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Age-related changes in natural killer cell receptors from childhood through old age

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ABSTRACT

Most studies on natural killer (NK) cells and aging have focused on overall cell numbers and global cytotoxic activity. NK cell functions are controlled by surface receptors belonging to three major families: killer cell immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs), and C-type lectins. The expression of these receptors was investigated from childhood through old age in T, NKT- and NK cells and also in the $CD56^{dim}$ (cytotoxic) and $CD56^{bright}$ (responsible for cytokine production) NK cell subsets. A decrease in the expression of activating receptors (NKp30 and NKp46) was observed in NK cells in elderly individuals. KIR expression was increased only in the $CD56^{bright}$ subset. Children presented similar results regarding expression of NKp30 and KIR, but not NKp46. NKG2D expression was decreased in T cells of elderly subjects. Analysis of KIR genotype revealed that KIR2DL5 and KIR2DS3 were significantly associated with old age. Cytotoxic activity was preserved from childhood through old age, suggesting that the increase of the absolute number of $CD56^{dim}$, observed in elderly, may represent a compensatory mechanism for the receptor expression alterations. This initial study provides the framework for more focused studies of this subject, which are necessary to determine whether the changing balance of NK receptor expression may influence susceptibility to infectious, inflammatory, and neoplastic diseases.

1. Introduction

Immunosenescence is defined as a state of dysregulated immune function that contributes to an increased susceptibility to infection, cancer and autoimmune diseases in aged organisms, including humans [1]. Although the T-cell immune response is affected most dramatically by aging, age-associated immunologic changes also occur in the phenotype and function of natural killer cells [2].

NK cells are defined by their ability to kill cancer cells and virally infected cells without prior sensitization [3,4]. Two distinct populations of human NK cells can be identified based on the cell surface density of CD56. These subpopulations differ in function, phenotype, and tissue localization. $CD56^{bright}$ NK cells have a unique functional role in the innate immune response as the primary source of NK cell-derived immunoregulatory cytokines. This subtype is rare in peripheral blood (~5%) and more frequent in lymph nodes (~95%). The majority of peripheral blood NK cells (~95%) belong to the $CD56^{dim}$ NK subset, which produces low levels of NK-derived cytokines and potently mediates natural cytotoxicity [5,6].

Progress in the field of NK cell receptors has led to the concept that NK cells distinguish between normal healthy cells and abnormal cells by using a sophisticated repertoire of cell surface receptors that control NK cells activation, proliferation and effector functions. Each NK cell appears to express its own repertoire of activating and inhibitory receptors, and cytotoxicity is ultimately regulated by the balance of signals from these receptors [7]. Major families of cell surface receptors that inhibit and activate NK cells to lyse target cells have been characterized, including killer cell immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs), and C-type lectins [4,7,8].

The immune system is functionally immature at birth and undergoes a sequential development additionally stimulated by antigen exposure [9]. NK cells originate in the bone marrow, where they develop and proliferate; however, further maturation processes and

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the homeostasis of NK cells in peripheral blood are not well understood. To better understand this process, it is important to study NK cells during childhood. It has been hypothesized that the innate immune system, including NK cells, are especially important during early life, when adaptive immunity is immature, and that NK cells must thus be more easily activated during childhood [10].

Changes in NK cell subsets and expression of NK cell receptors seem to be important in the pathogenesis of different diseases, such as viral infection [11–13] and cancer [14–17]. Preservation of NK cell cytotoxicity may be critical for resistance to age-related diseases and for healthy aging [2,18].

Most studies on NK cells and aging have focused on overall NK cell numbers and global NK cytotoxic activity. The reported decline in NK cell reactivity based on cytotoxicity assays may be caused by changes in inhibitory and stimulatory receptor expression. Little is known about the changes in NK receptors that recognize target cells during aging. As far as we know, there are three major published reports in this area. First, Lutz et al. [19] compared elderly (aged >65 years) and young adults (aged 21–30 years) and observed an age-related decrease in CD94 and NKG2A expression and a reciprocal age-related increase in KIR expression. However, the expression of other receptors, such as NKG2D and NCR, was not examined. Sundström et al. has examined the expression of NK receptors at 3 time points during early childhood, *i.e.*, cord blood, 2 and 5 years of age [10]. They found that the proportion of $CD94^+$ $NKG2C^-$ ($NKG2A^+$) NK cells and the level of expression of NKG2D, NKp30, and NKp46 was increased in cord blood. Garff-Tavernier et al. [20], comparing NK cells derived from cord blood, middle-aged (18–60 years), old (60–80 years), and very old (80–100 years) subjects, observed that NK cells in cord blood displayed specific features associated with immaturity, including poor expression of KIR and high expression of NKG2A. NK cells from older subjects, by contrast, preserved their major phenotypic and functional characteristics in this study. None of these three studies have separated evaluated the expression of NK receptors in $CD56^{dim}$ and $CD56^{bright}$ subsets or studied KIR and HLA genotype.

In the current study, we investigated possible age-related changes in the expression of the three main families of NK receptors: KIRs, NCRs and C-type lectins comparing children (≤18 years), adults (19–59 years), and elderly (≥60 years) subjects. The expression of these receptors was examined not only in the total NK cell population but also in the T- and NKT-cell populations. Furthermore, to our knowledge, for the first time the expression of NK cells receptors was separately evaluated in the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets. KIR and HLA genotype and cytotoxic activity were also studied in these three age groups.

2. Subjects and methods

2.1. Study Subjects

We studied 73 healthy subjects with ages ranging from 5 to 77 years. Our study population was divided into three groups according to age: children (ages ≤18), adults (ages 19–59), and elderly adults (ages ≥60). The children group consisted of seven males and eight females. The samples from this group were obtained from sibling bone marrow donors for stem-cell transplantation at the Bone Marrow Transplantation Center, National Institute of Cancer, Brazil. The adult group (17 females and 20 males) consisted of healthy donors from the Blood Bank of the National Institute of Cancer (Rio de Janeiro, Brazil). The elderly group (12 females and nine males) was recruited at the National Institute of Traumatology and Orthopedics (Rio de Janeiro, Brazil). Individuals presenting conditions that could affect the immune system were excluded according to the criteria proposed by the SENIEUR (from SENIor EURopean) protocol, Ligthart et al. [21]. Subjects were prescreened by a questionnaire, and blood samples were collected when there was no declaration of infections within 6 months, diabetes, cancer, immunologic illness, or current use of immunomodulatory medications. A second screening was performed based on laboratory examinations and clinical records. Samples from 33 elderly individuals were collected but 12 were excluded because individuals presented increased glucose or alkaline phosphatase, rheumatoid arthritis, gonarthrosis or Paget disease. This study was approved by the Ethics Committee of the National Institute of Cancer (090/07) and the National Institute of Traumatology and Orthopedics (0074-08). All samples (15 ml of peripheral blood) were collected with written informed consent.

