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Immunophenotypic Characterization of Normal Blood CD56^{+lo} Versus CD56^{+hi} NK-Cell Subsets and Its Impact on the Understanding of Their Tissue Distribution and Functional Properties

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ABSTRACT: In the present study we have compared the immunophenotypic characteristics of the CD56^{+lo} and CD56^{+hi} NK-cell subsets in a group of normal healthy adults. Our results show that CD56^{+hi} NK-cells display greater light-scatter properties than CD56^{+lo} NK-cells at the same time they have higher levels of CD25 and CD122 IL-2 chains, together with a higher reactivity for HLA-DR and CD45RO and lower levels of CD45RA, supporting that, as opposed to the majority of the CD56^{+lo} population, CD56^{+hi} NK-cells might correspond to a subset of activated circulating NK-lymphocytes. Higher expression of the CD2 and CD7 costimulatory molecules found for the CD56^{+hi} NK-cells would support their greater ability to respond to various stimuli. In addition, CD56^{+hi} NK-cells expressed higher levels of several adhesion molecules such as CD2, CD11c, CD44, CD56, and CD62L compared to CD56^{+lo} NK-cells, supporting a particular ability of these cells to migrate from blood to tissues and/or a potential advantage to form conjugates with target cells. Interestingly, CD56^{+lo} and CD56^{+hi} NK-cells showed a different pattern of expression of killer receptors that might determine different activation requirements for each of these NK-cell subsets. For instance, absence or low levels of CD16 expression might explain the lower antibody-dependent cytotoxicity activity of CD56^{+hi} NK-cells. On the other hand, the virtual absence of expression of the CD158a and NKB1 immunoglobulin-like and the greater reactivity for the CD94 lectin-like killer receptors on CD56^{+hi} in comparison to CD56^{+lo} NK-cells might determine different MHC-class I specificities for both NK-cell subsets, a possibility that deserves further studies to be confirmed.

Key Words: NK-cells; immunophenotype; flow cytometry; CD56; blood.

INTRODUCTION

Natural killer (NK) cells are commonly defined as CD3$^-$/TCR$^-$ large granular lymphocytes that express CD56 and/or CD16 and mediate non-MHC restricted cytotoxic functions (1). Two major NK-cell subsets differing on the expression of CD56 on the cell surface have been described so far: CD56^{+lo} and CD56^{+hi} NK cells (2). CD56^{+lo} NK-cells predominate in blood whereas CD56^{+hi} NK-cells are more frequently detected in various organs and tissues including the uterine placenta decidua (3–5), as well as in the lymphatic fluid (5). Although it was initially suggested that CD56^{+hi} could represent precursors of the most numerous CD56^{+lo} NK-cells (6), it was recently shown that CD56^{+hi} NK-cells represent a functionally distinct subset of mature NK-cells which are primarily responsible for cytokine production in response to monokines (7). In spite of this, it still remains unclear whether CD56^{+lo} and

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$CD56^{+hi}$ NK-cells correspond to different types of NK-cells or alternatively they represent different maturational/activation states of the same NK-cell population. Preliminary studies indicated that $CD56^{+lo}$ and $CD56^{+hi}$ NK-cells show different patterns of expression several surface markers (6, 8), suggesting that a detailed characterization of the molecules expressed on these NK-cell subsets could potentially contribute to clarify the relationship between them; nevertheless, at present information on the immunophenotypic features of both NK-cell subsets is not detailed enough to provide a comprehensive view of their tissue distribution and functional properties.

To gain further insights into the relationship between these subsets of blood NK-cells in the present study we have compared the immunophenotypic characteristics of $CD56^{+hi}$ with those of the predominating $CD56^{+lo}$ NK-cells, as regard to the expression of killer receptors, activation-related markers, cell adhesion molecules and homing receptors; based on the differences observed, we discuss on the functional relevance of each of these groups of molecules in determining NK-cell function and migratory properties.

MATERIALS AND METHODS

Samples

The enumeration of $CD56^{+lo}$ and $CD56^{+hi}$ NK-cells and the immunophenotypic analysis of these NK-cell subsets were performed in fresh EDTA-K3 anti-coagulated blood samples from six healthy adult individuals (blood donors)—three males and three females—aged from 24 to 38 years (median age of 32 years) who gave informed consent to enter in this study.

Immunophenotypic Studies

Immunophenotypic studies were performed using a whole blood stain-lyse-and-then-wash method and a direct immunofluorescence technique. Briefly, 100 μl of whole blood containing between 0.5 and 2×10^6 nucleated cells was incubated for 15 min at room temperature (RT) in the darkness, with saturating amounts of the appropriate monoclonal antibodies (MoAb). Lysis of red blood cells and fixation of the leukocytes was then performed by adding 2 ml of FACS lysing solution [Becton/Dickinson Biosciences (BD), San Jose, CA) diluted 1/10 (v/v) in distilled water and incubating the cells for another 10 min at RT. Cells were then washed once by centrifuging at 540*g* and resuspended in 0.5 ml of phosphate-buffered saline (PBS) for acquisition in the flow cytometer.

