

# The “*ex Vivo*” Patterns of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR Expression Identify Acute/Early and Chronic/Late NK-Cell Activation States

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Margarida Lima,<sup>1</sup> Julia Almeida,<sup>2</sup> Maria dos Anjos Teixeira,<sup>1</sup> Maria Luís Queirós,<sup>1</sup> Benvindo Justica,<sup>1</sup> and Alberto Orfão<sup>2</sup>

**ABSTRACT:** To define a dynamic sequence of phenotypic changes related to early and late phases of NK-cell activation, we have analyzed by four-color flow cytometry the immunophenotype of normal blood NK-cells from 12 healthy individuals and compared it with those from 15 patients with acute viral infections and 15 patients with either chronic infections or tumors. Although a great interindividual variability was found, nonstimulated CD56<sup>+</sup> NK-cells, present in normal blood samples, usually were CD2<sup>-/+lo</sup>, CD7<sup>+hi</sup>, HLA-DR<sup>-</sup>, CD11b<sup>+</sup>, CD38<sup>+</sup>, CD11a<sup>+hi</sup>, CD45RA<sup>+hi</sup>, and CD45RO<sup>-</sup>, the expression of CD11c and CD57 being heterogeneous and variable. Recently activated NK-cells, herein corresponding to NK-cells from patients with acute viral infections, displayed a pattern of expression of CD2/CD7 similar to that referred to above, but they typically showed higher levels of CD11a, CD38, and HLA-DR, as well as downregulation of CD11b and CD45RA, accompanied in some cases by coexpression of CD45RO; in addition, these NK-cells were CD11c<sup>+</sup> and CD57<sup>-/+lo</sup>. Late-activated NK-cells, represented by NK-cells present in patients with chronic infections and tumors, converted into a CD2<sup>+hi</sup>/CD7<sup>-/+lo</sup> immunophenotype and expressed heterogeneously low levels of CD38 and CD11b; moreover, they were CD57<sup>+</sup> and CD11c<sup>-/+</sup>. At this stage, most NK-cells had already reverted into their original CD45RA<sup>+</sup>/CD45RO<sup>-</sup>/HLA-DR<sup>-</sup> phenotype. In summary, we show that the patterns of expression of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR may help us to define the immunophenotypic profiles associated with early and late NK-cell activation phases in “*in vivo*” models. © 2002 Elsevier Science (USA)

**Key Words:** NK-cells; activation; infection; neoplasm; immunophenotype; flow cytometry.

## INTRODUCTION

Increased numbers of circulating NK-cells have been described in various physiological and pathological conditions (1–4), in addition to NK-cell leukemias/lymphomas (5, 6). In the former cases, they are believed to represent reactive cells that proliferate in response to a stimulus and that play an important role in host defense by exhibiting cytotoxicity against tumor and virus-infected cells, whereas in the latter case the malignant NK-cell population shows autonomous proliferation (7, 8).

Several phenotypic changes have been shown to occur after “*in vitro*” stimulation of NK-cells, including acquisition of molecules that are not expressed on resting NK-cells and up- or downregulation of receptors that are constitutively expressed on this leukocyte population (9–29). Most of these molecules proved to have an important role in determining migration of NK-cells into tissues, as well as in regulating NK-cell proliferation, target cell recognition, conjugate formation, and effective killing. Nevertheless, information on the immunophenotypic changes occurring after NK-cell activa-

Correspondence and reprint requests to: Margarida Lima, Serviço de Hematologia, Unidade de Citometria, Hospital Geral de Santo António, Rua D Manuel II, s/n, 4099-001 Porto, Portugal. Fax: 351-22-6004808. E-mail: mmc.lima@clix.pt.

<sup>1</sup> Service of Clinical Hematology, Unit of Cytometry, Hospital Geral de Santo António, Porto, Portugal.

<sup>2</sup> Servicio de Citometría, Universidad de Salamanca and Centro de Investigación del Cáncer, Salamanca, Spain.



tion “*in vivo*” is scanty and the few studies that have been published to date on this subject are restricted to the evaluation of a limited number of activation-related markers (30–32). The fact that blood NK-cells are heterogeneous (33) and that the typical immunophenotypic patterns observed in normal non-stimulated and stimulated NK-cells are far from being well-characterized, makes it difficult to distinguish between resting/activated normal polyclonal NK-cells and their neoplastic counterparts. Moreover, in the diagnosis of NK-cell leukemia/lymphoma molecular studies are not as useful as for T- and B-cell disorders—NK-cells do not rearrange the TCR genes and other alternatives such as X-linked molecular analysis frequently are either not applicable or not available (34, 35)—imposing additional difficulties in distinguishing normal from neoplastic NK-cells. Therefore, in patients displaying persistent NK-cell lymphocytosis in the absence of an obvious underlying disease the assessment of NK-cell clonality still remains a challenge. A comparative study of the immunophenotypic properties of normal blood NK-cells versus those of blood NK-cells from patients with pathological conditions potentially associated to NK-cell activation would not only contribute to a better understanding of the biological changes occurring during NK-cell activation, but it would also have immediate clinical application, since it could set the basis for the discrimination between normal and neoplastic NK-cells.

