Chemokine production by natural killer cells from nonagenarians

Erminia Mariani[1,2], Alessandra Meneghetti[1], Simona Neri[1], Giovanni Ravaglia[3], Paola Forti[3], Luca Cattini[1] and Andrea Facchini[1,2]

[1] Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla-Putti, IOR, Bologna, Italy
[2] Dipartimento di Medicina Interna e Gastroenterologia, University of Bologna, Bologna, Italy
[3] Dipartimento di Medicina Interna, Cardioangiologia ed Epatologia, University of Bologna, Bologna, Italy

In this study we investigated whether purified NK cells, derived from a group of nonagenarian healthy subjects, were able to produce the chemokines MIP-1α, RANTES and IL-8, and also characterized the effect of IL-12 or IL-2 immunomodulatory cytokines (that are among the most effective inducers of NK lytic activity and soluble factor secretion) on the induction, *in vitro*, of these chemokines and on the modulation of the corresponding receptors. This study provides evidence that human NK cells from healthy subjects over 90 years old retain the ability to synthesize MIP-1α, Rantes and IL-8 chemotactic cytokines, that NK cells isolated from these subjects can be activated to significantly up-regulate the production of these chemokines in response to stimulation by IL-12 or IL-2 cytokines (even though production remains lower than that observed in young subjects), and that NK cells express the corresponding chemokine receptors.

Received	30/7/01
Received	14/2/02
Accepted	14/3/02

Key words: Chemokine / NK / Ageing / IL-12 / IL-2

1 Introduction

Chemokines are a complex family of small regulatory proteins, produced by different cell types, involved in the recruitment, activation and chemotaxis of effector leukocytes to the inflammation sites [1], in integrin- and selectin-mediated adhesion and in the exocytosis of granule enzymes [2]. Based on their structural and genetic characteristics, four families of these proteins have been so far identified [3]. They appear to act *in vitro* via family-specific shared receptors that also are divided into four groups [2].

Recently, it was described that natural killer (NK) cells also produce some chemokines and, on the other hand, that chemokines are important soluble chemoattractants for NK cells [4].

NK cells are among the main effector cells of the innate immune system that target intracellular pathogens as well as cancer cells [5, 6]. The role of NK cells in early antiviral immune defense mechanisms seems to be crucial, in fact any defect in NK cell function would leave the host vulnerable to several major viral infections and can be correlated with a history of recurrent infections and virus-associated diseases [5, 6].

We demonstrated a decreased lytic activity of NK cells from the elderly [5, 7], possibly due to a pronounced age-related decrease in the ability to generate inositol triphosphate during spontaneous cytolytic activity against K562 targets [8]. The decreased functional activity of NK cells may also be involved in the increased incidence of infections and neoplastic diseases, the most frequent causes of morbidity and mortality in the elderly, through an impaired production of some chemokines.

IL-12 and IL-2 cytokines are among the most effective inducers of NK lytic activity and soluble factor secretion and play an important regulatory role in the initiation and maintenance of the immune response [9, 10].

In this study we investigated whether purified NK cells, derived from a group of nonagenarian healthy subjects, were able to spontaneously produce the chemokines MIP-1α, RANTES (representative of the CC family) and IL-8 (representative of the CXC family). We also characterized the effect of IL-12 or IL-2 stimulation on the induction, *in vitro*, of these chemokines and on the modulation of the corresponding receptors.

[I 22287]

Abbreviations: MIP-1α: Macrophage inflammatory protein-1α **RANTES:** Regulated on activation, normal T cells expressed and secreted

