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Novel method for isolating untouched rat natural killer cells with higher purity compared with positive selection and fluorescence-activated cell sorting

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Summary

Natural killer (NK) cells are important effectors of both innate and adaptive immune responses. Although human and mouse NK cells are extensively characterized, much less is known about the rat cells, partly because of the current lack of reliable isolation techniques. We aimed to develop a method for isolating highly pure 'untouched' rat NK cells by negative selection from splenocytes. Thereafter, we characterized them phenotypically and functionally in comparison with those isolated by positive selection targeting the NKR-P1 receptor. Our novel method isolated highly pure untouched NK cells reproducibly with $97 \pm 0.7\%$ ($n = 7$), $96.6 \pm 0.8\%$ ($n = 3$) and $88.3 \pm 1.5\%$ ($n = 9$) in LEWIS, Fischer and athymic nude rats, respectively. The positively selected NK cells were less homogeneous and exhibited undesired method-related activation profiles. Resting negatively selected NK cells were less proliferative and less robust compared with positively selected NK cells. Although resting positively selected NK cells were more cytotoxic, interleukin-2 (IL-2) activation increased the cytotoxicity of negatively selected cells three-fold. The negatively selected NK cells responded to cross-linking of the NKR-P1 receptor by calcium mobilization from intracellular stores. However, combined IL-2 and IL-12 activation resulted in significantly more interferon-γ release from positively selected NK cells. This new NK-cell isolation method will allow a deeper insight into rat NK-cell phenotypes and the roles of their receptors in the biology of these cells.

Keywords: natural killer cell; negative selection; NKR-P1; rat

Introduction

Natural killer (NK) cells are important actors in innate immunity. These cells are capable of killing virus-infected cells[1] and susceptible tumour cells[2] without prior activation. Their effector function is regulated by a balance between activating and inhibitory receptor signals that are triggered by specific major histocompatibility complex (MHC) class I or non-MHC ligands. It has been established that NK cells can influence the adaptive immune response by secreting cytokines and chemokines.[3] A new role of NK cells in adaptive immunity has recently emerged where it has been demonstrated that a subset of rat bone-marrow-derived NK cells can regulate T-cell

activation.[4] In addition, conventional NK cells play a role in maintaining immune homeostasis by eliminating inadvertently activated autoreactive T cells.[5]

Rat NK cells are phenotypically defined as NK receptor protein 1 (NKR-P1, also called CD161) -positive, CD3-negative (i.e. NKR-P1^{+} CD3^{−}) lymphocytes. The NKR-P1^{+} is a disulphide-linked homodimer expressed by all NK cells and a small subset of T cells.[6] In rats and humans, myeloid cells, including granulocytes, monocytes and dendritic cells were also demonstrated to express NKR-P1 weakly.[7–9] Several properties of NKR-P1 indicate that it may play a role in physiological NK-cell activation. Indeed, antibodies to the NKR-P1 molecule (clone 10/78 or clone 3.2.3) mediate redirected lysis by interleukin-2 (IL-2) -activated NK cells

against the FcR-expressing target cells P815.[8] Moreover, the stimulation of NKR-P1 on RNK-16 rat leukaemia cells with features of NK cells activates phosphoinositide turnover and calcium mobilization.[10]

The study of cellular immune responses in animal models of disease demands detailed knowledge of the development, function and regulation of different subsets of immune cells, such as NK cells. Whereas many studies use mouse NK cells, rat-derived cells can be a better model for human diseases such as cardiovascular diseases, diabetes, arthritis and many autoimmune disorders.[11] In particular, this model will be useful in developing pre-clinical adoptive NK-cell transfer immunotherapy against human brain tumours. Compared with that of mice, the rat brain is the organ of choice because of the particular advantages of its larger size and distribution of white matter tracts.

Several strategies for purification of splenic rat NK cells were previously described. The first method, proposed by Vujanovic et al.,[12] involved the isolation of lymphocytes from total splenocytes by gradient centrifugation on Ficoll–Hypaque, followed by passage over nylon wool columns. These cells were used directly as effector cells in cytotoxicity assays.[8] Another method consisted of NK-cell enrichment by depletion of B cells and T cells using magnetic bead separation by targeting CD45RA (OX-33) and CD3 (G4.18) antigens,[13] respectively. Both of these methods yielded NK cells of very low purity. The positive selection of NK cells labelled with anti-NKR-P1 antibodies by fluorescence-activated cell sorter (FACS) or magnetic column[14] are the most recently developed methods. However, positively selected NK cells are not always the best choice and in some applications 'untouched' or negatively selected cells may be more appropriate. Indeed, Stehling et al.[15] proposed a method for isolating 'untouched' NK cells from LEWIS rats by the Dynabeads™ system with a purity range of 70–85%. Moreover, Lautenbach et al.[16] devised their own biotin cocktail to purify NK cells from Fischer (F344) rats using the Miltenyi system with an assumed purity of 72·5%.