The following four-color combinations of MoAb directed against T- and NK-associated antigens conjugated with fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/PE-Cyanine 5 (PE-Cy5) and/allophycocyanin (APC) were used: CD2/CD7/CD56/CD3, CD5/CD7/CD56/CD3, CD57/CD11c/CD56/CD3, CD38/CD11b/CD56/CD3, CD16/NKB1/CD56/CD3, CD158a/CD161/CD56/CD3, CD94/CD8/CD56/CD3, CD122/CD25/CD56/CD3, CD44/CD40L/CD56/CD3, CD11a/HLA-DR/CD56/CD3, CD45RA/CD45RO/CD56/CD3, and CD62L/CD28/CD56/CD3. The source and specificity of each of the MoAb reagents are shown in Table 1.

Data acquisition was carried out in a FACSCalibur flow cytometer (BD) equipped with a 15-mW air-cooled 488-nm argon laser and a 625-nm neon diode laser, using the Cell QUEST software program (BD). Information on a minimum of 2×10^5 events was acquired for each staining and stored as list mode data. For data analysis the Paint-a-Gate PRO software program (BD) was used. As a first step, NK-cells were selected based on their $CD3^-/CD56^+$ phenotype and the proportion of $CD56^{+lo}$ and $CD56^{+hi}$ NK-cells within the total $CD56^+$ NK-cell compartment calculated. Then, each NK-cell subset was separately analyzed for both its light-scatter—side scatter (SSC) and forward scatter (FSC)—characteristics and expression of the surface antigens recognized by the MoAb referred above. For each antigenic determinant, the following characteristics were recorded: (i) percentage of positive cells, evaluated as the percentage of cells stained above the negative control value; (ii) intensity of expression, evaluated by the mean fluorescence intensity (MFI), expressed in arbitrary relative linear units of fluorescence scaled from 0 to 10,000; (iii)

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TABLE 1

Specificities, Clones, and Sources of the Monoclonal Antibodies Used in the Present Study

Specificity	Clone	Conjugate	Source[a]
CD2	SFCI3Pt2H9	FITC	BC
CD3	SK7	APC	BD
CD5	L17F12	PE	BD
CD7	3A1/1,7F3; 3A1E-12H7	FITC; PE	CLB; BC
CD8	DK25	PE	DK
CD11a	CLB-LFA-1/2	FITC	CLB
CD11b	D12	PE	BD
CD11c	S-HCL-3	PE	BD
CD16	3G8	FITC	BC
CD25	2A3	PE	BD
CD28	L293	PE	BD
CD38	LD38	FITC	CYT
CD44	L178	FITC	BD
CD45RA	L48	FITC	BD
CD45RO	UCHL-1	PE	BD
CD56	NCAM16.2	PE	BD
CD57	HNK-1	FITC	BD
CD62L	SK11	FITC	BD
CD94	HP-309	FITC	PH
CD122	MIK-b	FITC	CLB
CD154	TRAP1	PE	PH
CD158a	HP-3E4	FITC	BD
CD161	DX12	PE	BD
HLA-DR	L243	PE	BD
NKB1	DX9	PE	BD

[a] BC, Beckman Coulter (Miami, FL); BD, Becton/Dickinson BioSciences (San José, CA); CLB (Amsterdam, The Netherlands); CYT, Cytognos (Salamanca, Spain); DK, Dako A/S (Gostrup, Denmark); PH, Pharmingen (San Diego, CA).

pattern of antigen expression—homogeneous versus heterogeneous—evaluated by the coefficient of variation (CV). In addition, the relative intensity of expression of each antigen on $CD56^{+hi}$ NK-cells compared to $CD56^{+lo}$ NK-cells was evaluated as the ratio between the MFI values for these NK-cell subsets in each individual sample.

Statistical Analysis

For all variables under study, median, mean, standard deviation, minimum and maximum were calculated. To establish the statistical significance of the immunophenotypic differences observed between $CD56^{+lo}$ and $CD56^{+hi}$ NK-cells, the Mann–Whitney U and the χ^2 tests were used, for continuous and dichotomic variables (SPSS 9.0, SPSS, Chicago, IL). P values less than 0.05 were considered to be associated with statistically significant differences.

RESULTS

$CD56^+$ NK-cells accounted for a mean of $17 \pm 8\%$ of the blood lymphocyte compartment in the blood from the 6 normal individuals included in this study, the mean absolute NK-cell count being of $264 \pm 183 \times 10^6$/L. From the NK-cells, the majority showed dim CD56 expression ($CD56^{+lo}$) (mean of $93 \pm 7\%$, ranging from 80 to 99%) while a minor fraction displayed a high reactivity for CD56 ($CD56^{+hi}$) ($7 \pm 7\%$, ranging from 1 to 20%). These values did not differ significantly from those found in a group of 70 healthy adult individuals used for determination of normal reference values in our lab ($93 \pm 6\%$ versus $7 \pm 6\%$).