0014-2980/02/0606-1524$17.50+.50/0

2 Results

2.1 Lymphocyte phenotype and cytolytic activity

A reduction of $CD3^+$ T lymphocytes (mean ± SEM, old: 63±1%, young: 72±3%, $p<0.05$), an increase of NK cells (expressed as mean ± SEM of $CD56^+CD3^-$ cells, old: 18±4%, young: 12±3%, not reaching statistical significance) and a similar low number of NK lymphocytes with IL-12R (old: 6±1%, young: 5±2%, mean ± SEM of electronically gated $CD56^+$ cells), and with IL-2R, (old: 3±1%, young: 4±1%, mean ± SEM of electronically gated $CD56^+$ cells), were observed in the old compared to young subjects, confirming our previous data. Cytokine-induced activation of NK cells was revealed by the increment of cytolytic activity against Daudi and K562 target cell lines. The optimal dose of interleukins and the time required to induce the activation of NK cells were previously determined to be similar in young and old subjects (not shown). As expected, 1 day of incubation with IL-12 (1 ng/ml) or IL-2 (100 U/ml) significantly increased the cytolytic activity of NK cells from both young and old donors against Daudi and K562 target cells.

2.2 Effect of IL-12 and IL-2 on chemokine production and mRNA expression by NK cells

A similar time course of chemokine production in the culture supernatant was shown by NK cells from young and old subjects. MIP-1α and RANTES production peaked after 1 day of IL-12 or IL-2 stimulation, but NK cells from young subjects produced more chemokines than those from old ones. A slight increase or even a decrease for MIP-1α and RANTES (Fig. 1a–d), and a progressive increase for IL-8 (Fig. 1e, f) resulted after 5 days.

Within each group of subjects, a comparable amount of chemokines was induced by each stimulus after 1 day, the only exception being MIP-1α production where IL-2 incubation (Fig. 1b) was more effective than IL-12 ($p<0.005$) (Fig. 1a). Also after 5 days, a similar production was observed for RANTES (Fig. 1c, d) and for IL8 (Fig. 1e, f), while MIP-1α secretion was more induced by IL-2 ($p<0.05$) (Fig. 1a, b) in both groups.

mRNA expression of MIP-1α, Rantes and IL-8 by human NK cells was variable among individuals. In general, in unstimulated NK cells chemokine mRNA were less evident in the young group than in the old one (not shown). Following stimulation, both cytokines induced an evident and similar up-regulation of the expression of all the three mRNA (Fig. 2). The only difference was a more evident IL-2-induced up-regulation of MIP-1α mRNA in young and of RANTES mRNA in old subjects (in both cases without reaching statistical significance).

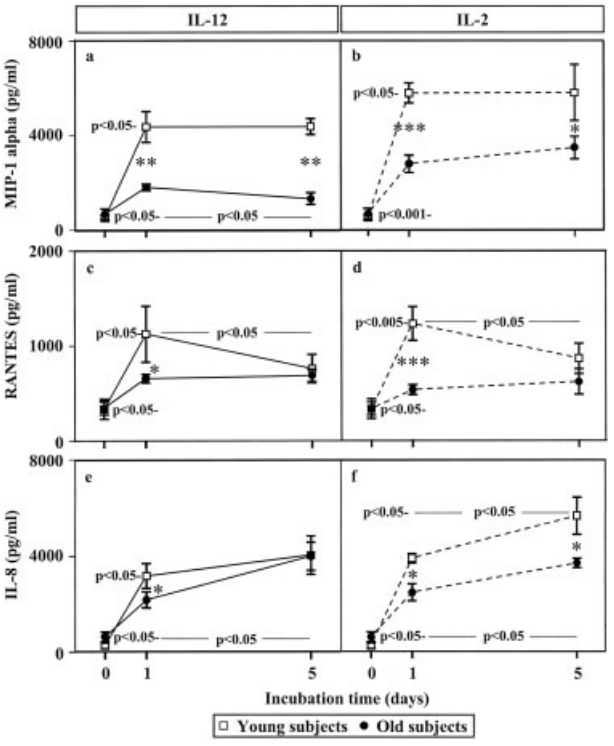

Fig. 1. MIP-1α, Rantes and IL-8 production in NK cell supernatants following IL-12 and IL-2 stimulation for 1 and 5 days in old and young subjects. Results are expressed as mean pg/ml ± SEM. Asterisks indicate statistically significant differences between old and young subjects: $*p<0.05$, $**p<0.01$; $***p<0.005$.