Few methods exist for the reliable isolation and purification of rat NK cells. This is largely because of the lack of species-specific antibody cocktails that recognize NK cells with high accuracy and efficiency. In the present paper, we propose a new method for negative selection of rat NK cells from splenocytes that differs from previously described techniques[16,17] in the cocktail of antibodies. We devised a novel biotinylated antibody cocktail that can be reliably used to isolate rat NK cells with high purity and efficiency. We investigated the antigenic phenotype and compared the functional characteristics of NK cells after positive selection [by cell sorting or by Miltenyi magnetic antibody cell sorting (MACS) separation system] or negative selection with our novel method. Using a range of assays investigating phenotype, proliferation, cytotoxicity, viability and interferon-γ (IFN-γ) production we showed that negatively selected NK cells were more homogeneous in comparison with positively selected NK-cell populations. In this study, we used athymic nude rats and immune-competent LEWIS and F344 rats. In athymic nude rats, the opportunity to study NK-cell activity in the absence of thymus-dependent cellular immune responses was increased.

Materials and methods

Chemicals and buffers

RPMI-1640 GLUTAMAX™ I medium, phosphate-buffered saline (PBS), HEPES, β-mercaptoethanol, fetal bovine serum (FBS), penicillin–streptomycin–neomycin mixture (PSN), 7-amino-actinomycin D (7-AAD), Fura Red, Fluo-4, propidium iodide (PI), probenecid, pluronic acid, ionomycin and TO-PRO-3 were purchased from Invitrogen GmbH (Karlsruhe, Germany). AutoMACS rinsing solution and MACS bovine serum albumin (BSA) stock solution were obtained from Miltenyi Biotec B.V. (Utrecht, the Netherlands). Sodium chloride, calcium chloride, Triton × 100, sodium azide and 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) were provided by Sigma-Aldrich (Munich, Germany). Nylon wool fibre was distributed by G. Kisker GbR (Steinfurt, Germany). Golgi Plug and Cytofix Cytoperm solution were purchased from BD Biosciences (Erembodegem, Belgium) and ACK Lysing Buffer was from Lonza (Basel, Switzerland). The recombinant human IL-2 and IL-12 were purchased from R&D Systems (Abingdon, UK).

Animals

Male athymic nude (Hsd : RH-Foxn1^{rnu} rnu/rnu), LEWIS (LEW/Han™Hsd) and Fischer (F344/NCrHsd) rats were purchased from Harlan Laboratories (Horst, the Netherlands) and kept under specific pathogen-free conditions, provided with standard pellet diet and sterilized water *ad libitum*. Animals were killed between the ages of 8 and 10 weeks by carbon dioxide inhalation and decapitation. All animal experiments were conducted in accordance with the current European and Luxembourgish ethical guidelines for use of laboratory animals.

Cell culture

YAC-1, a mouse lymphoma cell line, was purchased from European Collection of Cell Cultures (ECAAC, Salisbury, UK). Cells were grown in suspension in flasks (Nunc GmbH & Co. KG, Langenselbold, Germany), in complete RPMI-1640 medium (RPMI-1640 supplemented with PSN, 10% FBS, 1 mM HEPES and 50 μM β-mercaptoethanol). Only cells in the exponential growth phase were used for cytotoxicity assays.

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## Antibodies and extracellular staining for flow cytometry

Cells were stained using fluorescein isothiocyanate-
(FITC), phycoerythrin- (PE) and Alexa 647-conjugated
anti-rat monoclonal antibodies (mAbs) against CD3
(G4.18), anti-HLA class II (HIS19), from eBioscience Inc.
(San Diego, CA), anti-NKR-P1 A/B (10/78), anti-CD8α
(OX-8) from Biolegend (San Diego, CA), anti-CD172a
(OX-41), anti-CD11b/c (OX-42) from Immunotools
(Friesoythe, Germany). 7-AAD was used as viability dye
and 10^6 cells were stained in 50 μl of staining buffer (PBS
with 1% BSA and 0·1% NaN$_3$) for 30 min at 4° and
washed twice before analysis. Immunophenotyping was
performed by four-colour multiparameter flow cytometry
using a FACSCanto (Becton Dickinson, Aalst, Belgium).
Data analysis was performed using FACSDIVA Software
version 6.1.2 (Becton Dickinson). At least 50 000 events
per sample were acquired and the viable cells were gated
according to their negativity for 7-AAD and to their for-
ward-scatter (FSC) and side-scatter (SSC) characteristics.

Preparation of splenocytes for NK purification

Single cell rat splenocytes were obtained after dissocia-
tion of the spleen through a cell strainer. Erythrocytes
were lysed with ACK Lysing Buffer (Fig. 1a–c). Then
splenocytes were pre-purified over a nylon wool column
to remove the majority of B cells (Fig. 1d,e). Here we
used a modification of the procedure originally
described by Havenith *et al.*[18] Briefly, 10 ml syringes
were packed with 0·5 g of nylon wool. The nylon col-
umn was rinsed with complete RPMI-1640 medium
before 1 hr incubation at 37°. Post-incubation period,
the nylon column was loaded drop wise with cell sus-
pension. The column was incubated for 1 hr at 37°.
Non-adherent cells were collected by flushing the col-
umn with pre-warmed complete RPMI-1640 medium.
This effluent served for positive, negative and FACS
purification of NK cells. All subsequent steps were per-
formed at 4°.