2.2. Reagents

Anti–CD3-FITC, anti–CD56-PC5, and mAbs specific for NK-cell receptors were obtained from Beckman Coulter (Fullerton, CA). Anti-KIR mAbs included PE-labeled anti-CD158a/h, anti-CD158b1/b2,j, anti-CD158e1/e2 and anti-CD158i. KIR expression was assessed separately and also by using a mixture of PE-labeled anti-KIR monoclonal antibodies (anti-CD158a/h, anti-CD158b1/b2,j, and anti-CD158e1/e2) as described by Lutz et al. [20]. The expression of C-type lectin family members was detected using anti-CD94-PE and anti-CD314-PE antibodies. Anti–CD335-PE, anti-CD336, and anti-CD337 were used for the analysis of NCR family member expression. Anti–CD4-FITC, anti–CD8-PE, anti–CD45-PercP, and murine isotype control monoclonal antibodies were purchased from Becton Dickinson (BD Biosciences, San Jose, CA). Carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and 7-aminoactinomycin D (7AAD; Becton Dickinson) were used to assess NK cytolitic activity by flow cytometry.

2.3. Flow cytometry

Multi-color FACS analysis was performed on freshly harvested blood cells. Whole blood samples were stained with specific monoclonal antibodies, and red blood cells were lysed with commercial solution (BD Biosciences, San Jose, CA). At least 50,000 lymphocytes were acquired using a FACScan flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using Paint-a-Gate software (BD Biosciences, San Jose, CA). Isototypics controls were used to minimize the electronic noise. Using side scatter (SSC) and forward scatter (FSC), a gate was draw in lymphocytes population. Anti-CD3 and anti-CD56 mAb (present in all tubes) were used to define three cells populations: conventional T cells ($CD3^+CD56^-$), conventional NK cells ($CD3^-CD56^+$), and NKT cells ($CD3^+CD56^+$). NK cells were further divided based in CD56 expression in two subsets: $CD56^{dim}$ and $CD56^{bright}$. Receptor expression (KIR, NCR and C-type lectins) was assessed separately on each of the five populations. Gates strategies were designed based on Lutz et al. [19] and Hayhoe et al. [22], as exemplified in Fig. 1.

2.4. NK cytolytic activity assay

NK cytolytic assay was adapted from the described by Cholujová et al. [23]. Briefly, peripheral blood mononuclear cells were isolated by density gradient (Histopaque-1077, Sigma, St. Louis, MO). PBMNCs were frozen in fetal calf serum (Gibco) with 10% DMSO. Thawed PBMNCs were washed once with complete medium (RPMI 1640 cell supplemented with 10% FCS and 2 mmol/l glutamine) and added in different concentrations to target cells: effector/target (E/T) cells ratios of 25/1, 12.5/1, 6.25/1 were used. Target K562 cells were maintained in complete medium. Target cells were loaded in serum-free medium with 1 μmol/l CFSE (green fluorescent dye) and further incubated for 15 minutes at 37°C in the dark. The CFSE staining was stopped by addition of an

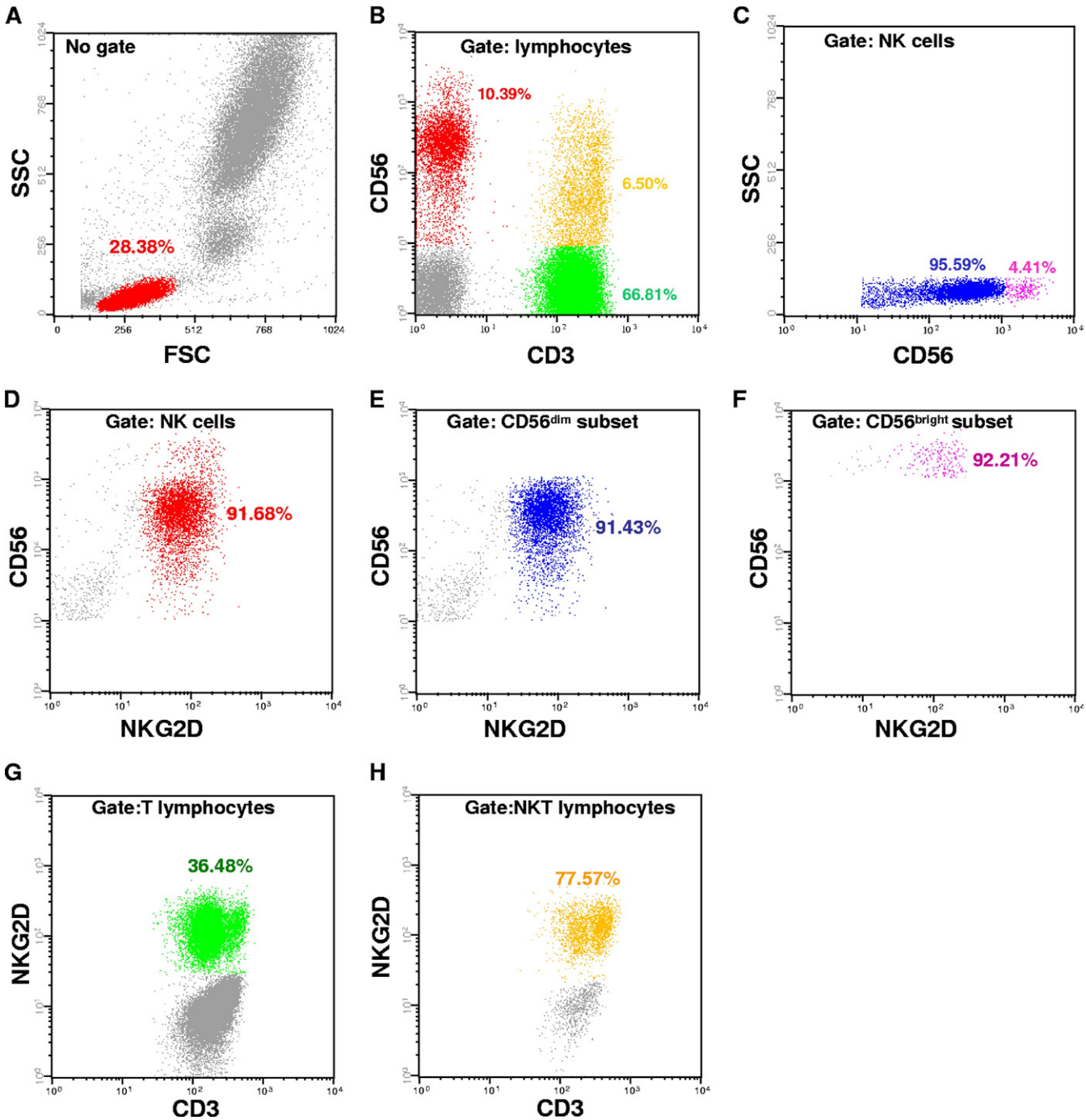

Fig. 1. Gate strategy and representative example of flow cytometric analysis of a healthy donor (male, age 25 years). Gate strategy (A–C) consisted of gating lymphocyte population based on FCS and SSC (A) and identification of cells populations of interest in lymphocyte gate based on CD3 and CD56 expression (B): red, NK cells ($CD3^-CD56^+$); green, classical T cells ($CD3^+CD56^-$); and yellow, NKT cells ($CD3^+CD56^+$). Identification of NK-cell subpopulations based on CD56 expression (C): Low density of CD56 characterizes the $CD56^{dim}$ subset (blue), whereas high density of this molecule identifies the $CD56^{bright}$ subset (pink). Frequency of each cell population is represented beside each population. Receptor expression was evaluated in each of these five cells populations (D–H), as exemplified by NKG2D expression in NK cells (D), $CD56^{dim}$ subset (E), $CD56^{bright}$ subset (F), T cells (G), and NKT cells (H). Frequency of $NKG2D^+$ cells in each cell population is represented inside each graphic.

equal volume of 10% FCS RPMI medium. Duplicates of effector and CFSE-stained target cells were coincubated for 4 hours at 37°C. Then, the samples were transferred into cytometric tubes and 7AAD was added to mark dead cells. Samples were acquired using a FACScan flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using Cell Quest software (BD Biosciences, San Jose, CA). The percentage specific lysis was calculated at each E/T ratio as follows: % specificlysis = (CT–TE/CT) × 100 (where CT is the percentage of viable CFSE target cells in control tubes and TE is the percentage of viable CFSE target cells in test tubes).