Apart from their differential expression of CD56 these NK-cell subsets displayed distinct light-scatter (FSC and SSC) characteristics. Accordingly, $CD56^{+hi}$ NK-cells showed higher FSC ($P = 0.041$) and SSC ($P = 0.026$) values than those observed for $CD56^{+lo}$ NK-cells, with identical FSC and SSC mean ratios (1.1 ± 0.1).

From the phenotypic point of view, $CD56^{+hi}$ NK-cells expressed CD56 at levels that were 6.7-$\pm$ 1.5-fold higher than those observed for $CD56^{+lo}$ NK-cells ($P = 0.002$) (Fig. 1), which in turn exceeded those of $CD56^+$ T-cells by 3.3-$\pm$ 1.5-fold. Interestingly, CD56 expression was extremely homogeneous on $CD56^{+hi}$ compared to the $CD56^{+lo}$ NK-cells ($P = 0.002$).

Besides the differential expression observed for CD56, the two NK-cell populations under study also differed in the percentage of cells that stained positively (Table 2) and/or on the intensity and pattern of expression (Table 3 and Fig. 1) of a large number of other antigens. Such phenotypic differences are illustrated in Fig. 2 and described below in more detail.

T-Cell-Associated Markers

None of the two blood NK-cell subsets showed surface expression of CD3. By contrast, both NK-cell subsets expressed the CD2 and CD7 antigens,

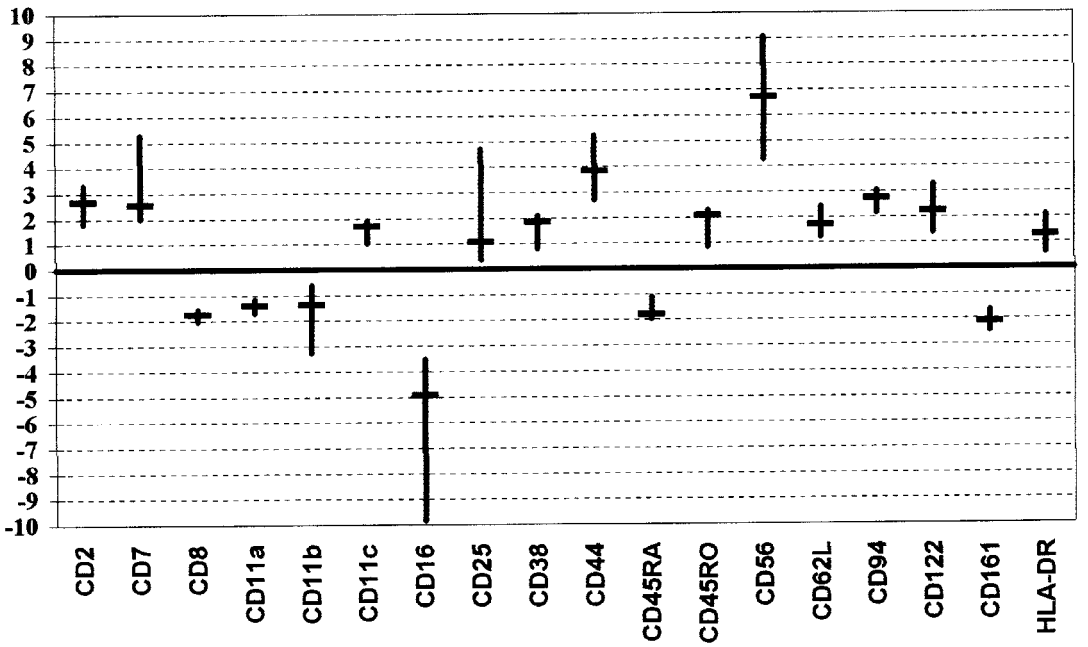

FIG. 1. Ratio between the intensity of antigen expression (MFI) obtained on $CD56^{+hi}$ and $CD56^{+lo}$ blood NK-cell subsets from six adult healthy individuals. Horizontal lines represent median values and vertical lines extend the minimum and maximum values.