Negative selection of NK cells by magnetic beads

The B cell depleted splenocytes were washed with auto-
MACS rinsing solution supplemented with 0·2% vol-
ume/volume (v/v) MACS BSA stock solution (MACS
buffer solution). Cells were incubated for 15 min at 4°,
with the biotinylated mAb cocktail composed of
12·5 μg/ml anti-rat CD5 (OX-19) and 5 μg/ml anti-
CD45RA (OX-33), both from Biolegend, 5 μg/ml anti-
endothelium (OX-43), 5 μg/ml anti-erythrocyte (OX-83),
12·5 μg/ml anti-CD172a (OX-41) from Cedarlane
(Ontario, Canada) and finally 5 μg/ml anti-granulocyte
(HIS48) from eBioscience Inc (Fig. 1f). Afterwards, the
cells were washed with MACS buffer solution followed
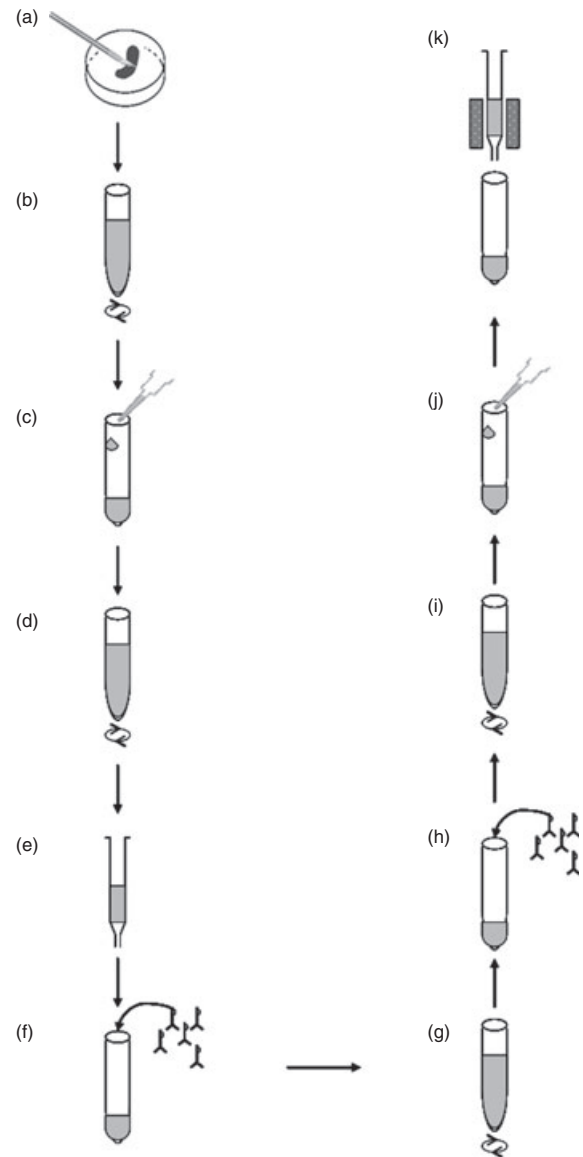
Figure 1. Procedure of negative purification of rat natural killer
(NK) cells. (a) Prepare a splenocyte suspension. (b) Pellet cells by
centrifugation. (c) Lyse erythrocytes. (d) Wash and pellet cells by
centrifugation. (e) Incubate cells on nylon wool column. (f) Collect
enriched fraction by washing nylon wool column and pellet cells by
centrifugation. Label cells with biotin antibody cocktail. (g) Wash
and pellet cells by centrifugation. (h) Add anti-biotin microbeads. (i)
Wash cells and pellet cells by centrifugation. (j) Resuspend cells in
separation buffer. (k) Apply into the magnetic column. Collect nega-
tive cell fraction by washing the column in a magnetic field: this
fraction corresponds to the untouched NK-cell fraction.

by an incubation step of 15 min with anti-biotin
microbeads from Miltenyi Biotec B.V. (Fig. 1g–i). After
this washing step, the pellet was resuspended in MACS
buffer solution and the cell suspension was applied on
a magnetic column to remove non-NK cells. Flow-
through corresponded to the purified fraction of
untouched NK cells (Fig. 1j,k).

Positive selection of NK cells by magnetic beads

The positive selection of NK cells using magnetic beads was performed in two steps. In a first step, CD3$^+$ cells were depleted and this was followed by a positive selection of cells expressing the NKR-P1 receptor. Briefly, splenocytes were incubated with 5 μg/ml of monoclonal biotinylated anti-rat CD3 (G4.18) mAb from eBioscience and then with an anti-biotinylated mAb coupled to microbeads. Selection columns placed in a magnetic field were used to deplete the CD3$^+$ cells. In a second step, the flow-through, corresponding to the CD3$^-$ fraction, was incubated with 5 μg/ml of biotinylated anti-NKR-P1 mAb (10/78) from Biolegend and was followed by anti-biotinylated secondary mAb coupled to microbeads. Finally, the NK-cell fraction was retained on the positive selection columns placed in a magnetic field. The percentage of NK-cell purity was analysed by flow cytometry.