In view of this purpose, we have recently characterized the immunophenotypic profile of normal blood CD56<sup>+lo</sup> and CD56<sup>+hi</sup> NK-cell subsets in that concerning the expression of a number of functionally relevant receptors (36). In the present paper we focus on the immunophenotypic changes occurring *in vivo* in conditions in which NK-cell activation is likely to occur, such as in patients with acute viral infection (AI) or in patients with chronic infection or neoplasias (CI/N) and compare the phenotypic patterns of blood CD56<sup>+</sup> NK-cells from these patients with those of normal healthy individuals.

## MATERIALS AND METHODS

### *Patients and Controls*

A total of 15 patients with acute viral infection irrespectively of having or not NK-cell lymphocytosis (8 males and 7 females, median age of 30 years, range: 11 to 58 years) and 15 patients with chronic infection or hematological neoplasias (11 males and 4 females, median age of 61 years, range: 11 to 83 years) in which persistent blood NK-cell lymphocytosis was an occasional finding in immunophenotypic studies performed as part of the investigation of the associated condition were studied.

All patients with acute infection had acute infectious mononucleosis due to recent Epstein–Barr virus ( $n = 6$ ), cytomegalovirus ( $n = 5$ ) or hepatitis C-virus ( $n = 1$ ) infection or infection of unknown origin ( $n = 3$ ). Among cases with chronic NK-cell lymphocytosis, four patients suffered from chronic infections, whereas the remaining 11 patients had an associated B-cell hematological disorder (non-Hodgkin lymphoma in 6 cases, Waldenstrom’s macroglobulinemia in 1 case, multiple myeloma in 2 cases and monoclonal gammopathy of uncertain significance in the remaining 2 cases).

In addition, 12 normal healthy volunteers (blood donors)—7 males and 5 females aged from 24 to 48 years—were studied as controls. In all cases, samples were obtained after informed consent.

### *Immunophenotypic Studies*

All studies were done in K3-EDTA anticoagulated blood samples. Immunophenotypic analyses were performed using a whole blood stain-lyse-and-then-wash direct immunofluorescence technique and four-color staining as previously described in detail (36). PE-cyanine 5-conjugated anti-CD56 and allophycocyanin-conjugated anti-CD3 were used in combination with the following pairs of fluorescein isothiocyanate/phycoerythrin-conjugated monoclonal antibodies (MoAb) directed against T- and NK-associated antigens: CD2/CD7, CD57/CD11c, CD38/CD11b,

CD11a/HLA-DR, CD45RA/CD45RO, CD7/CD5, CD16/NKB1, CD158a/CD161, and CD94/CD8.

NK-cells from controls and CI/N individuals were characterized for the expression of all the markers described above whereas NK-cells from AI patients were studied only for the expression of CD2/CD7, CD57/CD11c, CD38/CD11b, CD11a/HLA-DR, CD45RA/CD45RO, and CD16/NKB1.

The anti-CD3, -CD5, -CD8, -CD11b, -CD11c, -CD45RA, -CD45RO, -CD56, -CD57, -CD94, -CD158a, -CD161, -HLA-DR and -NKB1 reagents were purchased from Becton/Dickinson Biosciences (BD, San Jose, CA); the anti-CD7 and anti-CD16 MoAb were from Beckman Coulter (Miami, FL), anti-CD11a was from CLB (Amsterdam, The Netherlands) and anti-CD38 from Cytognos (Salamanca, Spain). Anti-CD7 was either from BC (PE) or from CLB (FITC) and anti-CD8 was from BD or from Dako A/S (Glostrup, Denmark).

Data acquisition was carried out for all samples in a FACSCalibur flow cytometer (BD) equipped with a 15-mW air-cooled 488-nm argon ion laser and a 625-nm neon diode laser, using the Cell QUEST software program (BD). Information on a minimum of  $2 \times 10^5$  events was acquired and stored as list mode data files for each staining. For data analysis the Paint-a-Gate PRO software program (BD) was used. In all samples analyzed, total NK-cells were firstly identified in the CD2/CD7/CD56/CD3 tube by their positivity for CD2 and/or CD7 in the absence of reactivity for CD3, in order to calculate the percentage of NK-cells within the lymphocyte population, as well as to establish the distribution of the CD56<sup>-</sup>, CD56<sup>+lo</sup> and CD56<sup>+hi</sup> NK-cell subsets in each case. Afterward, the phenotypic characterization of the major CD56<sup>+lo</sup> NK-cell population for the remaining markers included in the study was performed after specifically selecting for the CD3<sup>-</sup>/CD56<sup>+lo</sup> lymphocytes. For each antigenic determinant analyzed, the following characteristics were recorded: (i) percentage of positive cells; (ii) intensity of expression [evaluated as mean fluorescence intensity (MFI) expressed in arbitrary relative linear units scaled from 0 to 10,000]; (iii) pattern of antigen expression [homogeneous ver-

sus heterogeneous, evaluated by the coefficient of variation (CV)].

### Statistical Analysis

For all variables under study, mean, standard deviation, median and range values were calculated. The statistical significance of differences observed between groups was evaluated using the Mann–Whitney *U* test (SPSS 9.0., SPSS, Chicago, IL). *P* values less than 0.05 were considered to be associated with statistical significance.