although in both cases a $CD2^-$ NK-cell population was identified which represented a mean of 23 ± 11 and 6 ± 3% of $CD56^{+lo}$ and $CD56^{+hi}$ NK-cell subsets, respectively ($P = 0.004$). Upon comparing the reactivity for both antigens in the $CD56^{+hi}$ and $CD56^{+lo}$ NK-cell subsets it was observed that the former expressed CD2 and CD7 at higher intensities than those observed on $CD56^{+lo}$ cells (ratio of 2.6 ± 0.6 and 2.9 ± 1.2, respectively; $P = 0.002$ in both cases). In addition, both CD2 and CD7 were more heterogeneously expressed on $CD56^{+low}$ than on $CD56^{+hi}$ NK-cells ($P = 0.004$ and 0.041, respectively). A variable proportion of $CD56^{+lo}$ NK-cells showed dim CD5 expression (5 ± 7%, ranging from 0 to 18%), a $CD56^{+lo}/CD5^{+lo}$ NK-cell population being clearly identified in 3 out of 6 cases, where they represent 2, 9, and 18% of total $CD56^{+lo}$ NK-cells; in these cases, CD5 expression was homogeneous, and present at levels 9.6- ± 1.2-fold lower than those observed for normal blood T-cells. In contrast, $CD56^{+hi}$ NK-cells were consistently negative for CD5 ($P = 0.001$).

The mean fraction of $CD8^+$ cells within the $CD56^{+lo}$ (55 ± 16%) and the $CD56^{+hi}$ (54 ± 13%) NK-cell subsets were similar, CD8 expression being rather heterogeneous in both cases.

Adhesion Molecules and Homing Receptors

Virtually all NK-cells constitutively expressed homogeneously high levels of CD11a when compared to other circulating lymphocytes, its intensity of expression being significantly lower on $CD56^{+hi}$ NK-cells compared to the predominating $CD56^{+lo}$ NK-cell subset (ratio of 1.4 ± 0.2; $P = 0.002$). In contrast, CD11b was heterogeneously expressed on both cell subsets at similar levels ($P > 0.05$) whereas CD11c expression was significantly higher on the latter NK-cell subset (ratio of 1.6- ± 0.3-fold; $P = 0.041$). Additionally, most $CD56^{+hi}$ NK-cells were $CD11c^+$ (92 ± 6%) whereas its expression on $CD56^{+lo}$ NK cells was more variable, with a mean fraction of 72 ± 24% $CD11c^+$ cells ($P = 0.065$). Expression of CD11c was relatively heterogeneous on both NK-cell subsets, although lower coefficients of variation were found on $CD56^{+hi}$ than on $CD56^{+lo}$ NK-cells ($P = 0.009$). In contrast, CD57 was expressed in large fraction of $CD56^{+lo}$ NK whereas $CD56^{+hi}$ were virtually negative for CD57 (mean fraction of $CD57^+$ cells of 66 ± 14 and 1 ± 1%, respectively; $P = 0.002$).

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TABLE 2

Immunophenotypic Characteristics of $CD56^{+lo}$ and $CD56^{+hi}$ Peripheral Blood NK-Cell Subsets from Adult Healthy Individuals ($n = 6$)

	$CD56^+$ NK-cells		
	$CD56^{+lo}$	$CD56^{+hi}$	*P* value
CD2	77 ± 11 (64–91)	94 ± 3 (91–98)	**0.004**
CD5[a]	5 ± 7 (0–18)	0 ± 1 (0–1)	0.180
CD7	99 ± 1 (98–100)	94 ± 5 (85–99)	**0.015**
CD8	55 ± 16 (40–72)	54 ± 13 (41–66)	1.000
CD11a	100 ± 0 (99–100)	100 ± 0 (100–100)	1.000
CD11b	92 ± 13 (68–100)	84 ± 8 (71–90)	0.151
CD11c	72 ± 24 (28–93)	92 ± 6 (84–100)	0.065
CD16	98 ± 2 (95–100)	28 ± 16 (10–47)	**0.009**
CD25	1 ± 1 (0–2)	22 ± 9 (9–32)	**0.002**
CD38	99 ± 2 (95–100)	99 ± 2 (97–100)	0.584
CD44	100 ± 1 (97–100)	100 ± 2 (94–100)	1.000
CD45RA	100 ± 0 (100–100)	98 ± 2 (96–100)	1.000
CD45RO	4 ± 4 (1–12)	12 ± 5 (9–17)	**0.026**
CD57	66 ± 14 (52–91)	1 ± 1 (0–2)	**0.002**
CD62L	14 ± 7 (4–22)	100 ± 0 (99–100)	**0.002**
CD94	59 ± 11 (40–75)	98 ± 3 (90–100)	**0.000**
CD122	100 ± 0 (100–100)	100 ± 0 (100–100)	0.937
CD158a	18 ± 8 (10–25)	1 ± 0 (0–1)	**0.037**
CD161	83 ± 11 (63–95)	59 ± 25 (31–96)	0.132
HLA-DR	23 ± 13 (10–46)	83 ± 8 (74–92)	**0.002**
NKB1	16 ± 16 (0–47)	0 ± 1 (0–1)	**0.026**

Note. Results are expressed as means ± standard deviation of percentage of positive cells; range is shown in parentheses. CD3, CD28, and CD40L were constantly absent in NK-cells from the samples analyzed.