2.3 Analysis of surface, intracellular and mRNA expression of chemokine receptors

A large number of NK cells express CXCR1 (50±8% in the young and 27±5% in the old), only 4–6% (in both groups) express CCR3 and CCR5 (Fig. 3), but none expresses CCR1 (not shown), as determined by surface staining. Only CXCR1 appeared more densely distributed in young subjects (as suggested by mean fluorescence channel 176±11 in young and 145±4 in old, $p<0.05$), while no receptor appeared significantly modulated by IL-12 or IL-2 cytokines either in the young or the old group.

Intracellular staining (not shown) demonstrated that CCR1, CCR5 and CXCR1 were similarly expressed as on the cell surface, whereas CCR3 was present on at least four times more NK cells (21–25%) and was about 30% more densely distributed in the two groups (not shown).

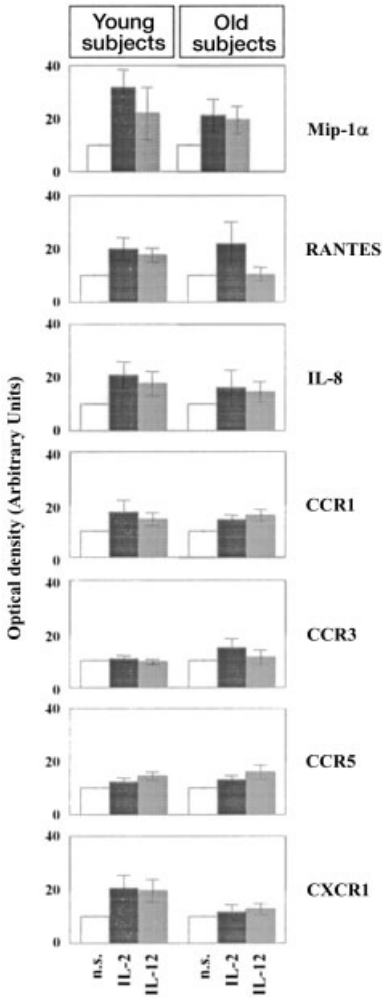

Fig. 2. MIP-1α, Rantes, IL-8 chemokine and CCR1, CCR3, CCR5, CXCR1 mRNA expression by human IL-2 or IL-12 stimulated NK cells from old and young subjects. The expression was evaluated by semi-quantitative RT-PCR. Densitometric analysis of band intensity is presented (mean ± SEM of seven different subjects for each group).

Even taking into account the variation between different donors, the expression on NK cells and the intracellular density of CXCR1 was higher in the young than in the old ($p<0.05$), similarly to surface distribution. No intracellular receptor was modulated by cytokine incubation for one day (not shown).

The RT-PCR analysis (Fig. 2) showed that NK cells contained the mRNA for all these receptors, including CCR1 (negative by FACS analysis) that gave the most intense signal on the gel (not shown). All receptor mRNA were only slightly modulated by cytokine treatment in both groups, except for CXCR1 mRNA whose up-regulation was more evident in the young group than in the old one, without reaching statistical significance.