## RESULTS

The absolute number of total PB NK-cells was of  $226 \pm 151 \times 10^6/\text{L}$  (range:  $70\text{--}580 \times 10^6/\text{L}$ ) in controls,  $649 \pm 597 \times 10^6/\text{L}$  (range:  $94\text{--}2392 \times 10^6/\text{L}$ ) in AI, and  $1006 \pm 662 \times 10^6/\text{L}$  (range:  $487\text{--}2819 \times 10^6/\text{L}$ ) in CI/N patients. In these three groups of individuals, the majority of blood NK-cells were CD56<sup>+lo</sup>, representing  $86 \pm 7$ ,  $89 \pm 6$  and  $92 \pm 4\%$  of the total NK-cells, respectively ( $P > 0.05$ ). The mean intensity of CD56 expression in terms of MFI in CD56<sup>+lo</sup> NK-cells was of  $481 \pm 185$  in controls,  $420 \pm 229$  in AI and  $374 \pm 197$  in CI/N individuals ( $P > 0.05$ ). In each group, only a minority of NK-cells was considered to be either CD56<sup>-</sup> ( $7 \pm 5$ ,  $8 \pm 4$ , and  $6 \pm 4\%$  in controls, AI, and CI/N patients, respectively;  $P > 0.05$ ) or CD56<sup>+hi</sup> ( $7 \pm 6\%$ ,  $3 \pm 4\%$  and  $2 \pm 1\%$  in controls, AI and CI/N patients, respectively;  $P > 0.05$ ).

### *Patterns of Expression of CD2/CD7, CD57/CD11c, CD11b/CD38, and CD11a/HLA-DR on CD56<sup>+lo</sup> NK-Cells from Normal and Pathological Blood Samples*

The immunophenotypic data obtained in normal individuals, AI patients and CI/N patients are summarized in Tables 1 and 2. As may be seen in both tables, the vast majority of CD56<sup>+lo</sup> NK-cells from both controls and patients expressed CD7, although among CI/N patients the mean levels of CD7 expressed per cell were significantly lower (MFI of  $991 \pm 446$  in CI/N patients versus  $1460 \pm 402$  in controls and  $1764 \pm 644$  in

TABLE 1

Immunophenotypic Analysis of Blood CD56<sup>+</sup>lo NK-Cells: Relative Distribution of CD2<sup>+</sup>, CD7<sup>+</sup>, CD11c<sup>+</sup>, CD57<sup>+</sup>, CD11b<sup>+</sup>, CD38<sup>+</sup>, CD11a<sup>+</sup>, HLA-DR<sup>+</sup>, CD45RA<sup>+</sup>, and CD45RO<sup>+</sup> NK-Cells in Normal Individuals (Controls), Patients with Acute Viral Infection (AI), and Patients with NK-Cell Lymphocytosis Secondary to Infections or Tumors (CI/N)

	CD2	CD7	CD11c	CD57	CD11b	CD38	CD11a	HLA-DR	CD45RA	CD45RO
Controls ( <i>n</i> = 12)	74 ± 11	99 ± 1	74 ± 17	56 ± 18	89 ± 7	89 ± 17	100 ± 0	16 ± 14	100 ± 0	5 ± 5
AI ( <i>n</i> = 15)	79 ± 9*	99 ± 1	83 ± 11 <sup>+</sup>	21 ± 8***,+++	66 ± 18***,++	100 ± 0***,+++	100 ± 0	69 ± 12***,+++	100 ± 0	23 ± 12***,+++
CI/N ( <i>n</i> = 15)	84 ± 15*	98 ± 2	60 ± 28 <sup>+</sup>	72 ± 16*,+++	79 ± 13*,++	80 ± 26 <sup>++</sup>	100 ± 0	30 ± 26 <sup>++</sup>	100 ± 0	7 ± 13 <sup>++</sup>

*Note.* Results are expressed as means ± standard deviation of the percentage of positive cells. The following results were obtained for other markers tested in this study: CD5 (controls, 3 ± 5; CI/N, 9 ± 21); CD8 (controls, 59 ± 17; AI, 37 ± 21; CI/N, 53 ± 20); CD16 (controls, 96 ± 3; AI, 91 ± 5; CI/N, 99 ± 1); CD94 (controls, 59 ± 11; CI/N, 56 ± 28); CD158a (controls, 18 ± 8; CI/N, 17 ± 17); CD161 (controls, 83 ± 11; CI/N, 64 ± 29); NKB1 (controls, 18 ± 12; AI, 11 ± 10; CI/N, 12 ± 9). Differences between groups were not statistically significant, except for the percentage of CD8<sup>+</sup> NK-cells: Controls versus AI patients (*P* < 0.001); AI patients versus CI/N patients (*P* < 0.001).

*P* values: Controls versus patients, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.01; AI versus CI/N patients, <sup>+</sup>*P* < 0.05, <sup>++</sup>*P* < 0.01, <sup>+++</sup>*P* < 0.001.

AI patients, *P* < 0.01) and more heterogeneous (mean CV of 87 ± 21, 71 ± 18 and 66 ± 13, respectively; *P* < 0.01) than in the other two groups of individuals. In contrast, the percentage of CD2<sup>+</sup> NK-cells was significantly higher (*P* < 0.05) in both groups of patients compared to controls (mean percentage of CD2<sup>+</sup> cells of 79 ± 9% in AI and 84 ± 15% in CI/N patients versus 74 ± 11% in controls). Moreover, although CD2 was expressed at variable levels in the different groups of individuals analyzed, the pattern of CD2 ex-

pression was more homogeneous in CI/N patients compared to the other groups of individuals (mean CV of 44 ± 13% in CI/N patients versus 64 ± 12% in controls and 60 ± 9% in AI patients; *P* < 0.001).