Positive selection of NK cells by FACS

After incubation on a nylon wool column, the enriched splenocyte fraction was washed once in staining buffer and incubated for 30 min with 10 μg 7-AAD, 0·25 μg of Alexa 647-conjugated anti-NKR-P1 and PE-conjugated anti-CD3 mAbs in 100 μl FACS buffer per 10^6 cells. After washing twice in staining buffer the cells were resuspended in PBS supplemented with 0·5% v/v FBS before sorting of 7-AAD$^-$ CD3$^-$ NKR-P1$^+$ cells by FACS with an acquisition rate of 1000–3000 events per second at 4° in complete medium. Cells were sorted using a FACSAria cell sorter (BD Biosciences). Sorted cells were determined to be > 99% pure by post-sort phenotyping.

CFSE cell proliferation assay

Before culture the freshly, positive or negative, selected NK cells, were labelled with CFSE using a modified technique, originally described by Lyons[19] First, purified NK cells were incubated with 2·5 μM CFSE for 15 min at 37°. Then labelled NK cells were cultured in complete RPMI-1640 medium supplemented with 1000 U/ml IL-2, during 4 days in a humidified incubator with 5% CO_2 at 37°. After culture the cells were recovered and stained with 10 μg 7-AAD, 0·25 μg Alexa 647-conjugated anti-NKR-P1 and PE-conjugated anti-CD3 mAbs. The analysis was performed on a FACSCanto flow cytometer.

Annexin V apoptosis assay

After 4 days of culture, cells were recovered and washed with binding buffer (10 mM HEPES, 140 mM NaCl, 2·5 mM CaCl$_2$, pH 7·4). Then cells were incubated with binding buffer supplemented with 2·5 μl Annexin V-allo-phycocyanin (eBioscience) for 30 min. Before analysis by flow cytometry, 0·25 μg PI was added.

Cytotoxicity assay

Cytotoxicity of purified NK cells was determined against the YAC-1 target cells that were labelled with 5 μM CFSE, before and after 72 hr of incubation in 1000 U/ml IL-2. Effector cells (E) were mixed with target cells (T) at E : T ratios ranging from 1 : 1 to 50 : 1. After 5 hr of incubation at 37° the cells were analysed on a FACSCanto flow cytometer. To identify dead cells, 15 μM of the dead cell marker TO-PRO-3 was added. At least 4000 target cells per sample were examined.

Intracellular detection of IFN-γ

Splenocytes obtained after nylon wool column separation and negatively selected NK cells were incubated for 18 or 36 hr with or without cross-linking of the NKR-P1 receptor in complete medium supplemented with 1000 U/ml IL-2. Positively and negatively selected NK cells were also incubated for 72 hr with 1000 U/ml IL-2. NK cells incubated with 10 μg/ml IL-12 overnight served as positive controls. Interferon-γ production was monitored by intracellular FACS staining using Golgi Plug for 4 hr. The extracellular antigens were first stained using 0·25 μg Alexa 647-conjugated anti-NKR-P1 and PE-conjugated anti-CD3 mAbs, followed by fixation and permeabilization using Cytofix Cytoperm solution. The immunostaining for IFN-γ was performed in PermWash solution (BD Biosciences) with an anti-IFN-γ (DB-1) FITC labelled mAb (Biolegend).

Calcium flux assay

To measure intracellular calcium mobilization, purified NK cells were loaded in 5 μM Fluo-4 AM and 10 μM Fura Red AM solution diluted in pluronic acid for 35 min at 37° in buffer containing 10 mM HEPES, 0.1% v/v BSA and 0.7 mg/ml probenecid at a pH of 7.4. The cells were stained with biotinylated anti-NKR-P1 mAb at room temperature for 10 min. Cells were washed and pre-warmed for 5 min at 37° before FACS acquisition. First, the baseline fluorescence for unstimulated NK cells was established and then the cross-linker, anti-biotin microBeads, was added. Ionomycin was used as positive control to check the efficiency of the loading. Data were analysed using FLOWJO software version 7.2.5 (Tree Star, Inc. Ashland, OR) and were presented as Fluo-4 against Fura Red intensity over time.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using unpaired Student's t-test. Differ-

ences were considered significant if $P < 0.05$. Data were analysed using GraphPad Prism version 4 (GraphPad Software, Inc. La Jolla, CA).

Results

Increased purity and yield of NK cells obtained by negative selection

By using negative purification methods on splenocytes from athymic nude rats, we obtained an NK-cell purity of $88.3 \pm 1.5\%$ ($n = 9$), with LEWIS rats this was $97 \pm 0.7\%$ ($n = 7$) and with F344 rats it was $96.6 \pm 0.8\%$ ($n = 3$). The NK-cell purity in the positively selected populations was lower in both athymic nude and LEWIS rat strains, [$66 \pm 6.4\%$ ($n = 4$) and $47.8 \pm 2.8\%$ ($n = 6$), respectively] (Fig. 2). The NK cells obtained from both strains of rats after FACS had purity > 99% (data not shown). Contamination of the NK-cell fraction by other splenocytes was negligible, both by negative purification and by cell sorting. In contrast, using the positive selection of NK cells by targeting the NKR-P1 receptor after CD3 depletion, we observed contamination by CD172a-positive cells, which were essentially monocytes, and also some B cells, which were not retained by the nylon wool column (Fig. 2). The cells were counted before and after the purification processes, and the NK-cell yield was given as the percentage obtained from the total number of enriched lymphocytes passed through the nylon wool column. Using LEWIS rats, FACS showed a significantly lower number of purified cells ($1.6 \times 10^6 \pm 0.4\%$) compared with both positive ($7.4 \times 10^6 \pm 4.2\%$) and negative ($4 \times 10^6 \pm 1.5\%$) selection with microbeads (data not shown).