[a] A $CD56^{+lo}/CD5^{+lo}$ NK-cell population was clearly identified in three of six cases, where they represent 2, 9, and 18% of total $CD56^{+lo}$ NK-cells, while $CD56^{+hi}$ were constantly $CD5^-$ (χ^2 test, $P = 0.001$).

All NK-cells were $CD44^+$, CD44 expression being significantly higher (ratio of 4.1 ± 1.1; $P = 0.002$) and more homogeneous ($P = 0.032$) on $CD56^{+hi}$ compared to $CD56^{+lo}$ NK-cells. In addition, nearly all $CD56^{+lo}$ NK-cells expressed CD62L whereas this molecule was negative or dimly positive in only a minor fraction of $CD56^{+lo}$ NK-cells (100 ± 0% versus 14 ± 7%; $P = 0.002$).

Killer Receptors

In contrast to the $CD56^{+lo}$ NK-cell compartment, in which the majority of cells (98 ± 16%) homogeneously expressed CD16, $CD56^{+hi}$ NK-cells either lacked CD16 or showed CD16 expression in a relatively small fraction of cells (mean of 28 ± 16%; $P = 0.009$) at an intensity 5.5- ± 2.3-fold lower than that observed for the $CD56^{+lo}$ NK-cell population ($P = 0.009$).

The expression of the immunoglobulin-like NKB1 and CD158a killer receptors on $CD56^{+lo}$ NK-cells was highly variable (mean percentage of positive cells of 16 ± 16 and 18 ± 8%, ranging from 0 to 47% and from 10 to 25%, respectively) whereas both were constantly negative on $CD56^{+hi}$ NK-cells ($P = 0.026$ and 0.037, respectively).

In that concerning lectin-like killer receptors, virtually all $CD56^{+hi}$ NK-cells expressed CD94 whereas only around half (59 ± 11%) of $CD56^{+lo}$ NK-cells showed dim CD94 expression, the intensity of expression for this molecule being 2.6- ± 0.3-fold higher ($P = 0.007$) and more homogeneous ($P < 0.001$) on $CD56^{+hi}$ compared to $CD56^{+lo}$ NK-cells. In contrast, reactivity for CD161 was relatively heterogeneous in both NK-cell subsets, although the intensity of CD161 expression was higher on $CD56^{+hi}$ NK-cells ($P = 0.004$).

Costimulatory Molecules, Cytokine Receptors, and Activation-Related Markers

All NK-cells constitutively expressed high levels of CD45RA. The intensity of CD45RA expression was 1.7- ± 0.7-fold higher ($P = 0.016$) and more homogeneous ($P = 0.026$) on $CD56^{+lo}$ than on $CD56^{+hi}$ NK cells. Although the vast majority of NK-cells were $CD45RO^-$, a few $CD45RA^+/CD45RO^+$ NK-cells were detected within both cell subsets, its percentage being higher among $CD56^{+hi}$ NK-cells (12 ± 5% versus 4 ± 4%; $P = 0.026$). In both cases $CD45RO^+/CD45RA^+$ NK-cells expressed lower levels of CD45RA than $CD45RA^+CD45RO^-$ NK-cells, expression of CD45RO being constantly dim and heterogeneous.

CD122 was constitutively expressed on virtually all NK-cells, its intensity of expression being 2.3- ± 0.6-fold higher on $CD56^{+hi}$ NK-cells ($P = 0.002$). Few $CD56^{+lo}$ NK-cells coexpressed CD25 (1 ± 1%) whereas a relatively high proportion of $CD56^{+hi}$ NK-cells was $CD25^{+lo}$

TABLE 3

Immunophenotypic Characteristics of $CD56^{+lo}$ and $CD56^{+hi}$ Peripheral Blood NK-Cell Subsets from Adult Healthy Individuals ($n = 6$): Intensity and Pattern of Expression of Each Antigen Analyzed