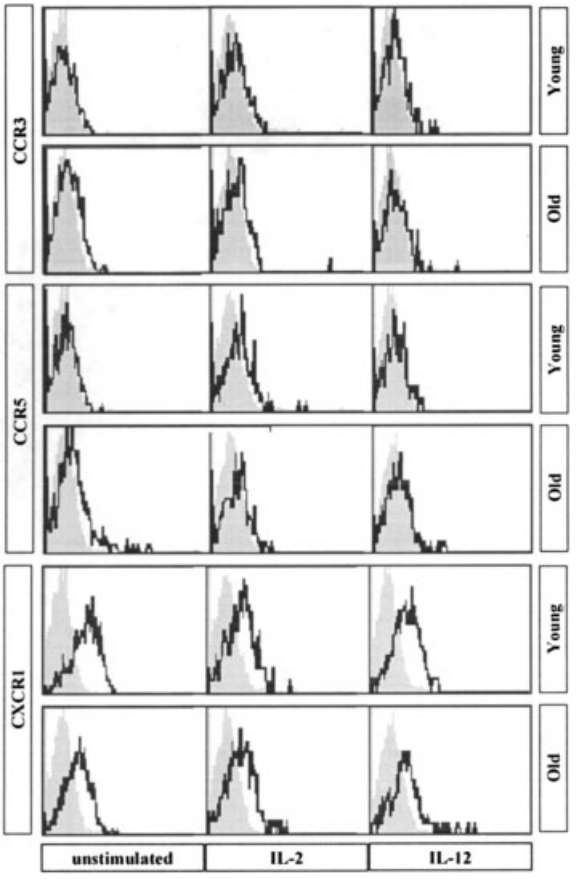

Fig. 3. Surface distribution of CCR1, CCR3, CCR5 and CXCR1 on NK cells from old and young subjects (results of one out of five different subjects analyzed for each group are presented). Negative control cells (gray histograms) were only incubated with FITC- or PE-conjugated immunoglobulin isotype controls.

3 Discussion

This study provides evidence that human NK cells from healthy subjects over 90 years old retain the ability to synthesize MIP-1α, Rantes and IL-8 chemotactic cytokines (confirming our previous preliminary observations [11]), that NK cells isolated from these subjects can be activated by IL-12 or IL-2 stimulation to significantly up-regulate the production of these chemokines (even if to a lower extent than in young subjects), and that NK cells express the corresponding chemokine receptors.

IL-12, a monocyte/macrophage-derived cytokine, is produced by these cells infected by intracellular pathogens. NK cells exert control on these infected monocytes through the synthesis of IFN-γ and MIP-1α that potentiate secretion of IL-1β and TNF-α inflammatory cytokines by IFN-γ-activated monocytes [12]. IL-2 is produced

mainly by $CD4^+$ T cells activated by antigens and its ability to induce chemokine production by T and NK cells and enhance the cytolytic activity of these cells suggests a possible role for the induced chemokines during the antigen-specific immune response [13, 14].

In general, in both groups of subjects, the mRNA expression and the production of MIP-1α, Rantes and IL-8 in culture supernatants were similarly induced by IL-2 and IL-12 incubation even if the total amount was higher following IL-2. Old subjects produced lower amounts of all three chemokines, but this was not evident at the mRNA level. The lack of correlation between protein secretion and mRNA expression does not allow specification of a transcriptional regulation of cytokine-induced chemokine production. The ability of IL-12 and particularly IL-2 to induce high levels of chemokines from NK cells (demonstrated also by others [12, 14]) is of interest in view of the potential for NK-derived chemokines to interfere with virus replication before the onset of antigen-specific immune responses. In addition, chemokines have been reported to stimulate cytolytic granule release [4], promote cytotoxic activity and regulate adhesiveness to target cells. Furthermore, the amount of chemokines released during NK–target interaction may influence the polar distribution of chemokine and adhesion receptors on NK cells that, acquiring migratory morphology, may be recruited (through the specific receptor) by other sources of chemokines such as activated monocytes and by cell-to-cell interactions during NK cell effector function [15].

Concerning specific chemokine receptors, these were similarly distributed in NK cells from both young and old subjects. We did not observe surface (in agreement with others [16]) or intracellular CCR1 receptors by FACS analysis, while the corresponding mRNA was detectable, excluding that CCR1 absence was due to a block of its transcription. The reason for CCR1 negativity is not clear: it might be due to its modulation and desensitization by ligands produced by NK cells (even if a different rate can be expected between young and old, because of the different amounts of these chemokines, respectively produced). Therefore, in young and old NK cells, the described chemotactic response towards MIP-1α and Rantes [17] and the ability of the same chemokines to enhance NK effector functions [18] may involve other shared receptors, such as CCR3 and/or CCR5. A low percentage of NK cells displayed surface CCR3 (according to others [16]) and CCR5; on the contrary, an evident expression of intracellular CCR3 was observed, suggesting that this receptor is accumulated in the cytoplasm. The absence of CCR1 and the presence of CCR5, respectively involved in cellular arrest and spreading [19], might mirror a differential NK cell recruitment. In fact, the lack of CCR1 (probably more involved in cell arrest), may be consistent with the spreading characteristics of NK cells. As concerning CXCR1, the intracellular and surface expression observed on NK cells (in agreement with previous data [20]), appeared reduced with age.