2.5. DNA extraction

Genomic DNA was isolated from 150 μl of buffy coat obtained from EDTA-treated peripheral blood using the PureLink Genomic DNA Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The DNA concentration was determined in Qubit fluorometer (Life Technologies, Carlsbad, CA).

2.6. KIR and HLA genotyping

The reverse sequence-specific oligonucleotide technique (One Lambda, Canoga Park, CA) with Luminex xMAP technology (Luminex Corp., Austin, Tx) was used for the typing of 14 KIR genes and two pseudosgenes: KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1, KIR3DL2, KIR3DL3, KIR3DS1, KIR2DP1, and KIR3DP1. This technique was also performed for HLA Class I and II alleles: HLA-A, HLA-B, and HLA-Cw according to the manufacturer's instructions. Additional tests for KIR genotyping was performed using commercial kits of sequence-specific primer technique KIR Genotyping SSP Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The individual KIR gene frequencies were estimated according to the following formula: $1 - \sqrt{(1-f)}$ [24].

2.7. Statistical analysis

Descriptive data are presented as medians and box plots. The evaluation of the influence of age and gender on the studied parameters was performed using the Mann–Whitney nonparametrical test. The χ^2 or Fisher exact probability tests were performed to assess the statistical significance of frequency differences of KIR and HLA genotype observed between the age groups. Statistical analysis was performed using SPSS version 18.0 software.

3. Results

3.1. Age-related changes in T-, NKT-, and NK-cell populations

Using flow cytometry, we defined the populations of interest based on CD3 and CD56 expression (Fig. 1). The influence of aging and gender on T-, NKT-, and NK-cell populations was analyzed. The percentage of classical T-cell population ($p = 0.035$) was reduced in the elderly group, but the percentage of CD4 and CD8 subpopulations were not affected by age (Table 1). No differences were observed in the percentage of NKT-cell population among different age groups (Table 1). The percentage of NK-cell population increased in the elderly group ($p < 0.001$), but no differences were observed in this population between children and adults (Table 1). The decreased frequency of T cells and the increased frequency of NK cells in the elderly subjects confirms previous findings and serves to validate the current study population [25]. The white blood cells (WBC) count was increased in children ($p = 0.009$) and elderly individuals ($p = 0.009$) as observed in Table 1. The increase of WBC count leaded to an augment of absolute number in children of T- ($p = 0.001$), CD4 T- ($p = 0.007$), CD8 T- ($p = 0.001$), and NK-cells ($p = 0.028$) populations, even though no statistic difference was observed in the percentage of these populations in children (Table 1). Regarding the elderly group, the absolute number of NK-cells was increased ($p < 0.001$), as observed in Table 1.

3.2. Aging is associated with changes in both NK-cell subpopulations

The relative expression of CD56 defines two phenotypically and functionally different subpopulations of NK cells: cytotoxic $CD56^{dim}$ cells, which express low surface levels of CD56, and cytokine-producing $CD56^{bright}$ cells, which express high surface levels of CD56. A representative FACS plot showing the identification of NK cells subsets is provided in Fig. 1C. We examined these NK-cell subpopulations during human senescence (Table 1) and found a slight but significant ($p = 0.001$) increase in the percentage of $CD56^{dim}$ NK cells in elderly subjects compared with young adults. Conversely, a significant decrease ($p = 0.001$) in the frequency of $CD56^{bright}$ NK cells was observed in elderly subjects. No gender-related changes were observed in NK-cell subsets (data not shown). The frequencies of the two NK-cell subpopulations in children were similar to those in adults (Table 1). Regarding absolute numbers, an increase in $CD56^{dim}$ subset was observed in elderly ($p < 0.001$) and also in children ($p = 0.049$).

3.3. NK activity is preserved from childhood through old age

We assessed a measure of functional ability of NK cell at different age groups performing flow cytometer analysis of cytolytic activity (Fig. 2). There was no significant difference in the percentage of lysis between children (median of 75.07% at 25/1 E/T) adults (median of 70.51%), and elderly persons (median of 65.35%).

3.4. Aging affects the expression of KIRs on $CD56^{bright}$ cells but not on the other populations analyzed

KIRs can be stimulatory or inhibitory and are expressed in a stochastic fashion on NK cells. Each anti-KIR monoclonal antibody recognizes more than one receptor because activating and inhibitory KIRs that recognize the same HLA molecule have identical extracellular segments. To approximate overall KIR expression on conventional NK cells, we used a mixture of three anti-KIR monoclonal antibodies that were labeled with the same fluorescent tag. This mixture recognizes six different KIRs and is referred to in the figures as KIRmix. KIR expression was investigated in T, NKT, NK cells, and NK-cell subpopulations. Very few conventional T cells

Table 1
Frequency of conventional T, NKT, NK, $CD56^{dim}$ NK, and $CD56^{bright}$ NK cells in blood from subjects in different age groups

	Median of cell population				*p* Value (Mann–Whitney test)	
	All subjects	Children	Adult	Elderly	Children vs adults	Adults vs elderly
Percentage (%)						
T cells	66.79	70.75	66.81	64.54	0.100	**0.035**
CD4 T cells	43.33	41.80	44.71	45.80	0.262	0.865
CD8 T cells	31.05	31.94	31.05	29.45	0.592	0.815
NKT cells	5.88	5.93	5.90	5.67	0.425	0.533
NK cells	9.58	7.52	8.93	14.85	0.294	**<0.001**
$CD56^{dim}$ NK subset	95.34	94.36	94.52	97.45	0.579	**0.001**
$CD56^{bright}$ NK subset	4.66	5.64	5.48	2.59	0.579	**0.001**
Absolute numbers (cells/mm^3)						
WBC count	6400	7620	5500	7545	**0.009**	**0.009**
T cells	1152	1561	1048	1163	**0.001**	0.622
CD4 T cells	815	979	702	879	**0.007**	0.296
CD8 T cells	584	779	466	657	**0.001**	0.139
NKT cells	106	119	94	113	0.333	0.921
NK cells	192	218	141	293	**0.028**	**<0.001**
$CD56^{dim}$ NK subset	184	209	136	279	**0.049**	**<0.001**
$CD56^{bright}$ NK subset	8	9	7	9	0.579	0.375

Percentage values represent the frequency of T cells, CD4 T cells, CD8 T cells, NKT cells, and NK cells among all peripheral blood lymphocytes or the frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells among the NK-cell population. Significant *p* values are highlighted in boldface type.