	MFI			CV		
	$CD56^{+lo}$	$CD56^{+hi}$	*P* value	$CD56^{+lo}$	$CD56^{+hi}$	*P* value
CD2	192 ± 70	476 ± 77	**0.002**	55 ± 8	38 ± 5	**0.004**
CD5	205 ± 33	Negative	—	42 ± 6	Negative	—
CD7	1585 ± 314	4409 ± 1300	**0.002**	81 ± 21	54 ± 15	**0.041**
CD8	423 ± 176	230 ± 66	0.200	115 ± 13	127 ± 6	0.400
CD11a	548 ± 109	343 ± 67	**0.002**	32 ± 2	31 ± 3	0.240
CD11b	91 ± 37	61 ± 18	0.222	103 ± 11	107 ± 18	0.541
CD11c	162 ± 34	258 ± 84	**0.041**	97 ± 12	72 ± 10	**0.009**
CD16	236 ± 161	47 ± 39	**0.009**	45 ± 12	65 ± 22	0.132
CD25	24 ± 20	24 ± 14	0.589	129 ± 42	107 ± 20	1.000
CD38	110 ± 42	146 ± 46	0.421	88 ± 23	79 ± 8	0.730
CD44	38 ± 20	145 ± 31	**0.002**	83 ± 22	48 ± 13	**0.032**
CD45RA	308 ± 35	194 ± 64	**0.016**	34 ± 7	57 ± 16	**0.026**
CD45RO	37 ± 7	63 ± 18	**0.016**	154 ± 80	114 ± 12	0.699
CD56	488 ± 148	3071 ± 461	**0.002**	53 ± 7	35 ± 6	**0.002**
CD57	698 ± 303	Negative	—	110 ± 27	Negative	—
CD62L	27 ± 7	49 ± 16	**0.015**	62 ± 11	51 ± 11	0.132
CD94	60 ± 9	161 ± 43	**0.007**	65 ± 5	38 ± 3	**0.000**
CD122	30 ± 10	63 ± 12	**0.002**	62 ± 12	44 ± 12	**0.093**
CD158a	27 ± 17	Negative	—	67 ± 8	Negative	—
CD161	48 ± 18	23 ± 7	**0.004**	65 ± 6	73 ± 6	**0.056**
HLA-DR	79 ± 23	93 ± 34	0.589	153 ± 63	127 ± 14	0.485
NKB1	225 ± 72	Negative	—	46 ± 9	Negative	—

Note. Results are expressed as means ± standard deviation of the mean fluorescence intensity (MFI) and the coefficient of variation (CV) of antigen expression on positive cells. MFI are expressed as arbitrary relative linear units scaled from 0 to 10,000. CD3, CD28, and CD40L were constantly absent in NK-cells from the samples analyzed.

(22 ± 9%) ($P = 0.002$). The mean proportion of $HLA\text{-}DR^+$ cells was also higher among $CD56^{+hi}$ NK-cells (83 ± 8% versus 23 ± 13%; $P = 0.002$). Both NK-cell populations displayed similar patterns of CD38 expression ($P > 0.05$).

The CD28 costimulatory molecule was constantly absent in the two NK-cell subsets here analyzed in all blood samples studied, as did the CD40 ligand (CD154).

DISCUSSION

In the present study we confirm and extend previous observations as regards the presence in the blood from normal individuals of two distinct populations of NK-cells defined by differential levels of CD56 expression. While the $CD56^{+lo}$ subset constantly represent the major fraction of NK-cells, the $CD56^{+hi}$ only accounts for a minor proportion of $CD56^+$ NK-cells present in blood. Interestingly, these NK-cell subsets showed distinct light scatter properties, higher FSC and SSC values being found for the $CD56^{+hi}$ NK-cells, supporting the existence of differences between these NK-cell subsets as regards both size and the cell internal complexity/granularity. Additionally, both cell subsets displayed distinct immunophenotypic patterns for a great part of the surface proteins analyzed, which may be related to distinct functional and migratory properties (9–15). As an example, receptors that regulate adhesion to vascular endothelium and extracellular matrix might contribute to a differential representation of these NK-cell subsets in blood as well as in tissues; in a similar way, differences on the expression of killer receptors, NK-cell costimulatory molecules and cytokine receptors may have a determinant role on the differential capacity of these NK-cell subsets to proliferate and being