These results indicate that NK cells either from young or old subjects are not uniform in terms of expression of chemokine receptors that may be more evident inside the cell. IL-12 and IL-2 very slightly modulated mRNA expression for all these receptors. This weak modulation, when observed, did not suggest a cytokine-induced transcriptional control for the synthesis of these molecules.

In conclusion, the decreased production of MIP-1α, Rantes and IL-8 chemokines during ageing may be involved in the lower lytic activity found in nonagenarians and in general in the lower response of old subjects to infections. Understanding the complex cytokine networks that involve human NK cells in the production of immunoregulatory cytokines and chemokines may provide novel therapeutic approaches for improving or redirecting the early innate immune response to infective diseases that represent important causes of morbidity and death in the elderly.

4 Materials and methods

4.1 Subjects

Sixteen elderly subjects (mean age ± SD: 97±3 years), 12 women and 4 men and 7 young healthy controls (mean age ± SD: 30±2 years), 5 women and 2 men, were selected following the suggestions of the Senieur protocol criteria [21]. Subjects gave their informed consent to enrol in the present study, which was approved by the local ethical committee.

4.2 Mononuclear cell separation and NK cell purification

Mononuclear cells (MNC) were separated from peripheral blood by conventional density gradient. NK cells were purified by negative selection using NK isolation kit and MACS magnetic cell separator [22]. The sum of the remaining $CD3^+$, $CD14^+$ and $CD19^+$ cells in the eluted NK fraction were for old: 1.89±0.43% and for young: 2.0±0.4% (mean ± SEM) as evaluated by flow cytometry.

4.3 Flow cytometric analysis

MNC were stained with FITC- or PE-conjugated anti-CD3, CD16, CD56, CD25 (Becton Dickinson, USA), CCR1, CCR3,

CCR5, CXCR1 monoclonal antibodies (mAb) and anti-IL-12R (2.4E6 clone supernatant – 7th HLDA) as previously described [7, 22]. The intracellular distribution of chemokine receptors was determined on fixed and permeabilized cells by the addition of anti-chemokine receptor mAb.

4.4 Stimulation of NK cells with IL-12 and/or IL-2

NK cells (1.25×10^6/ml) were incubated with recombinant human (rh) IL-12 (1 ng/ml final concentration; specific activity 5×10^6 U/mg) or rhIL-2 (100 UI/ml final concentration; specific activity 1.6×10^6 U/mg) in culture plates for 1 or 5 days at 37°C in 5% CO_2. Cell-free supernatants from unstimulated and stimulated NK cultures were stored at –80°C. NK pellets were used for chemokine receptor evaluation and RNA purification (after 1 day of activation) and as cytolytic effector cells (after both incubation times). K562 and Daudi lines were used as targets to reveal interleukin-induced activation of NK cells. A micro chromium release test was performed using a 12:1 effector/target cell ratio [22].

4.5 Chemokine production in the supernatant

MIP-1α, RANTES and IL-8 chemokine levels in cell culture supernatants were measured by commercial quantitative immunoassay. Sensitivities of ELISA kits were: 2 pg/ml for MIP-1α, 2.5 pg/ml for RANTES and 6 pg/ml for IL-8.

4.6 RT-PCR analysis

Total RNA from resting or activated NK cells was extracted using RNAwiz™ reagent and quantified. One microgram from each sample was reverse-transcribed using murine leukemia virus (MuLV) reverse transcriptase at 42°C for 15 min, heated to 99°C for 5 min and flash-cooled to 4°C. To compensate for initial quantitation errors and tube-to-tube variations in RT reaction, cDNA were analyzed for the expression of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) in the linear range of amplification, using the forward primer 5'-TGGTATCGTGGAAGGACTCATGAC-3' and the reverse primer 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'. Amplification conditions (25 μl): 1 μl of cDNA, 1 μM each primer, 2 mM $MgCl_2$, 200 μM each dNTP and 0.5 U AmpliTaq DNA polymerase in thermophilic magnesium-free buffer. Thermal profile: 94°C for 3 min, 28 cycles (20 s at 94°C, 20 s at 60°C, 20 s at 72°C), 72°C for 3 min.