Differences in both CD2 and CD7 expression on CD56<sup>+</sup>lo NK-cells between normal and pathological samples were due to differences in the relative representation of distinct subsets of NK-cells defined by the pattern of expression of these molecules: CD2<sup>-/+lo</sup>/CD7<sup>hi</sup> and CD2<sup>hi</sup>/

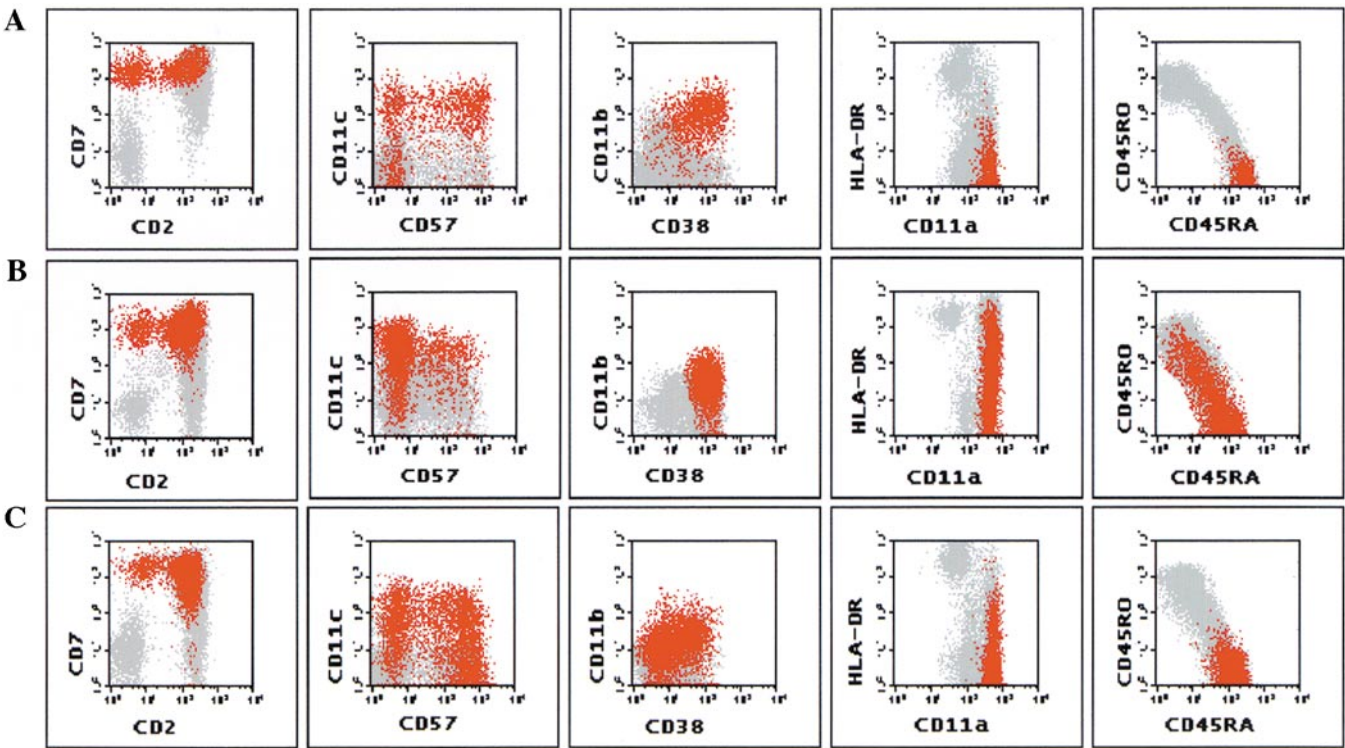
TABLE 2

Immunophenotypic Analysis of Blood CD56<sup>+</sup>lo NK-Cells: Levels and Patterns of Expression of CD2, CD7, CD11c, CD57, CD11b, CD38, CD11a, HLA-DR, CD45RA, and CD45RO on CD56<sup>+</sup>lo NK-Cells from Normal Individuals (Controls), Patients with Acute Viral Infection (AI), and Patients with NK-Cell Lymphocytosis Secondary to Infections or Tumors (CI/N)