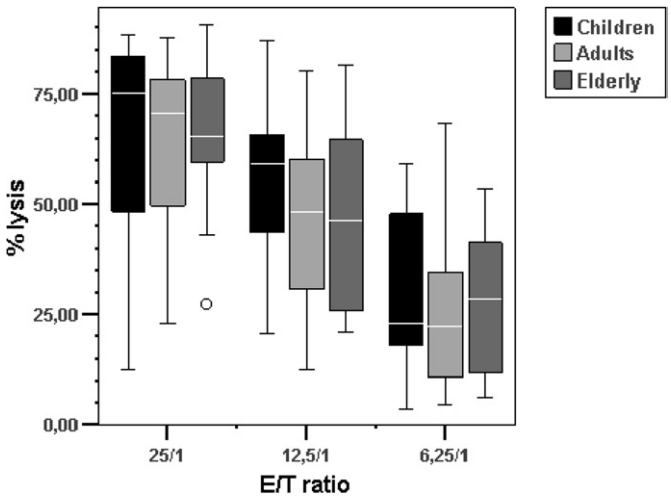

Fig. 2. Effect of age in NK cytolitic activity. Box plot shows percentage of lysis. There was no statistically significant difference among the three age groups.

expressed KIRs (<1.5%), and the effects of aging and gender were not examined in this population. A considerable proportion of the NKT-cell population expressed KIRs, but the frequency of NKT cells expressing KIRs was lower than that of NK cells. No age- or gender-related changes were observed in the overall expression pattern of KIRs or in the expression patterns of individual KIRs in NKT or NK cells (Table 2). We also analyzed the expression of these receptors in NK-cell subpopulations. KIRs were expressed by a greater proportion of $CD56^{dim}$ NK cells than $CD56^{bright}$ NK cells. Similar to the other cell populations examined, KIR expression was not altered with age or gender in $CD56^{dim}$ NK cells (Table 2 and data not shown). In contrast, we observed age-related changes in the expression of almost all KIRs evaluated in the $CD56^{bright}$ NK-cell subset (Fig. 3, Table 2). The frequencies of $CD56^{bright}$ NK cells expressing CD158a, CD158b, and KIRs stained with the anti-KIR antibody mixture were increased in children and elderly subjects compared with those in young adults. The frequency of $CD56^{bright}$ NK cells expressing CD158e ($p = 0.042$) was also increased in elderly subjects (Fig. 3, Table 2).

3.5. KIR and HLA genotype analysis

In the present study, diversity within KIR gene content was examined in 52 healthy individuals to determine its effect on aging. A high degree of variation was observed with a total of 17 different genotypes being detected (Table 3). Genotype 1 was the most frequent in children and adults, but was not present in the elderly group, whereas genotype 7 was the most frequent in elderly but was not found in any other group.

Table 2
Frequency of conventional T, NKT, NK, $CD56^{dim}$ NK, and $CD56^{bright}$ NK cells expressing various NK receptors in different age groups

	Median percentage of cell population expressing each receptor				*p* Value (Mann–Whitney test)	
	All subjects	Children	Adults	Elderly	Children vs adults	Adults vs elderly
T cells						
NKG2D	35.65	37.58	36.18	28.77	0.108	**0.034**
NKT cells						
CD158a	3.41	5.10	2.73	3.69	0.473	0.528
CD158b	12.03	9.78	12.18	12.62	0.621	1.000
CD158e	3.12	3.54	3.44	2.37	0.592	0.304
KIRmix	18.67	18.35	16.08	22.38	0.633	0.386
CD158i	5.24	3.86	7.55	5.18	0.117	0.865
CD94	28.13	28.13	31.48	15.90	0.538	**0.009**
NKG2D	86.47	88.28	85.06	91.10	0.327	0.533
NK cells						
CD158a	19.63	19.76	19.63	17.48	0.694	0.929
CD158b	26.42	25.55	26.42	31.46	0.880	0.698
CD158e	11.86	14.18	11.37	6.78	0.193	0.901
KIRmix	50.35	48.77	46.62	53.96	0.588	0.119
CD158i	7.73	4.08	14.54	5.79	0.107	0.704
NKp46	77.34	78.79	82.37	67.57	0.499	**0.003**
NKp30	46.44	36.85	60.96	33.24	**0.005**	**<0.001**
CD94	56.47	49.30	61.09	49.08	**0.028**	**0.014**
NKG2D	96.56	94.11	96.74	97.01	0.271	0.961
$CD56^{dim}$ subset						
CD158a	20.61	21.75	20.61	17.92	0.635	0.929
CD158b	28.30	25.74	28.30	32.64	0.912	0.777
CD158e	12.27	14.70	11.71	6.91	0.207	0.895
KIRmix	52.87	48.39	49.74	54.98	0.879	0.225
CD158i	7.27	3.60	14.56	4.88	0.106	0.633
NKp46	74.97	77.31	81.45	64.75	0.551	**0.003**
NKp30	44.47	33.70	59.24	31.67	**0.006**	**<0.001**
CD94	55.16	44.38	59.27	45.15	**0.012**	**0.016**
NKG2D	95.84	93.61	96.01	96.36	0.327	0.929
$CD56^{bright}$ subset						
CD158a	4.00	7.20	2.70	6.67	**0.007**	**0.011**
CD158b	5.71	9.12	4.38	9.78	**0.004**	**0.003**
CD158e	2.81	3.64	1.79	3.73	0.474	**0.042**
KIRmix	10.76	14.29	7.49	14.39	**0.018**	**0.001**
CD158i	5.33	5.00	4.76	10.16	0.391	0.059
NKp46	98.09	98.85	98.80	96.15	0.943	**0.034**
NKp30	65.30	45.00	91.94	48.73	**0.003**	**<0.001**
CD94	95.67	94.42	98.13	93.75	0.126	**0.009**
NKG2D	98.82	96.62	99.10	98.51	0.092	0.583

Values shown represent the median percentage of cells within each population that expressed a given NK receptor. Significant *p* values are highlighted in bold.