Adjusted amounts of cDNA were then analyzed by PCR for the expression of MIP-1α, Rantes, IL-8, CCR1, CCR3, CCR5, CXCR1 (Table 1). PCR reaction mixtures (25 μl) consisted of 1 μM each primer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 U AmpliTaq in thermophilic magnesium-free buffer. Thermal profile: 94°C for 3 min, 35 cycles (20 s at 94°C, 20 s at 58°C for chemokines and 54°C for receptors, 20 s at 72°C), 72°C for 3 min. In each PCR reaction, a positive (resting lymphocytes) and a negative (sample without template) control were included. Ten microliters of each PCR product were analyzed on 2% agarose gel, together with a 100-bp DNA ladder as molecular weight marker. Densitometric analysis of the bands was performed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 120.

Table 1: Primers sequences

MIP1α pF	5'-GAATCATGCAGGTCTCCAC-3'
MIP1α pR	5'-CGAAGCTTCTGGACCCCTC-3'
Rantes pF	5'-ATGAAGGTCTCCGCGGCAGCCC-3'
Rantes pR	5'-CTAGCTCATCTCCAAAGAGTTG-3'
IL-8 pF	5'-ATGACTTCCAAGCTGGCCGT-3'
IL-8 pR	5'-TATGAATTCTCAGCCCTCTTCAAAAACTTCTC-3'
CCR1 pF	5'-CACCACAGAGGACTATGACAC-3'
CCR1 pR	5'-GCATCACCAAAAACCCAG-3'
CCR3 pF	5'-CCATCTTCTGTCTCGTTCTCC-3'
CCR3 pR	5'-TCCGCTCACAGTCATTTCC-3'
CCR5 pF	5'-GCTCTCATTTTCCATACAGTC-3'
CCR5 pR	5'-TGCCTCTTCTTCTCATTTC-3'
CXCR1 pF	5'-CCTCAACCCCATCATCTAC-3'
CXCR1 pR	5'-TGGAAGAGACATTGACAGAC-3'

4.7 Statistical analysis

The experimental data were expressed as means ± SEM. Analysis was performed using Student's *t*-test for independent or paired data. Statistica for Windows was used.

Acknowledgements: This work was supported by grants from MURST (RFO fund), Ricerca Corrente IOR, FP from Health Ministry of Italy and ImAginE framework from EU (QLK6-CT-1999–02031).

References

1 **Baggiolini, M.,** Chemokines and leukocyte traffic. *Nature* 1998. **392:** 565–568.

2 **Rossi, D. and Zlotnik, A.,** The biology of chemokines and their receptors. *Annu. Rev. Immunol.* 2000. **18:** 217–242.

3 **Kunkel, S. L., Lukacs, N. W., Strieter, R. M., Standiford, T. and Chensue, S. W.,** The function of chemokines in health and disease. In **Rollins, B. J.** (Ed.) *Chemokines and cancer.* Humana Press, Totowa 1999, pp 3–19

4 **Taub, D. D.,** Natural killer cell-chemokine interactions. Biologic effects on natural killer cell trafficking and cytolysis. In **Rollins, B. J.** (Ed.) *Chemokines and cancer*. Humana Press, Totowa 1999, pp 73–93

5 **Pawelec, G., Solana, R., Remarque, E. and Mariani, E.,** Impact of aging on innate immunity. *J. Leukoc. Biol.* 1998. **64:** 703–712.

6 **Biron, C. A.,** Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol.* 1997. **9:** 24–34.

7 **Mariani, E., Roda, P., Mariani, A. R., Vitale, M., Degrassi, A., Papa, S. and Facchini, A.,** Age-associated changes in $CD8^+$ and $CD16^+$ cell reactivity: clonal analysis. *Clin. Exp. Immunol.* 1990. **81:** 479–484.