		CD2	CD7	CD11c	CD57	CD11b	CD38	CD11a	HLA-DR	CD45RA	CD45RO
Controls	MFI	167 ± 57	1460 ± 402	144 ± 42	554 ± 339	80 ± 38	85 ± 31	484 ± 53	76 ± 21	259 ± 88	45 ± 28
( <i>n</i> = 12)	CV	64 ± 12	71 ± 18	95 ± 7	115 ± 44	108 ± 16	81 ± 16	32 ± 2	166 ± 59	34 ± 5	115 ± 18
AI	MFI	182 ± 63	1764 ± 644	232 ± 105*,++	259 ± 188*,+++	49 ± 41**	177 ± 63***,+++	645 ± 185*	472 ± 221***,+++	189 ± 77	39 ± 7
( <i>n</i> = 15)	CV	60 ± 9 <sup>+++</sup>	66 ± 13	99 ± 20	113 ± 28 <sup>+++</sup>	118 ± 20	50 ± 9***,+++	32 ± 4	161 ± 30	59 ± 24***,+	85 ± 20*
CI/N	MFI	199 ± 86	991 ± 446***,+++	158 ± 97 <sup>+</sup>	653 ± 410 <sup>+++</sup>	42 ± 18**	51 ± 25***,+++	553 ± 77*	80 ± 36 <sup>+++</sup>	333 ± 116	46 ± 18
( <i>n</i> = 15)	CV	44 ± 13***,+++	87 ± 21*,++	103 ± 24	82 ± 21*,+++	120 ± 37	111 ± 59 <sup>+++</sup>	30 ± 6	158 ± 35	43 ± 19 <sup>+</sup>	114 ± 49

*Note.* Results are expressed as means ± standard deviation of the mean fluorescence intensity (MFI) and coefficient of variation (CV) of positive cells. MFI is expressed as arbitrary relative linear units scaled from 0 to 10,000. The following results were obtained for MFI of cells that stained positively for other markers tested in this study: CD5 (controls, 196 ± 26; CI/N, 127 ± 32); CD8 (controls, 343 ± 128; AI, 287 ± 119; CI/N, 366 ± 132); CD16 (controls, 269 ± 142; AI, 202 ± 133; CI/N, 209 ± 96); CD94 (controls, 60 ± 9; CI/N, 74 ± 28); CD158a (controls, 30 ± 13; CI/N, 38 ± 13); CD161 (controls, 48 ± 18; CI/N, 46 ± 20); NKB1 (controls, 252 ± 108; AI, 283 ± 136; CI/N, 203 ± 104). Differences between groups were not statistically significant. The following results were obtained for pattern of antigen expression (CV) on cells that stained positively for other markers tested in this study: CD5 (controls, 62 ± 10; CI/N, 76 ± 20); CD8 (controls, 110 ± 14; AI, 147 ± 69; CI/N, 99 ± 23); CD16 (controls, 44 ± 9; AI, 58 ± 19; CI/N, 53 ± 16); CD94 (controls, 65 ± 5; CI/N, 50 ± 13); CD158a (controls, 66 ± 8; CI/N, 47 ± 19); CD161 (controls, 65 ± 6; CI/N, 71 ± 9); NKB1 (controls, 44 ± 8; AI, 46 ± 13; CI/N, 57 ± 22). Differences between groups were not statistically significant except for a more heterogeneous CD16 expression in AI (*P* = 0.035) and a more homogeneous CD94 expression in CI/N patients (*P* = 0.001) compared to controls.

*P* values: Controls versus patients, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001; AI versus CI/N patients, <sup>+</sup>*P* < 0.05, <sup>++</sup>*P* < 0.01, <sup>+++</sup>*P* < 0.001.



**FIG. 1.** Representative CD2/CD7, CD57/CD11c, CD38/CD11b, CD11a/HLA-DR, and CD45RA/CD45RO dot plots illustrating the phenotypic patterns most frequently observed in normal blood samples (A), as well as in blood samples from patients with either acute viral infection (B) or NK-cell lymphocytosis associated with chronic infection or tumors (C).

CD7<sup>-/+lo</sup> NK-cells (Fig. 1). The majority of CD56<sup>+lo</sup> NK-cells from normal individuals and AI patients were CD2<sup>-/+lo</sup>/CD7<sup>+hi</sup> whereas CI/N individuals showed an increased proportion of CD2<sup>+hi</sup>/CD7<sup>-/+lo</sup> NK-cells (Table 3). In fact, CD2<sup>+hi</sup>/CD7<sup>-/+lo</sup> NK-cells were detected in only 4 out of 12 normal individuals (33%) and 3 of 15 AI patients (20%) whereas the majority of CI/N

patients (10 of 15; 67%) had CD2<sup>+hi</sup>/CD7<sup>-/+lo</sup> NK-cells. Moreover, when considering only the cases in which CD2<sup>+hi</sup>/CD7<sup>-/+lo</sup> NK-cells were identified, the percentage of these NK-cells (within the CD56<sup>+lo</sup> NK-cell subset) was lower in AI patients (36 ± 10%) than in CI/N patients (79 ± 13%), intermediate values being observed in controls (60 ± 17%). Interestingly, in those normal