Positive selection of NK cells by targeting the NKR-P1 molecule triggers their activation by cross-linking of the selective antibody

#### *The positive selection of NK cells increases cytotoxicity*

The cytotoxic capacity of purified NK cells was followed in a cytotoxicity assay using YAC-1 target cells. Positively selected NK cells from athymic nude rats exhibited significantly greater cytotoxicity at day 0 for the E : T ratio 20 : 1 ($P < 0.05$) compared with the negatively selected populations (data not shown). These differences in the cytotoxicity of NK cells isolated by positive versus negative selection were confirmed with immune-competent LEWIS rats. Indeed, positively selected NK cells were more cytotoxic against YAC-1 cells than negatively selected NK cells, and differences were significant at the E : T ratios 5 : 1 ($P < 0.01$), 10 : 1 and 25 : 1 ($P < 0.001$) (Fig. 3a). The cytotoxicity of positively and negatively selected NK cells was identical after incubation for 72 hr in 1000 U/ml of IL-2 (Fig. 3b). Interestingly, NK cells sorted by FACS were capable of lysing YAC-1 cells with a greater intensity than negatively selected NK cells at the ratio 5 : 1 and 25 : 1 ($P < 0.01$). Furthermore, at a 5 : 1 ratio, the sorted NK cells were more cytotoxic than positively selected NK cells ($P < 0.05$) (data not shown).

Positively selected NK cells exhibit increased proliferation and survival

Survival of cultured NK cells from LEWIS rats was investigated by double immunostaining with PI and Annexin
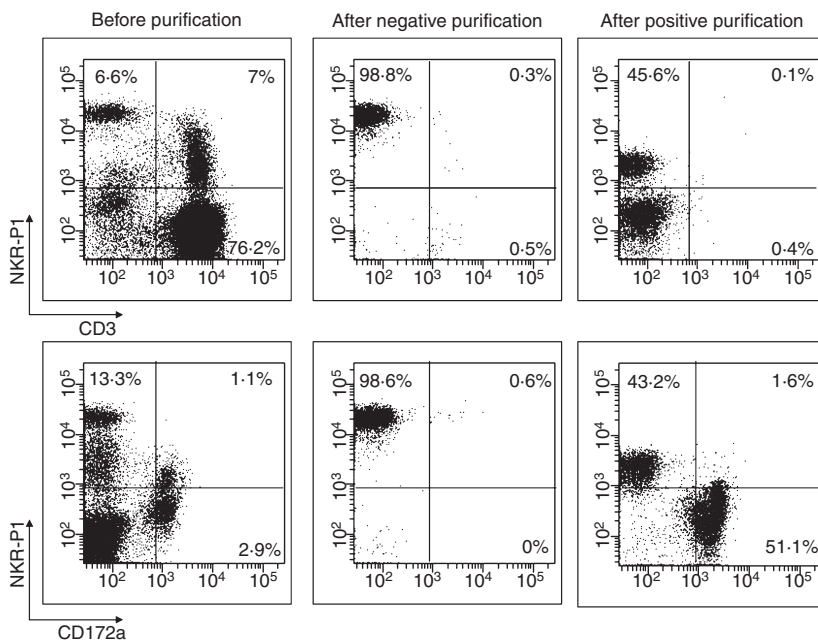
Figure 2. Purity of natural killer (NK) cells after negative or positive isolation methods. Fluorescence-activated cell sorting (FACS) analysis of NK cells before and after purification by positive or negative selection procedure on LEWIS rats. The upper panel presents the expression of NKR-P1 and CD3 and the lower panel represents the expression of NKR-P1 and CD172a. Cells were labelled with fluorescein isothiocyanate-conjugated anti-rat CD172a, phycoerythrin-conjugated anti-rat CD3 and Alexa 647-conjugated anti-rat NKR-P1 antibodies. Dead cells were excluded by gating out 7-amino-actinomycin D-positive cells. These pictures are representative of seven independent experiments on LEWIS rats.
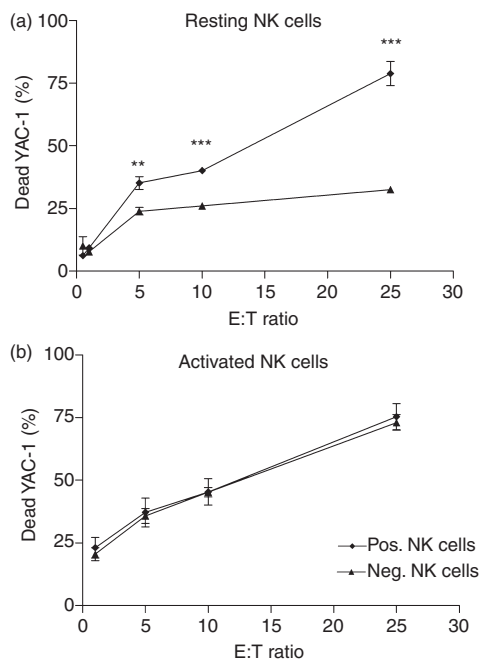
Figure 3. Comparison of the killing capacity of natural killer (NK) cells from LEWIS rats after positive (♦) or negative (▲) purification, in a 5-hr cytotoxicity assay towards CFSE-labelled YAC-1 target cells with different effector : target (E : T) ratios, using resting (a) and activated (b) cells. Cell viability was determined by flow cytometry using TO-PRO-3 as supravital dye. Data are plotted as mean ± SEM from three independent experiments. **$P < 0.005$, ***$P < 0.0001$.