8 **Mariani, E., Mariani, A. R., Meneghetti, A., Tarozzi, A., Cocco, L. and Facchini, A.,** Age-dependent decreases of NK cell phosphoinositide turnover during spontaneous but not Fc-mediated cytolytic activity. *Int. Immunol.* 1998. **7:** 981–989.

9 **Smith, K. A.,** Interleukin 2. *Annu. Rev. Immunol.* 1984. **2:** 319–333.

10 **Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U. and Presky, D. H.,** The interleukin-12/interleukin-12 receptor system: Role in normal and pathologic immune responses. *Annu. Rev. Immunol.* 1998. **16:** 495–521.

11 **Mariani, E., Pulsatelli, L., Neri, S., Dolzani, P., Meneghetti, A., Silvestri, T., Ravaglia, G., Forti, P., Cattini, L. and Facchini, A.,** RANTES and MIP-1α production by T lymphocytes, monocytes and NK cells from nonagenarians subjects. *Exp. Gerontol.* 2002. **37**: 219–226.

12 **Bluman, E. M., Bartynski, K. J., Avalos, B. R. and Caligiuri, M. A.,** Human natural killer cells produce abundant macrophage inflammatory protein-1α in response to monocyte-derived cytokines. *J. Clin. Invest.* 1996. **97:** 2722–2727.

13 **Trinchieri, G., Matsumoto-Kobayashi, M., Clark, S. C., Seehra, J., London, L. and Perussia, B.,** Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* 1984. **160:** 1147–1169.

14 **Oliva, A., Kintar, A. L., Vaccarezza, M., Rubbert, A., Catanzaro, A., Moir, S., Monaco, J., Ehler, L., Mizell, S., Jackson, R., Li, Y., Romano, J. W. and Fauci, A. S.,** Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication *in vitro*. *J. Clin. Invest.* 1998. **102:** 223–231.

15 **Nieto, M., Navarro, F., Perez-Villar, J. J., del Pozo, M. A., Gonzalez-Amaro, R., Mellado, M., Frade, J. M., Martinez-A, C., Lopez-Botet, M. and Sanchez-Madrid, F.,** Role of chemokines and receptor polarization in NK-target cell interactions. *J. Immunol.* 1998. **161:** 3330–3339.

16 **Inngjerdingen, M., Damaj, B. and Maghazaehi, A. A.,** Expression and regulation of chemokine receptors in human natural killer cells. *Blood* 2001. **97:** 367–375.

17 **Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M. and Moser, B.,** Activation of NK cells by CC chemokines. Chemotaxis, $Ca2^+$ mobilization, and enzyme release. *J. Immunol.* 1996. **156:** 322–327.

18 **Taub, D. D., Sayers, T. J., Carter, C. R. D. and Ortaldo, J. R.,** α and β chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J. Immunol.* 1995. **155:** 3877–3888.

19 **Weber, C., Weber, K. S., Klier, C., Wank, R., Horuk, R. and Nelson, P. J.,** Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruirment of monocytes and T(H)1-like/CD45RO(+) T cells. *Blood* 2001. **97:** 833A-833.

20 **Morohashi, H., Miyawaki, T., Nomura, H., Kuno, K., Murakami, S., Matsushima, K. and Mukaida, N.,** Expression of both types of human interleukin-8 receptors on mature neutrophils, monocytes, and natural killer cells. *J. Leukoc. Biol.* 1995. **57:** 180–187.

21 **Ligthart, G. J., Corberand, J. X., Fournier, C., Galanaud, P., Hijmans, W., Kennes, B., Muller-Hermelink, H. K. and Steinmann, G. G.,** Admission criteria for immunogerontological studies in man: the SENIEUR protocol. *Mech. Ageing Dev.* 1984. **28:** 47–55.

22 **Mariani, E., Corona, A. and Solana, R.,** Age-related alterations to natural killer function. In **Barnett, Y. A. and Barnett, C. R.** (Eds.) *Aging methods and protocols*. Humana Press, Totowa 2000, pp 311–320.

Correspondence: Erminia Mariani, Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla-Putti, IOR, Via di Barbiano 1/10, I-40136 Bologna, Italy
Fax: +39-051-6366807
e-mail: marianie@alma.unibo.it