**TABLE 3**

Immunophenotypic Analysis of Blood CD56 <sup>+lo</sup> NK-Cells: Relative Distribution of Different CD56 <sup>+lo</sup> NK-Cell Subsets Established According to Their Pattern of Reactivity for CD2/CD7 and CD57/CD11c in Normal Healthy Individuals (Controls), Patients with Either Acute Viral Infection (AI), and Patients with NK-Cell Lymphocytosis Associated with Chronic Infections or Tumors (CI/N)									
Group	CD2/CD7 (%)					CD57/CD11c (%)			
	CD2 <sup>-</sup>	CD7 <sup>+hi</sup>	CD2 <sup>+lo</sup>	CD7 <sup>+hi</sup>	CD2 <sup>+hi</sup> CD7 <sup>±lo</sup> #	CD11c <sup>+</sup> CD57 <sup>-</sup>	CD11c <sup>+</sup> CD57 <sup>+</sup>	CD11c <sup>-</sup> CD57 <sup>+</sup>	CD11c <sup>-</sup> CD57 <sup>+</sup>
Controls (n = 12)	26 ± 11		54 ± 24		20 ± 31	31 ± 14	42 ± 16	13 ± 14	
AI (n = 15)	21 ± 9		72 ± 12 <sup>*,+++</sup>		7 ± 16 <sup>++</sup>	63 ± 9 <sup>***,+++</sup>	19 ± 10 <sup>***,++</sup>	2 ± 2 <sup>***,+++</sup>	
CI/N (n = 15)	16 ± 15		32 ± 27 <sup>*,+++</sup>		53 ± 40 <sup>*,++</sup>	20 ± 12 <sup>*,+++</sup>	43 ± 22 <sup>++</sup>	27 ± 24 <sup>+++</sup>	

*Note.* Results are expressed as means ± standard deviation of the percentage of each NK-cell subset among total CD56<sup>+lo</sup> PB NK-cells. # CD2<sup>+hi</sup>/CD7<sup>±lo</sup> NK-cells were detected in 4 of 12 (33%) normal PB samples, 3 of 15 (20%) PB samples from AI, and 10 of 15 (67%) PB samples from CI/N patients, where they represented 60 ± 17, 36 ± 10, and 79 ± 13% of CD56<sup>+lo</sup> NK-cells, respectively.  
P values: Controls versus patients, \*P < 0.05; \*\*P < 0.01, \*\*\*P < 0.001; AI versus CI/N patients, +P < 0.05, ++P < 0.01, +++P < 0.001.

samples in which  $CD2^{+hi}/CD7^{-/+lo}$  NK-cells were identified, the absolute NK-cell counts were significantly higher than in those in which only  $CD2^{-/+lo}/CD7^{+hi}$  NK-cells were found ( $378 \pm 144 \times 10^6/L$  versus  $150 \pm 84 \times 10^6/L$ , respectively;  $P = 0.008$ ).

As shown in Table 1 and illustrated in Fig. 1, patients with acute infections showed a slightly higher percentage of  $CD11c^{+}$  NK-cells ( $83 \pm 11\%$ ;  $P = 0.152$ ) and a lower proportion of  $CD57^{+}$  NK-cells ( $21 \pm 8\%$ ;  $P < 0.001$ ) in comparison to healthy controls ( $74 \pm 17\%$  and  $56 \pm 18\%$ , respectively). These differences resulted from a marked increase in the proportion of  $CD11c^{+}/CD57^{-}$  NK-cells ( $63 \pm 9\%$  versus  $31 \pm 14\%$ ;  $P < 0.001$ ) and a decrease in both  $CD11c^{+}/CD57^{+}$  ( $19 \pm 10\%$  versus  $42 \pm 16\%$ ;  $P < 0.001$ ) and  $CD11c^{-}/CD57^{+}$  NK-cells ( $2 \pm 2\%$  versus  $13 \pm 14\%$ ;  $P < 0.001$ ) (Table 3 and Fig. 1). In addition, among AI patients the intensity of expression of CD11c on  $CD56^{+lo}$  NK-cells was higher (mean MFI of  $232 \pm 105$  versus  $144 \pm 42$ ;  $P < 0.05$ ) and the levels of CD57 were lower (MFI of  $259 \pm 188$  versus  $554 \pm 339$ ;  $P < 0.05$ ) than those observed in controls (Table 2). By contrast, CI/N patients showed significantly higher relative numbers of  $CD57^{+}$  NK-cells ( $72 \pm 16\%$ ) compared to controls ( $56 \pm 18\%$ ;  $P < 0.05$ ), this differences being even more evident in comparison to AI patients ( $21 \pm 8\%$ ;  $P < 0.001$ ) (Table 1). Moreover, although the mean CD57 levels displayed by  $CD56^{+lo}$  NK-cells from CI/N patients did not differ significantly from those observed in normal individuals (mean MFI of  $653 \pm 410$  versus  $554 \pm 339$ ;  $P > 0.05$ ), the pattern of CD57 expression was more homogeneous in this group of patients (mean CV of  $82 \pm 21\%$ ) compared to both controls (mean CV of  $115 \pm 44$ ;  $P < 0.05$ ) and AI individuals (mean CV of  $113 \pm 28\%$ ;  $P < 0.001$ ) (Table 2).

CD38 levels were significantly higher and more homogeneously expressed on  $CD56^{+lo}$  NK-cells from AI patients (MFI of  $177 \pm 63$  and mean CV of  $50 \pm 9\%$ ;  $P < 0.001$ ) while lower in CI/N patients (MFI of  $51 \pm 25$ ;  $P < 0.01$ ) in comparison to normal individuals (mean MFI of  $85 \pm 31$  and mean CV of  $81 \pm 16\%$ ) (Table 2). In contrast, CD11b levels were significantly de-

creased in  $CD56^{+lo}$  NK-cells from both groups of patients in comparison to controls (mean MFI of  $49 \pm 41$  in AI and  $42 \pm 18$  in CI/N patients versus  $80 \pm 38$  in controls;  $P < 0.01$ ) (Table 2).