V. The cellular proliferation was determined by loss of CFSE labelling over time during cell divisions (Fig. 4). Positively selected NK cells showed a modified morphology compared with NK cells from negative isolation, as reflected by distinct size and granularity co-ordinates on FSC/SSC dot plot (Fig. 4a,b). Moreover, positively selected NK cells had a significantly reduced proportion of cells in late phases of apoptosis (PI$^+$/Ann V$^+$) than negatively selected NK cells, $6·2 ± 3·6\%$ and $22·1 ± 7·4\%$ (respectively, $P < 0·05$, $n = 3$) (Fig. 4c,d). CFSE labelling was more rapidly depleted in positively selected NK populations, indicating that they grew faster than both the negatively selected (Fig. 4e,f) and FACS-sorted NK cells (data not shown). These differences in proliferation and survival of NK-cell populations isolated by the three methods were not significantly correlated with the surface expression levels of MHC class II, CD8α or CD11b/c (data not shown).

NK positive selection triggers a calcium flux that is not followed by IFN-γ secretion

To investigate the effect of the positive purification process on the function of NK cells, we cross-linked the NKR-P1 in resting or IL-2-activated negatively selected cells and examined calcium mobilization and IFN-γ
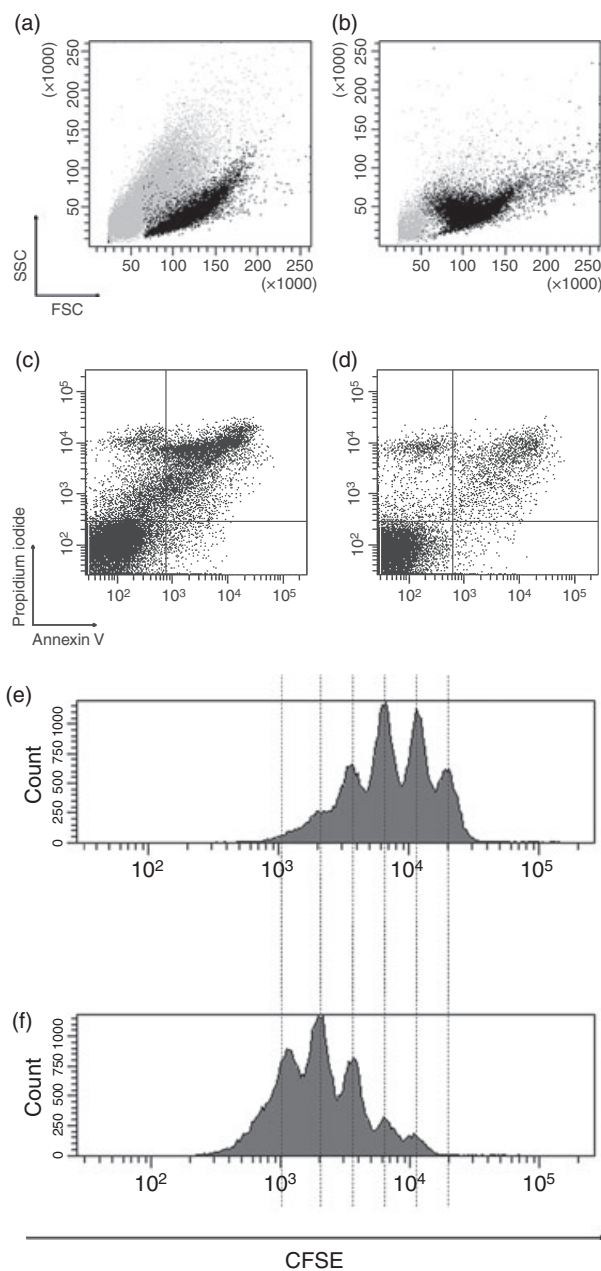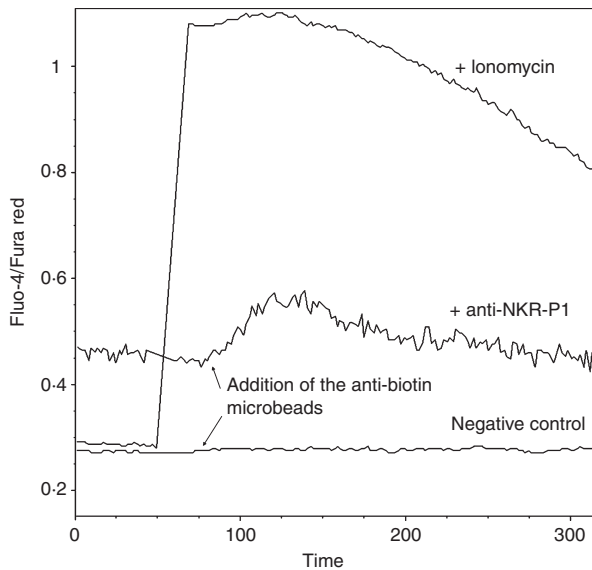