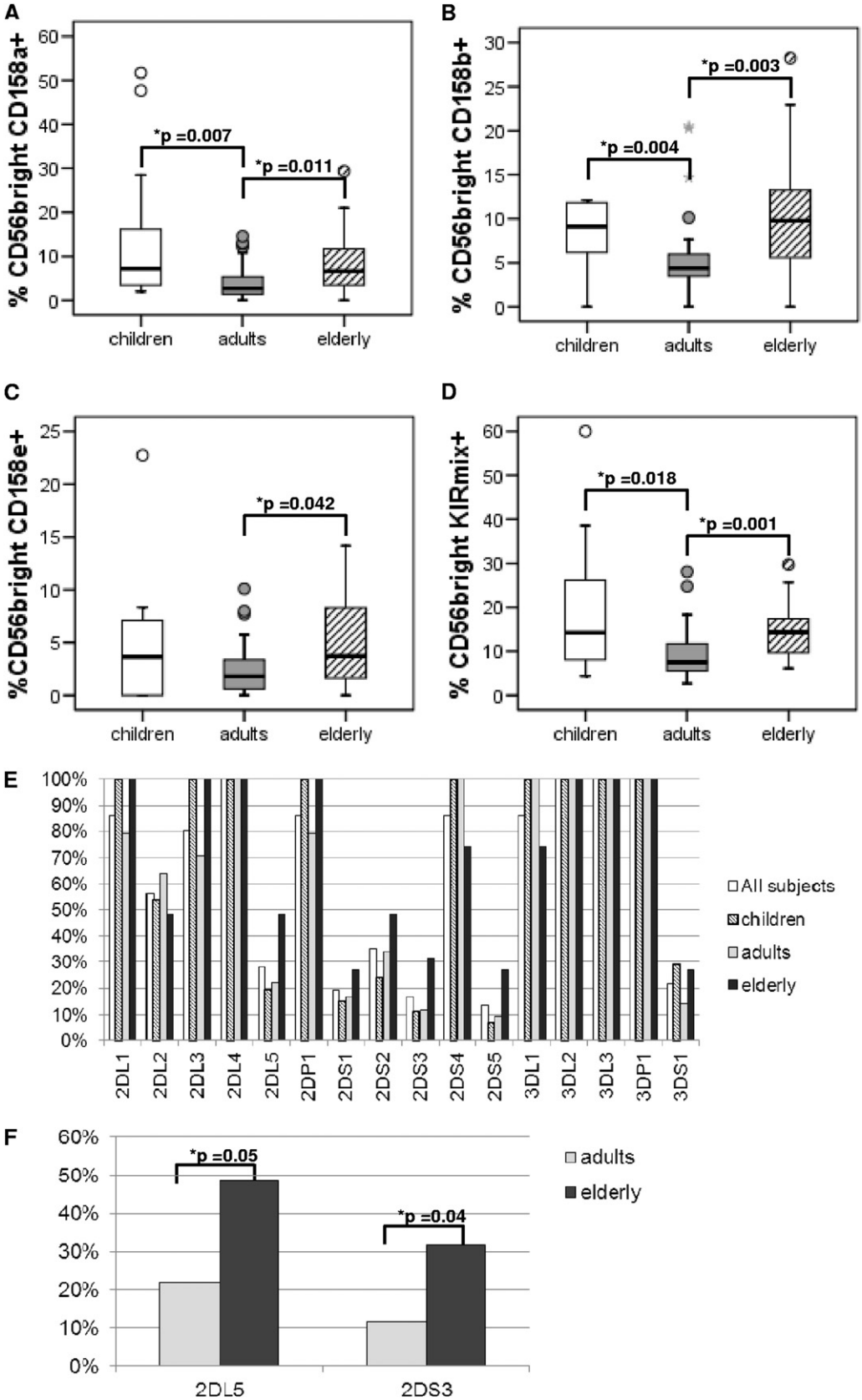

Fig. 3. Age-related changes in KIR expression in CD56^{bright} cells and KIR genotype. Box plots show frequency of cells expressing individual KIRs (A–C) and KIR mixture (D) in CD56^{bright} subset. (E) Individual frequency of KIR genes in all individuals and in the three age groups. (F) KIR2DL5 and KIR2DS3 are significantly more frequent in elderly individuals. *Statistically significant difference. Individual KIR gene frequencies were estimated according to the following formula: $1 - \sqrt{(1-f)}$.

Table 3
KIR gene content of the genotypes detected and KIR genotype frequency in different ages groups

Genotype no.	2DL1	2DL2	2DL3	2DL4	2DL5	2DP1	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DL3	3DP1	3DS1	Frequency (%)			
																	All subjects	Children	Adults	Elderly
1	+	+	+	+	−	+	−	−	−	+	−	+	+	+	+	−	10/52 (19.23)	3/14 (21.43)	7/23 (30.43)	0/15 (0.00)
2	+	+	+	+	−	+	−	+	−	+	−	+	+	+	+	−	9/52 (17.31)	2/14 (14.29)	5/23 (21.74)	2/15 (13.33)
3	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	5/52 (9.62)	2/14 (14.29)	2/23 (8.70)	1/15 (6.67)
4	+	−	+	+	−	+	−	−	−	+	−	+	+	+	+	−	5/52 (9.62)	2/14 (14.29)	1/23 (4.35)	2/15 (13.33)
5	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	4/52 (7.69)	2/14 (14.29)	1/23 (4.35)	1/15 (6.67)
6	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	−	3/52 (5.77)	0/14 (0.00)	1/23 (4.35)	2/15 (13.33)
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3/52 (5.77)	0/14 (0.00)	0/23 (0.00)	3/15 (20.00)
8	+	−	+	+	+	+	+	−	−	+	+	+	+	+	+	+	2/52 (3.85)	0/14 (0.00)	1/23 (4.35)	1/15 (6.67)
9	+	−	+	+	+	+	−	+	+	+	−	+	+	+	+	−	2/52 (3.85)	0/14 (0.00)	1/23 (4.35)	1/15 (6.67)
10	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	2/52 (3.85)	0/14 (0.00)	2/23 (8.70)	0/15 (0.00)
11	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	1/52 (1.92)	1/14 (7.14)	0/23 (0.00)	0/15 (0.00)
12	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	1/52 (1.92)	0/14 (0.00)	0/23 (0.00)	1/15 (6.67)
13	+	−	+	+	−	+	−	+	−	+	−	+	+	+	+	+	1/52 (1.92)	1/14 (7.14)	0/23 (0.00)	0/15 (0.00)
14	+	+	+	+	−	+	−	−	−	+	−	+	+	+	+	+	1/52 (1.92)	1/14 (7.14)	0/23 (0.00)	0/15 (0.00)
15	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	−	1/52 (1.92)	0/14 (0.00)	1/23 (4.35)	0/15 (0.00)
16	−	+	−	+	−	−	−	+	−	+	−	+	+	+	+	−	1/52 (1.92)	0/14 (0.00)	1/23 (4.35)	0/15 (0.00)
17	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	−	1/52 (1.92)	0/14 (0.00)	0/23 (0.00)	1/15 (6.67)

In agreement with previous studies [26], activating KIR were present at much lower frequency than genes encoding inhibitory receptors (Fig. 3E). Analysis of frequency or each KIR revealed that all KIR gene frequencies were comparable among the three age groups, with the exception of KIR2DL5 and KIR2DS3. The frequency of both of these KIR genes were significantly increased in the aged (KIR2DL5 48% and KIR2DS3 32%) compared with adults (KIR2DL5 22% and KIR2DS3 12%, $p = 0.05$), as observed in Fig. 3F. The frequency of known KIR ligands (HLA-C group 1, HLA-C group 2 and BW4) was also analyzed (data not shown), but no difference was observed between groups.

The antibodies used to analyze KIR expression do not distinguish activating and inhibitory KIR. Therefore, we used KIR genotype in an attempt to make this distinction by grouping samples according to described in Table 4. There was no significant difference between age groups (data not shown). Most cases presented only inhibitory KIR or both activating and inhibitory KIR. Only one individual presented activating KIR in absence of its counterpart inhibitory KIR.