In normal, as well as in pathological samples, virtually all  $CD56^{+lo}$  NK-cells were  $CD11a^{+hi}$ , the levels of expression of this adhesion molecule being higher in both AI and CI/N patients than in controls (mean MFI of  $645 \pm 185$  in AI and  $553 \pm 77$  in CI/N patients versus  $484 \pm 53$  in controls;  $P < 0.05$ ) (Table 2). In addition,  $CD56^{+lo}$  NK-cells displayed variable levels of HLA-DR expression ranging from  $16 \pm 14\%$  HLA-DR<sup>+</sup> cells in control samples to  $30 \pm 26\%$  in CI/N ( $P = 0.2$ ) and  $69 \pm 12\%$  in AI patients ( $P < 0.001$ ). As expected, in this later group of individuals significantly higher levels of HLA-DR molecules per NK-cell were found compared to both normal individuals and CI/N patients (mean MFI of  $472 \pm 221$  versus  $76 \pm 21$  and  $80 \pm 36$ , respectively;  $P < 0.001$ ).

In a similar way, most  $CD56^{+lo}$  NK-cells from both healthy individuals and CI/N patients showed a homogeneous and high CD45RA expression (MFI of  $259 \pm 88$  and  $333 \pm 116$  and mean CV of  $34 \pm 5\%$  and  $43 \pm 19\%$ , respectively;  $P > 0.05$ ) in the absence of CD45RO (Tables 1 and 2) while  $CD56^{+lo}$  NK-cells from AI patients displayed a slightly lower (mean MFI of  $189 \pm 77$ ,  $P = 0.2$ ) and more heterogeneous (mean CV of  $59 \pm 24\%$ ;  $P < 0.001$ ) CD45RA expression in the presence of an increased percentage of CD45RO<sup>+</sup> NK-cells (mean of  $23 \pm 12\%$  versus  $5 \pm 5\%$  in controls and  $7 \pm 13\%$  in CI/N patients;  $P < 0.001$ ).

### Other Markers

Differences between normal individuals and patients concerning the expression of other markers addressed in this studied were less evident (see notes to Tables 1 and 2). Accordingly, except for a lower percentage of CD8<sup>+</sup> cells ( $P < 0.01$ ) and a more heterogeneous expression of CD16 expression ( $P < 0.05$ ) among AI individuals, no significant differences were observed as regards the expression of the other antigens analyzed. Interestingly, in 2/15 AI patients and 2/15 CI/N

patients (whereas in none of the control individuals) two CD56<sup>+</sup> NK-cell populations were clearly distinguished based on the levels of CD16 expression, the one expressing the lowest levels of CD16 (CD16<sup>+</sup>) representing between 33 and 72% of total CD16<sup>+</sup>/CD56<sup>+</sup> NK-cells.

In that concerning the expression of immunoglobulin-like (CD158a and NKB1) and lectin-like (CD94 and CD161) killer receptors, the only statistically significant difference was a more homogeneous CD94 expression on CD56<sup>+</sup> NK-cells from CI/N patients compared to the control group (mean CV of 65 ± 5 and 50 ± 13%, respectively;  $P < 0.001$ ).

## DISCUSSION

Previous studies have shown that activated NK-cells express a number of molecules that are absent or present at very low levels in nonactivated NK-cells. Accordingly, NK-cells express “*de novo*” CD69 (9–14), HLA-DR (9, 10), CD40L (15), and CD25 and CD132 (16, 17) after activation with different stimuli at the same time they transiently acquire CD45RO (18). Besides this, NK-cell activation also results in either down or upregulation of different receptors constitutively expressed by resting mature NK-cells. Accordingly, it has been reported that upon *in vitro* activation, expression of CD2, CD11a/CD18, CD26, CD44, CD54, and CD58 increases (19–21), whereas the surface levels of CD7 (22), CD11b-CD11c/CD18 and CD16/TCRzeta chain complex decrease (9, 10, 19, 23, 24, 26), reactivity for other molecules such as CD8 remaining stable (25). Activation-related changes on the expression of some other molecules still remain controversial, probably depending on the type and intensity of the stimuli. In this sense, some reports showed a decrease on the expression of CD56 (27), whereas others observed an increase on the expression of this adhesion molecule (9, 10); the effect of NK-cell activation on the expression of other molecules, such as CD62L (28) and CD49a-f (29), seems also to be stimuli-dependent. In spite of all the information mentioned above, it should be noted that our knowledge on the modulation of the cellular surface phenotypes associ-

ated with NK-cell activation is based mostly on *in vitro* studies and that few data are available on documenting the phenotypic changes that occur after NK-cell activation *in vivo*.

In the present study, we have analyzed the phenotypic patterns of both normal and reactive NK-cells, using two different *in vivo* models of NK-cell activation: patients with acute and chronic processes that have been considered to be associated with activation of this leukocyte population. Interestingly, the use of CD2/CD7, CD57/CD11c, CD38/CD11b, and CD11a/HLA-DR double stainings allowed the identification of discrete subsets of NK-cells which are usually present in normal blood that change their relative distribution during both acute and chronic *in vivo* responses to stimulatory signals, changes in these subsets contributing to explain the different phenotypic patterns observed in the groups here analyzed.