Figure 4. Morphology (a, b), survival (c, d) and proliferation (e, f) comparison of negatively (a, c, e) and positively (b, d, f) selected natural killer (NK) cells from LEWIS rats after 4 days in culture with IL-2. Forward-scatter and side-scatter dot plots represent the difference of morphology between negative (a) and positive (b) NK-cell fraction. The viability of the different fractions of purified NK cells was detected by flow cytometry in a dot plot were the horizontal axis shows the intensity of fluorescence of allophycocyanin-Annexin V, while the vertical axis shows the intensity of fluorescence of propidium iodide. Proliferation of negatively (e) and positively (f) selected NK cells is represented by a histogram plot of CFSE fluorescence. Purified NK cells were loaded with CFSE dye after the purification process and put in culture during 4 days with interleukin-2. Data shown are representative of at least three similar experiments.
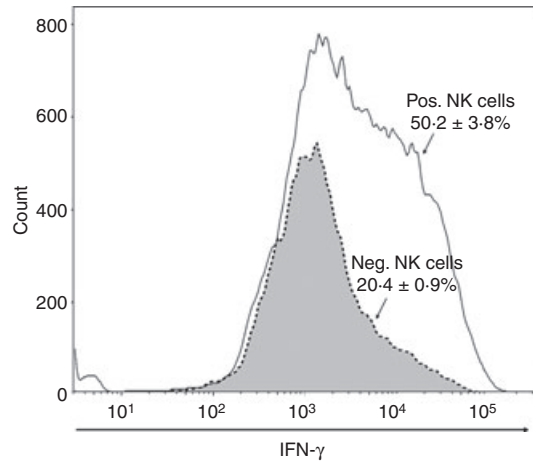
release from intracellular stores. Our results demonstrated that cross-linking of NKR-P1 in resting NK cells from the immunocompetent rats induced a calcium flux that was sustained over time (Fig. 5). Intriguingly, the activation of the NKR-P1 on negatively selected NK cells was not followed by concomitant intracellular IFN-γ production either after 12 hr or 36 hr of culture with IL-2. Moreover, no difference was observed after combined activation with IL-2 and IL-12 (data not shown). In contrast, when negatively and positively selected NK cells were cultured for 72 hr in IL-2 plus IL-12, the positively selected NK cells accumulated significantly more intracellular IFN-γ ($50.2 \pm 3.8\%$) than the negatively selected NK cells ($20.4 \pm 0.9\%$) ($P \leq 0.01$, $n = 3$) (Fig. 6).

Discussion

Natural killer cells are an important component of the innate immune response. They are involved in both immune surveillance and infection control. *In vitro* studies of NK cells are necessary to obtain fundamental information about their function and the mechanisms of their interaction with the other cells of the immune response. Currently no reliable protocol exists for isolating sufficient

Figure 6. Intracellular secretion of interferon-γ (IFN-γ) by natural killer (NK) cells upon activation for 72 hr in combination with IL-12. Histogram of intracellular IFN-γ on activated negative NK cells (Neg. NK cells) and positive NK cells (Pos. NK cells). Dead cells were excluded by gating out 7-AAD$^+$ cells. This result is representative of three individual experiments.

quantities of pure untouched rat NK cells for *in vitro* studies. Different methods have been previously developed to separate rat NK cells, such as the sorting of NKR-P1$^+$ CD3$^-$ cells by FACS or the positive selection of NKR-P1$^+$ cells after T-cell and B-cell depletion. Other research teams have developed a negative purification procedure for extracting untouched NK cells.[16,17] However, these strategies have several disadvantages in terms of purity, yield or activation profile.

The aim of the present study was to develop a suitable method for isolating untouched rat NK cells with a high purity and yield and to study their functions and activity compared with currently available methods.

Our novel protocol for negative purification of rat NK cells from LEWIS and F344 rats produced superior purity ($97 \pm 0.7\%$ and $96.6 \pm 0.8\%$, respectively) compared with those obtained by the group using the Dynabeads system with LEWIS rats ($85 \pm 7.3\%$)[17] or the Miltenyi system with F344 rats (72.5%).[16] Our antibody cocktail was more efficient in eliminating non-NK cells from the spleen than the previously published cocktails.[16,17] Using athymic nude rats we obtained a lower purity than with the other rat strains at $88.3 \pm 1.5\%$. Their lack of normal thymus and functionally mature T cells might explain why the purity was lower with this rat strain.[20] T-like cells could not be efficiently recognized by the anti-CD5 mAb present in the negative selection cocktail. Furthermore, we showed a better purity by our negative selection than after the positive procedure ($66 \pm 13\%$ pure NK cells from athymic nude and $47.8 \pm 2.8\%$ from LEWIS rats after positive selection). The lack of specificity for NK cells of the anti-NKR-P1 antibody can explain why we obtained lower purity after positive selection. As shown