3.6. NCRs NKp30 and NKp46 are affected differently by age

We also investigated the expression of three receptors belonging to the NCR family: NKp30, NKp44 and NKp46. None of these receptors were expressed at considerable levels in T cells or NKT cells. NKp44 is an activation marker and was not present in any healthy donor studied. We found a significant decrease in the frequency of NK cells expressing NKp30 ($p < 0.001$) and NKp46 ($p = 0.003$) in elderly subjects. Similarly, the frequency of NK cells expressing NKp30 was lower in children than in young adults ($p = 0.005$), but no significant difference was observed in the frequency of NK cells expressing NKp46 between these groups (Fig. 4A and B and Table 2). The expression of these receptors was also studied in $CD56^{dim}$ and $CD56^{bright}$ NK cells. Both NK-cell subsets expressed NKp30 and NKp46 and the frequency of cells expressing these receptors was decreased in both subpopulations in elderly subjects. In children, we found a decrease in the frequency of cells expressing NKp30 but not NKp46 in both NK-cell subsets (Fig. 4C–F and Table 2). NCR expression was not influenced by gender (data not shown).

3.7. Effect of age on receptors of the C-type lectin family

The CD94 molecule associates with several members of the NKG2 family; therefore, flow cytometric analysis of CD94 expression approximates the overall expression of these members of the C-type lectin family. CD94 was expressed by few conventional T cells (<3%). We observed a frequency of 28.13% of NKT cells and 56.47% of NK cells expressing CD94. A significant decrease in the frequency of cells expressing CD94 was observed in NKT cells ($p = 0.009$) and NK cells ($p = 0.014$) of elderly subjects. The frequency of cells expressing CD94 was decreased in NK cells ($p = 0.028$) but not in the NKT cells of children (Fig. 5A and B and Table 2). We also analyzed the expression of this molecule in NK-cell subpopulations. An old age-related decrease in the frequency of cells express-

Table 4
Subjects grouped according to relation between antibodies anti-KIR and KIR genotype

	Group	Frequency (%)			
		All subjects	Children	Adults	Elderly
CD158a (2DL2/2DS2)	1 $2DL2^-/2DS2^-$	1/52 (1.9)	0/14 (0.0)	1/23 (4.3)	0/15 (0.0)
	2 $2DL2^+/2DS2^-$	33/52 (63.5)	10/14 (71.4)	15/23 (65.2)	8/15 (53.3)
	3 $2DL2^-/2DS2^+$	0/52 (0.0)	0/14 (0.0)	0/23 (0.0)	0/15 (0.0)
	4 $2DL2^+/2DS2^+$	18/52 (34.6)	4/14 (28.6)	7/23 (30.4)	7/15 (46.7)
CD158b (2DL2/2DL3/2DS2)	1 $2DL2^-/2DL3^-/2DS2^-$	0/52 (0.0)	0/14 (0.0)	0/23 (0.0)	0/15 (0.0)
	2 $2DL2^+/2DL3^-/2DS2^-$, $2DL2^-/2DL3^+/2DS2^-$ or $2DL2^+/2DL3^+/2DS2^-$	22/52 (42.3)	8/14 (57.1)	10/23 (43.5)	4/15 (26.7)
	3 $2DL2^-/2DL3^-/2DS2^+$	0/52 (0.0)	0/14 (0.0)	0/23 (0.0)	0/15 (0.0)
	4 $2DL2^+/2DL3^-/2DS2^+$, $2DL2^-/2DL3^+/2DS2^+$ or $2DL2^+/2DL3^+/2DS2^+$	30/52 (57.7)	6/14 (42.9)	13/23 (56.5)	11/15 (73.3)
CD158e (3DL1/3DS1)	1 $3DL1^-/3DS1^-$	0/52 (0.0)	0/14 (0.0)	0/23 (0.0)	0/15 (0.0)
	2 $3DL1^+/3DS1^-$	32/52 (61.5)	7/14 (50.0)	17/23 (73.9)	8/15 (53.3)
	3 $3DL1^-/3DS1^+$	1/52 (1.9)	0/14 (0.0)	0/23 (0.0)	1/15 (6.7)
	4 $3DL1^+/3DS1^+$	19/52 (36.5)	7/14 (50.0)	6/23 (26.1)	6/15 (40.0)

Group 1: antibody cannot recognize any KIR of the set; group 2: antibody can only recognize inhibitory KIR of the set; group 3: antibody can only recognize activating KIR of the set; group 4: antibody can recognize both activating and inhibitory KIR of the set.

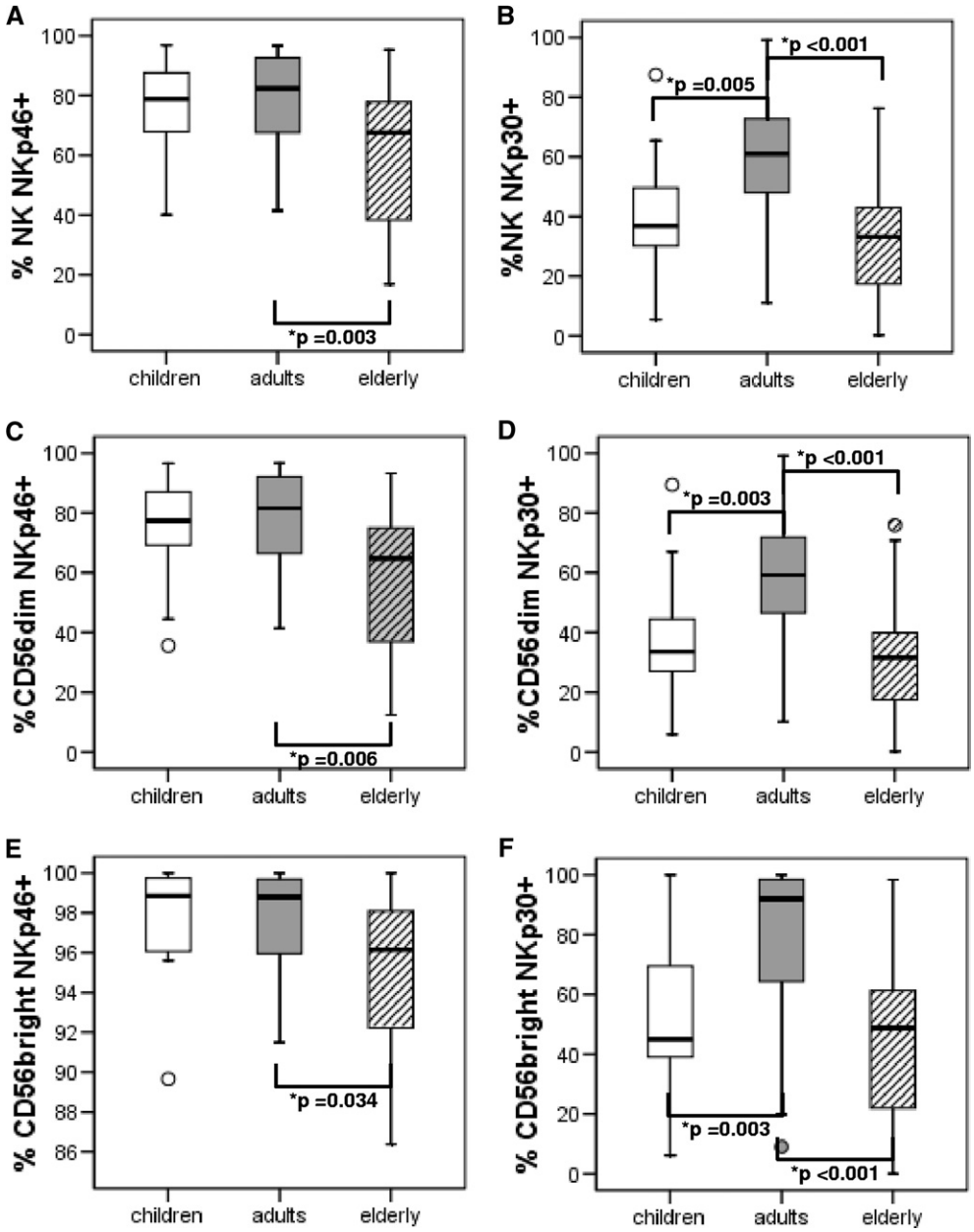

Fig. 4. Age-related changes in natural cytotoxicity receptors (NCR) expression. Box plots show the frequency of cells expressing NCRs among NK cells (A and B), $CD56^{dim}$ NK cells (C and D) and $CD56^{bright}$ NK cells (E and F). *Statistically significant difference.