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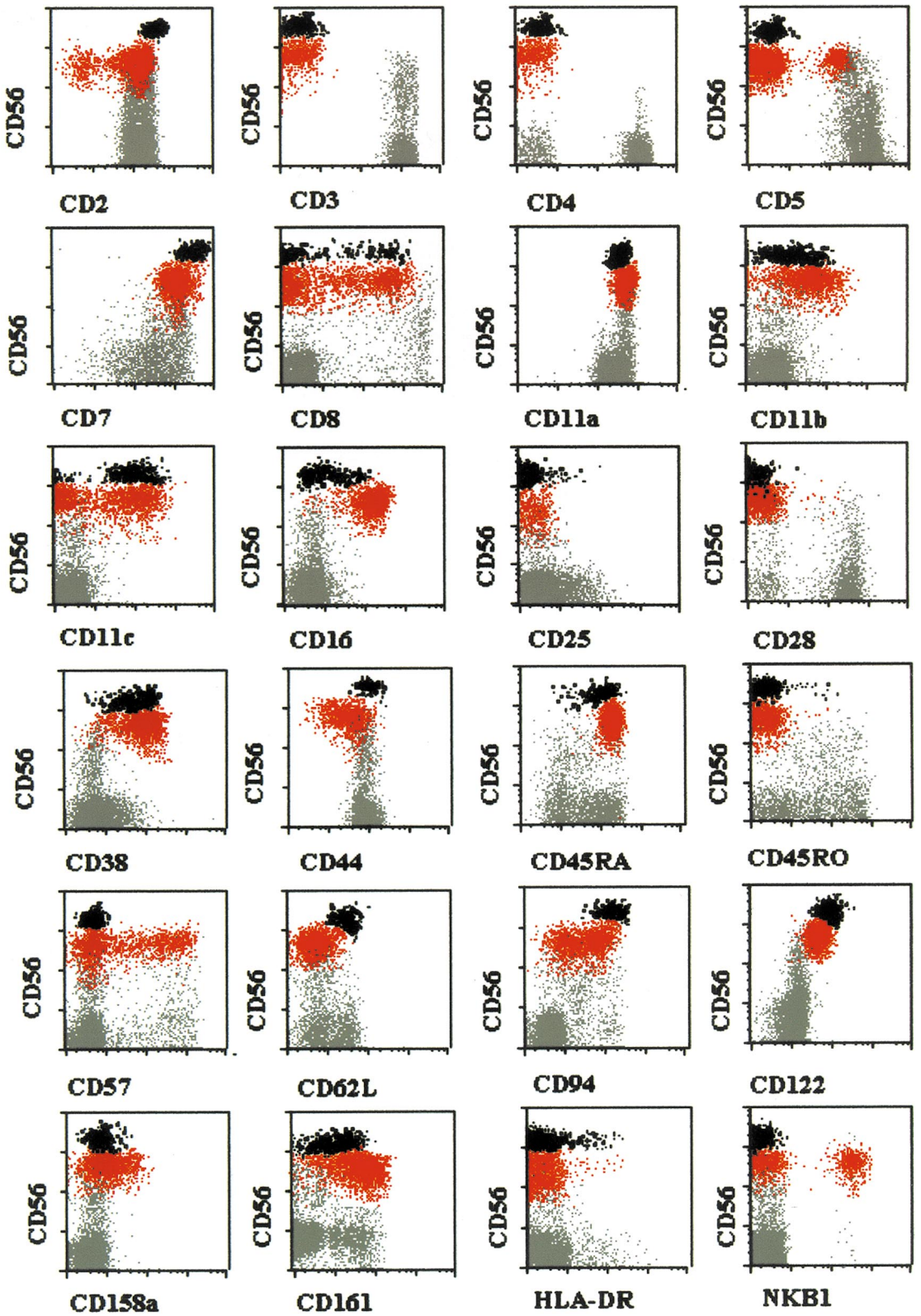

FIG. 2. Representative dot plots illustrating the distinct immunophenotypic patterns observed for the $CD56^{+lo}$ (red dots) and $CD56^{+hi}$ (black dots) blood NK-cells subsets from an adult healthy individual. Events painted in gray correspond to blood T-cells present in the sample.

either activated or inhibited in response to stimuli that trigger and regulate cytotoxic functions.

In that concerning to adhesion molecules that may favor homing of NK-cells to different tissues, it should be noted that CD62L—the L-selectin adhesion molecule that mediates interaction of leukocytes with peripheral lymph node high endothelial venules (HEV) (16, 17)—and CD44—a molecule that mediates adhesion by acting as the hyaluronate receptor and promotes NK-cell homing by binding to mucosal HEV (18, 19)—were preferentially expressed in $CD56^{+hi}$ compared $CD56^{+lo}$ NK-cells in accordance to previous observations (8, 20). These findings might at least in part explain why $CD56^{+hi}$ more efficiently bind to HEV than $CD56^{+lo}$ NK-cells (20) and could contribute to a preferential homing of $CD56^{+hi}$ NK-cells into different human tissues where they are found at higher frequencies (3–5).

Other adhesion molecules, such as the β1-integrins (21) have also been shown to be involved in the adhesion to vascular endothelium and transmigration across endothelial cells by means of different interactions, including those between CD11a and CD11b and their ligands (CD54, CD102, and fibrinogen) (22–30). In the present study we show, in accordance to previous studies (8), that CD11a is expressed on NK-cells at higher levels than those observed in other blood cells, a characteristic that probably contributes to the greater migratory properties of NK-cells compared to other circulating lymphocyte subsets. Nevertheless, differences on the expression of CD11a cannot explain the preferential localization of $CD56^{+hi}$ compared to $CD56^{+lo}$ NK-cells in tissues, as the intensity expression of CD11a is slightly lower in the former NK-cell subset. For the same reasons, differences on the expression of CD11b can neither account for the higher migratory properties of $CD56^{+hi}$ NK-cells. The meaning of the finding that CD11c is preferentially expressed on $CD56^{+hi}$ NK-cells is not known. However, in a similar way to that occurring for CD11b (31, 32), both C3bi (33, 34), fibrinogen (35) and CD54 (36) have been proposed as ligands for CD11c and this molecule has been claimed to be involved in adhesion to the endothelium and to the extracellular matrix (36), as well as to play a role in conjugate formation between NK-cells and target cells (37). The significance of the exclusive expression of CD57—an oligosaccharide with sulfated glucoronic acid residues that is expressed on T-cells during advanced phases of immune responses and that presumably also mediates cell adhesion (38)—on $CD56^{+lo}$ NK-cells is neither clear.