Figure 5. Calcium mobilization determined by the ratio of Fluo-4 to Fura Red fluorescence over time. Negatively selected natural killer (NK) cells from LEWIS rats were loaded with the calcium-sensitive dyes Fluo-4 and Fura Red, and incubated with (middle line, ± anti-NKR-P1) or without (lower line, negative control) biotin anti-NKR-P1 antibody. After a washing step the cells were analysed for 20 seconds to take the baseline, the tube was removed to add the anti-biotin microbeads and the tube was replaced to follow the effect on the calcium flux. Purified cells activated with ionomycin served as positive control (upper line, ± ionomycin). This result is representative of three individual experiments.

in Fig. 2(a) and by other teams[21,22] this antibody also recognized NKT cells, monocytes, granulocytes and dendritic cells that are known to have NKR-P1 dimly expressing subpopulations. On the other hand, our negative selection gave the highest yield of purified NK cells ($4 \times 10^6 \pm 1.5\%$) compared with the FACS procedure ($1.6 \times 10^6 \pm 0.4\%$). Hence, we confirmed previous studies that demonstrated that the isolation of eosinophils or liver stem cells by magnetic sorting was more efficient than by FACS.[23,24]

Previous studies clearly demonstrated that the activation of the NKR-P1A molecule can increase the cytotoxicity of rat NK cells from the PVG rat strain against Chinese hamster ovary cells transfected with one of the ligands of this receptor, the Clr11.[25] We confirmed this observation in a cytotoxicity assay against YAC-1 targets using positively selected NK cells compared with negatively selected NK cells. Indeed, we show that the cross-linking of the mAb 10/78 epitope significantly increases the capacity of rat NK cells to lyse target cells (Fig. 3a). Interestingly, this difference was abrogated after 72 hr of culture in IL-2 (Fig. 3b), indicating a difference in the initial activation profiles of these NK-cell populations. We also corroborated the fact that if cross-linked, the mAb 10/78 epitope can induce a calcium flux in NK cells (Fig. 5).[10,26] It has been shown that the cross-linking of the NKR-P1 receptor by the anti-NKR-P1 antibody (clone 10/78) on the human NK cell line YTSeco NKR-P1A$^+$ (engineered to express the rat NKR-P1A)[26] and on RNK-16, a rat NK cell line,[10] triggers an intracellular calcium flux after cross-linking. Remarkably, this calcium flux was not followed by IFN-γ secretion after 18, 36 or 72 hr in culture with IL-2 (data not shown and Fig. 6). It is possible that IFN-γ secretion requires a much longer activation time to occur, or it could mean that stimulation of IFN-γ secretion is not linked to that particular signal transduction cascade. Moreover, Kurago et al.[27] have shown that the increase of IFN-γ production is not always correlated with the elevation of cytotoxicity on mouse NK cells, so the inverse can also be true. Nevertheless, only after 72 hr in culture with combined IL-2 and IL-12 were the positively selected NK cells more capable of IFN-γ secretion in contrast to negatively selected NK cells (Fig. 6). These data suggest that the NKR-P1 receptor can regulate IFN-γ release via IL-12 signalling.

We demonstrated for the first time that activation of the NKR-P1A receptors by cross-linking with mAb 10/78 increased NK-cell viability and proliferation (Fig. 4c–f). In addition, cross-linking modified cell morphology (Fig. 4a,b) but not the cell surface expression of CD8α, CD11 b/c and MHC class II molecules (data not shown) in cell culture over 4 days with IL-2. This can mean that the NK-cell subpopulation stays unchanged in culture but the activation stage can be modified as a result of the purification process. Further work is required to elucidate

these findings. The expression of the CD62 ligand antigen could be a good marker for determining the activation profile. Indeed, activation of the mouse isoform of the NKR-P1A by an anti-NK1.1 mAb in the presence of IL-2 was shown to induce IFN-γ secretion[28] and proliferation.[29] NK1.1 shares 70% homology with NKR-P1A. Furthermore, the cytoplasmic domain of the rat NKR-P1 receptor was shown to interact with leucocyte-specific protein tyrosine kinase (lck)[30] and heterotrimeric G proteins.[31] These intracellular proteins mediate downstream signalling events that result in phosphoinositide turnover, mobilization of intracellular calcium, tyrosine kinase activation, leading to proliferation, degranulation and release of cytokines.[32,33]

In addition to the inadvertent activation of the NK cells that we observed associated with the positive purification procedures, the NKR-P1 receptors remained occupied by antibody (Fig. 2). This can interfere with further studies on the purified cells, such as phenotyping subpopulations or study of physiological effects of drugs on this receptor.

In conclusion, our novel negative selection method produced increased purity and yield of NK cells compared with all previously published methods. Moreover, this method is rapid, simple and inexpensive compared with FACS. The resting NK cells remained inactivated but could be significantly activated by IL-2 stimulation and therefore are well suited for functional studies or for adoptive transfer therapy.

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Disclosures

The authors have no conflicts of interests to declare.

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