ing CD94 was detected in both the cytotoxic $CD56^{dim}$ subset ($p = 0.016$) and the cytokine-producing $CD56^{bright}$ subset ($p = 0.009$). Similarly, we observed a reduction in the frequency of cells expressing CD94 in the $CD56^{dim}$ subpopulation ($p = 0.012$) but not in the $CD56^{bright}$ subpopulation in children (Fig. 5C and D and Table 2). In general, gender did not affect CD94 expression; however, a greater proportion of NKT cells expressed CD94 in males than in females ($p = 0.008$, data not shown).

The expression of NKG2D, an activating member of the C-type lectin family that does not interact with CD94, was also examined. All populations analyzed expressed this receptor, but the frequency of its expression differed among the cell populations. Approximately 36 percent of conventional T lymphocytes, 86% of NKT cells and 97% of NK cells expressed NKG2D. Aging reduced the frequency of expression of this molecule in T cells ($p = 0.034$, Fig. 5F) but not in NKT cells, NK cells or NK-cell subsets (Table 2). No differences were found in the frequency of cells expressing NKG2D between children and young adults in any of the populations analyzed. Males presented an increased frequency of T cells expressing NKG2D ($p = 0.012$, data not shown).

4. Discussion

The study of immunosenescence is crucial to understanding the mechanisms underlying the development of age-related diseases. Conversely, during childhood, the adaptive immune system is not yet mature, and the innate immune system is thus more important. Because specific diseases are associated with each phase of life, it is important to study the influence of aging on immunity. In this study, we analyzed the effect of aging on T, NKT, and NK cells with a focus on the expression patterns of the NK-related receptors responsible for the recognition of target cells.

Increases in the expression of NK-related receptors, such as CD16, CD56, CD57, and CD94, on the T cells of elderly persons have been reported [25,27,28]. Abedin et al. [29] proposed that this is a

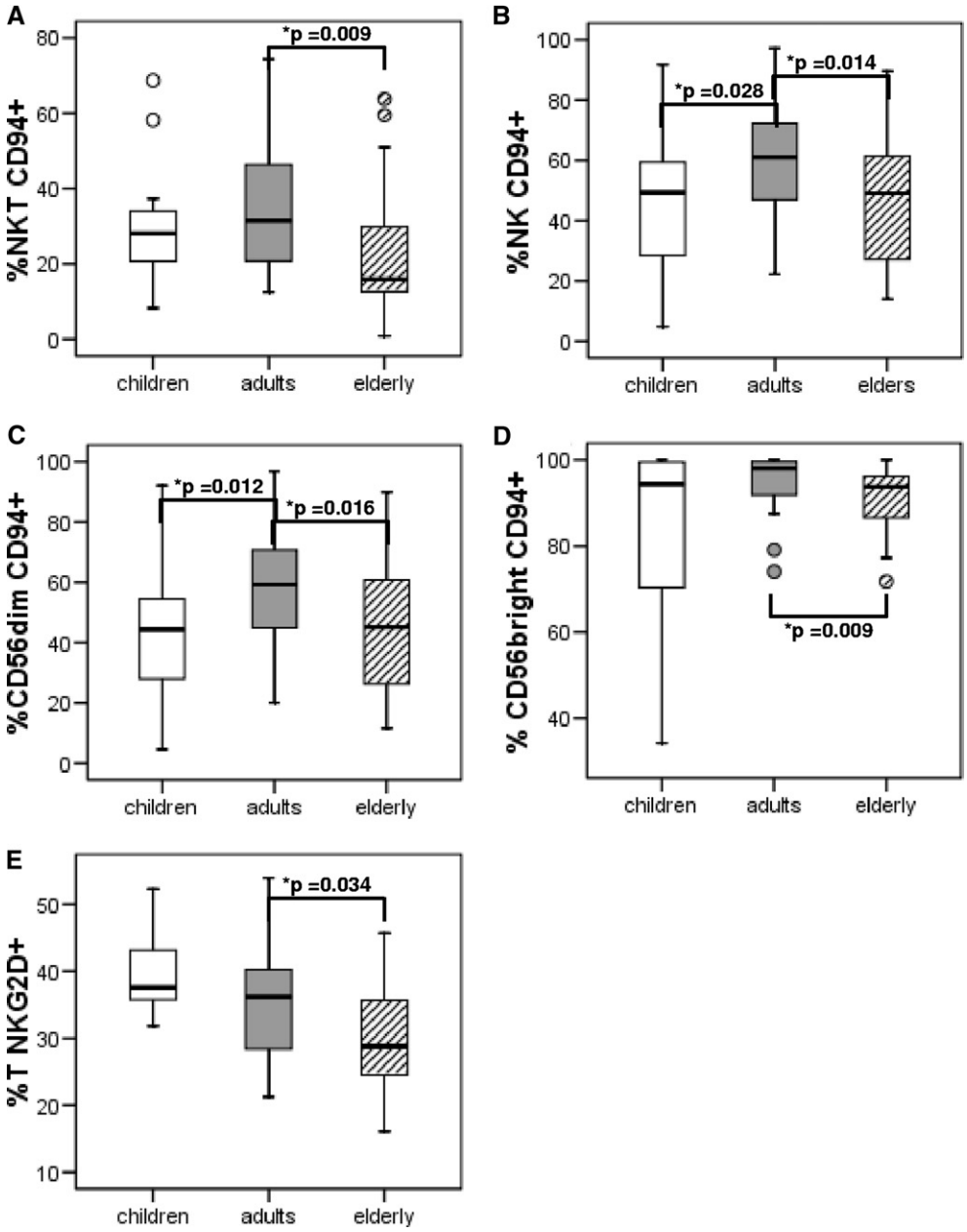

Fig. 5. Age-related changes in C-type lectin family. Box plots show the frequency of cells expressing CD94 among NKT cells (A), NK cells (B), $CD56^{dim}$ NK cells (C), and $CD56^{bright}$ NK cells (D). (E) Frequency of T cells expressing NKG2D. *Statistically significant difference.

compensatory adaptation allowing for the maintenance of immune competence despite the overall decrease in T-cell receptor diversity during aging. We report for the first time an age-related decrease in NKG2D expression in conventional T cells. It has been suggested that NKG2D acts as a costimulatory receptor in T cells, enhancing TCR-dependent responses [30]. The reduction in NKG2D observed in T cells of elderly subjects may thus contribute to the impairments in function that occur in these cells during aging.