Besides determining adhesion properties and migratory capabilities, adhesion molecules may also be crucial for conjugate formation between NK-cells and target cells, a process that depends on the homophilic binding mediated by CD56 (39, 40) and on other pairs of receptors such as CD11a/CD54 (41, 42), CD2/CD58 (43, 44), and CD8/MHC-class I antigens (45, 46). Accordingly, the higher expression of CD56 and CD2 found on $CD56^{+hi}$ NK-cells may favor a closer interaction between these NK-cells and their targets.

Cytotoxicity depends on a delicate balance between different groups of receptors that either activate or inhibit the NK-cell killer functions (47–49) connecting to adapter molecules and turning on/off different signal-transducing pathways (50–53).

CD16—the α-chain of the low-affinity receptor for the Fc portion of IgG (FcγRIII)—activates cytotoxicity on NK-cells through its binding to immune-complexed IgG and plays a determinant role in NK-cell proliferation and both antibody-dependent and antibody-independent cell cytotoxicity (54–59). Absence or low intensity of expression of CD16 may explain at least to a certain extent why both blood and decidual $CD56^{+hi}$ NK-cells are less efficient in mediating non-MHC-restricted cytotoxicity than the $CD56^{+lo}$ NK-cells (2, 60, 61). Antibody-independent NK-cell cytotoxic activity is usually related to signals mediated by multiple immunoglobulin-like (CD158, NKB1; NKp44; NKp46) and lectin-like (CD94, CD161) receptors which may act through an MHC class I dependent (CD94, CD158, and NKB1) or independent (CD161) pathway (62–66). Positive stimulation may be initiated through the activation forms of some of the MHC-dependent receptors, such as CD158 and CD94, and other non-MHC restricted triggering molecules, such as CD161, whereas suppresser signals derive

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from inhibitory forms of the MHC-dependent receptors mentioned above. As a consequence of these regulatory mechanisms, differences on the expression of killer receptors may determine different MHC-dependent activator/suppressive requirements for $CD56^{+lo}$ and $CD56^{+hi}$ NK-cell subsets. For instance, the fact that $CD56^{+hi}$ NK-cells do not express CD158a and NKB1 whereas they strongly express CD94, may determine specificity for non-classical HLA-E molecules, whereas the opposite pattern of expression of CD158a and NKB1 with low reactivity for CD94 usually observed on $CD56^{+lo}$ NK-cells would suggest a preferential recognition of classical MHC class I A, B, and C molecules by this cell subset (67–69).

Higher levels of other molecules that exhibit NK-cell costimulatory functions on $CD56^{+hi}$ NK-cells such as CD2 (70–72), CD7 (73, 74), CD38 (75, 76), and CD44 (77, 78), may also be related to a higher ability of $NK56^{+hi}$ NK-cells to respond to different stimuli. By contrast, and in accordance to previous reports, we were not able to detect surface expression of neither the CD28 (79) nor the CD40L (CD154) (80) costimulatory molecules on NK-cells.

Interestingly, $CD56^{+hi}$ NK-cells exhibited a phenotype suggestive of cell activation, as reflected by a higher fraction of $HLA\text{-}DR^{+}$ and $CD25^{+}$ cells (81, 82). The lower intensity of expression of CD45RA and the highest proportion of $CD45RA^{+}/CD45RO^{+}$ cells among $CD56^{+hi}$ NK-cells may also be a signal of NK-cell activation, since previous studies have shown that activated NK-cells transiently acquire CD45RO, a phenomena that is accompanied by a decrease on CD45RA expression (83).

NK-cells proliferate and display enhanced cytotoxic activity in response to various cytokines and growth factors, including IL-2 (84–89). Consequently, differences on the expression of cytokine receptors would probably contribute to the different ability of these NK-cell subsets to be activated and to proliferate in response to specific cytokines. The fact that CD122—IL-2R β-chain—is constitutively expressed with a higher intensity on $CD56^{+hi}$ NK-cells at the same time that a higher fraction of these cells express CD25—IL-2R α-chain—could probably explain why $CD56^{+hi}$ NK-cells display a preferential response to low doses of IL-2 with increased cytolytic activity and proliferation compared to the $CD56^{+lo}$ NK-cell subset (90–94).

In summary, our results show that $CD56^{+hi}$ and $CD56^{+lo}$ NK-cells display distinct phenotypic characteristics suggesting that the former cells may correspond to a previously activated NK-cell subset with a different pattern of recognition of MHC molecules and different requirements to undergo proliferation and activation of its cytotoxic functions at the same time that they would explain its higher ability to migrate into different human tissues.

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