Accordingly, resting mature CD56<sup>+</sup> NK-cells, corresponding to the majority of the NK-cells present in normal blood, have a CD2<sup>-/+lo</sup>, CD7<sup>+</sup><sup>hi</sup>, HLA-DR<sup>-</sup> and CD45RO<sup>-</sup> phenotype. Most of these cells are CD11b<sup>+</sup>/CD38<sup>+</sup> and homogeneously express high levels of CD11a and CD45RA, whereas expression of CD11c and CD57 is heterogeneous and variable.

Recently activated NK-cells, herein represented by those NK-cells present at high frequency in the blood of patients with acute viral infection, have a pattern of expression of CD2 and CD7 similar to that observed in resting mature CD56<sup>+</sup> NK-cells, i.e., they are CD2<sup>-/+lo</sup>/CD7<sup>+</sup><sup>hi</sup>, except that a higher percentage of CD2<sup>+</sup> NK-cells was found. CD2<sup>+</sup><sup>lo</sup>/CD7<sup>+</sup><sup>hi</sup> NK-cells probably originate from CD2<sup>-</sup>/CD7<sup>+</sup><sup>hi</sup> NK-cells upon stimulation, through *de novo* expression of CD2 molecules on the cell surface. In line with this hypothesis, CD2<sup>+</sup><sup>lo</sup> CD56<sup>+</sup> NK-cells express higher levels of HLA-DR compared to CD2<sup>-</sup> CD56<sup>+</sup> NK-cells (data not shown). This would also be supported by previous studies in which it has been shown that CD7<sup>+</sup>/CD2<sup>-</sup>/CD3<sup>-</sup>/CD16 & CD56<sup>+</sup> NK-cells from human umbilical cord blood proliferate in response to interleukin 2 and phytohemagglutinine at the same time they become CD2<sup>+</sup> (37) and that CD2<sup>-</sup>/



CD16<sup>+</sup> peripheral blood NK-cells have less cytotoxic ability than their CD2<sup>+</sup>/CD16<sup>+</sup> counterparts (38). Recently activated NK-cells typically show strong reactivity for CD38 and express high levels of HLA-DR. At that phase, there is also downregulation of CD45RA, a phenomenon that is transiently accompanied by co-expression of CD45RO in variable proportions of NK-cells. CD11a levels are increased, whereas expression of CD11b may be either normal or decreased. Moreover, these NK-cells are CD11c<sup>+</sup> while they are either negative or dimly positive for CD57.

In patients with chronic infections and tumors, the former NK-cell subset is substantially replaced by chronically activated NK-cells. These cells shown unique phenotypic features, including upregulation of CD2, which becomes homogeneous (CD2<sup>hi</sup>), and downregulation of CD7, which becomes heterogeneous (CD7<sup>+/lo</sup>). In contrast to recently activated CD2<sup>+/lo</sup>/CD7<sup>hi</sup> NK-cells, CD2<sup>hi</sup>/CD7<sup>+/lo</sup> NK-cells are CD38<sup>+/lo</sup> and CD11b<sup>+/lo</sup> and express high and homogeneous levels of CD57, while CD11c expression is dim and heterogeneous. At this stage, most NK-cells have already reverted into their original HLA-DR<sup>-</sup>/CD45RA<sup>+</sup>/CD45RO<sup>-</sup> phenotype.

Interestingly, it should be noted that NK-cells displaying an early-activation phenotype were found in all patients with acute viral infection whereas in only a minor proportion of patients with chronic infections and tumors and none of the normal healthy individuals. Similarly, chronically-activated NK-cells were overrepresented in patients with NK-cell lymphocytosis associated to conditions in which the underlying stimuli persist for a long time, such as chronic infections and neoplastic diseases, although they could also be found at lower numbers in a few percentage of patients with acute infections as well as in normal individuals.

Changes involving other surface molecules occurring after *in vivo* NK-cell activation included a decrease on the percentage of CD8<sup>+</sup> CD56<sup>hi</sup> NK-cells in AI patients, a more heterogeneous CD16 expression on CD56<sup>hi</sup> NK-cells in a subgroup of both AI and CI/N patients and a more homogeneous expression of the CD94 lec-

tin-like NK-cell receptor on CD56<sup>hi</sup> NK-cells from CI/N patients, while no significant changes were found as regards the expression of other NK-cell receptors analyzed (CD158a, CD161, and NKB1).

Our results not only show that the majority of changes that have been documented upon NK-cell activation *in vitro* are also reproducible *in vivo*, but also allow us to establish a dynamic sequence for these immunophenotypic changes, defining the phenotypic profiles that characterize both the early/acute- and late/chronic- phases of NK-cell activation. Curiously, changes that occur at early stages of NK-cell activation mimic those that were previously described for T-cells (36), with the exception of upregulation of CD2 and downregulation of CD7, which seems to take place at a latter stage during NK-cell activation. In line with that occurring during T-cell activation (39), CD57 expression also behaves as a late activation marker for NK-cells.

In summary, in the present study we show that the pattern of expression of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR, contribute to the precise identification of blood NK-cell subsets which display immunophenotypic patterns associated with acute/early and chronic/late NK-cell activation. These findings would contribute to a better understanding of the functional role of NK-cells, as well as to establish the basis necessary for distinguishing between normal/reactive and neoplastic NK-cells, in the absence of clonality markers.

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