting subpopulation of NK cells is stimulated to differentiate by target cell interaction since the NK cell targets K562 and RPMI-8866 do not stimulate (H. S. Warren, unpublished data), whereas activated T cells which are not NK cell targets are potent stimulator cells.[4]

The cell-surface molecule(s) involved in triggering differentiation of resting NK cells have yet to be identified. We previously proposed[5] that resting NK cells and resting gamma/delta T cells may use a common cell-surface molecule for cell activation, since both resting cell populations were activated by the same stimulator cells in the presence of IL-2-CM. NK cells have recently been shown to express the zeta chain of the TcR–CD3 complex in the absence of any of the chains of that structure.[28] The zeta chain co-associates with several different proteins on NK cells, and one of these has recently been identified as CD16.[29] The zeta chain is required for cell-surface expression of the TcR–CD3 complex and for signal transduction in T cells[30] and allows expression of CD16 on COS-7 cells.[29] It is likely, therefore, that the zeta chain or a homologous structure will be a common signal-transducing molecule for other cell-surface molecules, including those necessary for triggering differentiation of the resting subpopulation of NK cells.

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