NKT cells represent a heterogeneous group of lymphocytes that nearly always have an invariant Vα14-Jα18 rearrangement and reactivity to the glycosphingolipid α-galactosylceramide (αGalCer) when presented by the class I–like molecule CD1d. NKT cells may express T-cell markers such as CD3, CD4, CD8, and TCR as well as NK-cell markers such as CD56, CD16, CD69, CD94 and KIR [31–35]. Similar to previous reports, we found that neither age nor gender significantly affected NKT-cell numbers [36,37]. Although the thymus is the main site of NKT cell maturation, these cells may directly migrate from the bone marrow to extrathymic sites (liver) for their development. Thus, the extrathymic development of NKT cells becomes more prominent to compensate for thymic failure during aging [2]. In accordance with the findings of Lutz et al. [19] and in contrast to those of Borrego et al. [27] and Hayhoe et al. [22], we found a significant decrease in CD94 expression in NKT cells of elderly subjects and a gender bias (males expressed more CD94 than females).

Similar to previous reports, we found an age-related increase in NK cells [19,27,38,39]. Furthermore, the increase in the frequency of NK cells observed in aged subjects was mainly due to the expansion of the $CD56^{dim}$ subset and reduction of $CD56^{bright}$ subset, as supported by previous studies [20,27,40,41]. Human $CD56^{bright}$ NK cells have a unique functional role in the innate immune response as the primary source of NK cell-derived immunoregulatory cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor–β (TNF-β), granulocyte macrophage-colony-stimulating factor, interleukin-10

(IL-10) and IL-13 [5,6]. Overall cytokine production has been reported to be altered in the elderly. The production of IL-2, IFN-α and IFN-γ has been shown to be decreased in the elderly, while IL-4 and IL-10 are produced in greater amounts in elderly subjects. Furthermore, leukocytes of elderly persons produce higher amounts of IL-1, IL-6, IL-8, and TNF-α after induction with lipopolysaccharide [42]. Previous reports have demonstrated age-related changes in cytokine production by NK cells; for example, the activated NK cells of elderly subjects secreted reduced levels of IFN-γ [43].

NK-cell functions, such as cytokine production and natural cytotoxicity, are controlled by the surface receptors responsible for target-cell recognition. Therefore, age-related changes in NK-cell functions may be due to alterations in NK receptors. Until now, few studies have addressed this subject [10,19,20] and none studied the influence of age in receptor expression in $CD56^{dim}$ and $CD56^{bright}$ subsets.

In contrast to Lutz et al. [19] and similar to Garff-Tavenier [20], we found no age-related changes in overall KIR expression on NK cells. However an increase in KIR expression was observed in the $CD56^{bright}$ subset of elderly subjects. Surprisingly, we found similar changes in childhood. Some reports suggested that $CD56^{bright}$ cells may represent activated $CD56^{dim}$ cells [44,45]. More recently, was demonstrated that $CD56^{bright}$ are immature NK cells and can be differentiated in $CD56^{dim}$, both *in vitro* and *in vivo*. This process may occur at sites of inflammation on contact with fibroblasts or upon cytokine activation [46,47]. More importantly, it was shown that KIR expression can be induced in $CD56^{bright}$ cells upon cytokine activation [47] and the cytokine milieu of elderly is altered. This could explain the increase of KIR expression in $CD56^{bright}$ subset observed in the present study.

It has been suggested that phenotyping of KIR must be carried out in parallel with genetic characterization for meaningful interpretations of the data [48]. Grouping KIR genotype according to anti-KIR antibody recognition, we observed that most individuals presented only the inhibitory KIR or both inhibitory and activating KIR counterparts. Therefore, the increase in KIR expression observed in $CD56^{bright}$ cells observed in children and elderly is probably related to inhibitory KIR with some lesser contribution from activating KIR. As far as we know, this is the first time that KIR genotype and KIR expression have been evaluated together in healthy aging individuals. More focused studies are necessary to better understand this subject.

Interestingly, KIR2DS3 and KIR2DL5 genes were significantly more frequently in elderly subjects. Maxwell et al. [26] also observed association of KIR2DS3 and KIR2DL5 with older age in their first cohort; however, when they analyzed a second cohort the significance was lost. Ligand specificities of activating KIR2DS3 remain mysterious. Although the ligand of KIR2DL5 is still undetermined, KIR2DL5 is considered one of the functional inhibitory receptors [49,50]. So far, the expression of these two receptors was not examined in older individuals.

We also analyzed the expression of NCRs in NK cells. NKp46 has been previously reported to trigger NK cytotoxicity, Ca^{2+} mobilization, and cytokine production. The second member of the NCR family, NKp30, shares several common features with NKp46 [51]. Garff-Tavernier et al. [20] reported no effect of age in NKp46 and NKp30 expression. Our findings provide the first evidence of a decline of NKp46 and NKp30 expression in NK cells and NK-cell subsets in the elderly. Interesting, we observed that NKp30, but not NKp46, was also reduced in children.

NKp30 appears to play a central role in the interaction of NK cells with dendritic cells. This crosstalk between NK cells and dendritic cells contributes to the coordination of innate and adaptive immune responses [52–54]. Therefore, the age-related decline in NKp30 expression in NK cells may be related to changes in other immune cells. Aging also affects many aspects of lymphocyte [55] and dendritic cell function [56].

Regarding C-type lectins family, as previously described [19,22], we found a reduction in the frequency of NK cells expressing CD94 in elderly subjects. Regarding NK-cell subsets, Borrego et al. [27] found no age-related changes in CD94, whereas we and Hayhoe et al. [22] found that the frequency of cells expressing CD94 decreased with age in both the $CD56^{dim}$ and $CD56^{bright}$ subsets. During childhood, we found a decrease in the frequency of cells expressing CD94 among the cytotoxic $CD56^{dim}$ subset but not among the cytokine-producing $CD56^{bright}$ subset.

We also studied NKG2D, an activating member of the C-type lectin family that recognizes MICA/B. According to previously reported [20], our results demonstrated that NKG2D expression is preserved in old age. Importantly, because NKG2D signals through a pathway that is distinct from those of the activating KIR and C-type lectin NK receptors, NKG2D-mediated cell activation is likely less susceptible to inhibitory receptor signaling [7]. We thus suggest that sustained NKG2D expression is important for the maintenance of NK-cell function. In fact, we observed that NK cytotoxic activity was preserved in childhood and old age. The preservation of this NK function contributes to the prevention of some age-related diseases and to healthy aging [2,18]. NK cell cytotoxicity may be preserved in healthy elderly subjects because of the ability of increased numbers of NK cells to compensate for potential decrements in NK cell cytotoxicity on a per-cell basis [2]. Our finding that the $CD56^{dim}$ subset increases with age, supports this hypothesis.

In conclusion, we have demonstrated several age-related changes in the expression of NK-related receptors in different cell populations. Interestingly, we showed that NK cells and $CD56^{dim}$- and $CD56^{bright}$-subsets from children and elderly display specific features regarding receptor expression. Cytotoxic activity was preserved from childhood through old age. We also observed some KIR genes significant associated with healthy elderly individuals. This initial study provides the framework for more focused studies of these subjects, which are necessary to determine whether the changing balance of NK receptor expression may influence susceptibility to infectious, inflammatory, and neoplastic diseases.

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