Natural killer cell alterations correlate with loss of renal function and dialysis duration in uraemic patients

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Abstract

Background. Natural killer (NK) cells provide a first line of immune defence towards infections and tumours, and participate in atherosclerosis and pregnancy diseases, of which there is a higher incidence in uraemic patients. Still, their relative contribution to the immunodeficient state associated with renal failure is poorly documented.

Methods. A multivariate and comparative analysis of lymphocyte subsets in haemodialysed (HD) and undialysed (UD) uraemic patients in comparison to healthy donors (HC) is provided in this article. NK-mediated cytotoxicity, degranulation and interferon secretion were compared in HD and HC.

Results. Evaluation of NK cells in 210 HD patients concluded with a decrease in NK cell counts in comparison to HC. Multivariate analysis associated lowered NK cell counts in UD patients with decreased renal clearance and higher NK counts HD with male gender and age. The 32% NK cell count decrease observed in sex- and age-matched groups (n = 88) was associated with B- and CD8+T-lymphocyte defects. NK cell functions were similar in subgroups of HD and HC matched for NK cell counts. Longer dialysis duration was associated with improved NK cytotoxic activity. While the expression of receptors modulating NK cytotoxicity were not modified, expression of the activation markers CD69 and NKP44, CD94 and chemokine receptors CX3CR1 and CXCR4 was altered in HD.

Conclusions. This study is the first to associate decrease in renal function with selective fading of NK cell number and identify haemodialysis duration as a factor influencing NK cell function. It further shows that lower cell counts rather than intrinsic NK cell dysfunction per se characterize immune disorders in HD.

Keywords: lymphocyte subsets; NK; renal clearance; cytotoxicity; end-stage renal disease; haemodialysis

Introduction

Reduced lymphocyte counts were identified as predictors of mortality in haemodialysis patients (HD) [1], who die mainly of cardiovascular diseases and infections. Furthermore, chronic renal failure (CRF) induces an immunodeficiency state and is associated with a 30% increase in malignant tumours [2,3], diminished serological responses to vaccination and anergy in cutaneous delayed hypersensitivity tests [4]. Young women with renal failure also exhibit a marked reduction in fertility and increased risk of pregnancy-associated disorders, including prematurity and intrauterine growth restriction [5]. Various lymphocyte subsets participate in the control of these clinical disorders. Among immune effector cells, NK cells participate in atherosclerosis [6] and represent a first line of defence of the immune system towards viral infections and tumours [7]. NK cells are also thought to play a role in autoimmune diseases, transplant rejection [8] and fetal implantation where they are essential to vascular trophoblast invasion [9]. Reported immunological alterations occurring in HD include defective antigen presentation and phagocytic potential of monocytes/macrophages, reduced B-lymphocyte antibody production and impaired T-cell number and functions. The immunodeficient state of renal failure patients has been related to both uraemic toxins and haemodialysis [10]. These studies, mainly conducted in the 1980–1990s, focused on T- and B-cell adaptive immunity, thus leading to an incomplete view of the status of lymphocyte subsets in HD, in particular for the natural killer cell (NK) compartment [11–13].
The NK cell compartment, identified by flow cytometry as cells expressing CD56 and CD16 cell surface receptors in the absence of the CD3 T-cell receptor complex (CD3-CD56+CD16+), represents about 10% of peripheral blood lymphocytes [14]. In contrast to adaptive responses, NK activation provides an immediate immune response to pathogen-induced changes. Through their activating and inhibitory receptors, NK cells sense cellular target ligands that modulate their potential to kill target cells [7,15]. Moreover, activated NK cells deliver cytokines/chemokines that are crucial in the stimulation of antigen presentation, and consequent modulation of adaptive T-cell-mediated immunity [16]. Only few studies analysed NK cells in HD, and none were conducted on undialysed patients in relation to renal function. These reports, conducted in small cohorts, did not associate functional and quantitative analysis, and led to conflicting reports with regard to the identification of NK alterations in uraemic patients [13,17–20]. This may be related to the technical evolution improving dialysis efficiency and biocompatibility, or to the introduction of recent therapeutic approaches such as statins [21], which motivated our evaluation of NK cells in HD.

The aim of the present study was thus to provide an extensive quantitative, functional and phenotypic multivariate analysis of NK parameters in relation to other lymphocyte subsets and clinical characteristics of end-stage renal diseased patients. A group of undialysed renal failure patients (UD) was also analysed to evaluate the impact of uraemia per se on NK cell counts.

Subjects and methods

Patients
This study was conducted in accordance with the Declaration of Helsinki and approved by the independent local ethic committee. Lymphocyte subsets from 210 HD evaluated for kidney transplantation were retrospectively analysed. Most blood samples were obtained at random time points between two haemodialysis sessions; none were collected on undialysed patients during or just after dialysis. From this cohort, 41 patients were evaluated for NK cell function. Phenotypic analysis of NK cells was also conducted in 25 patients. Clinical parameters including age, gender, body mass index, haemodialysis duration, CMV serology status, kidney disease, anterior kidney transplantation, antibody anti-HLA in panel-reactive antibody, statin treatment and diabetes were noted.

Lymphocyte subset counts were analysed in 39 UD patients followed for chronic renal failure in our nephrology unit. All patients included were undialysed and clinically stable. Clinical parameters including serum creatinine, serum urea, phosphorus, age, gender, weight, kidney disease, statin and diabetes were noted. The control group consisted of 88 unpaid adult volunteer healthy donors. None of the patients had infection, cancer or immunosuppressive therapy.

Phenotypic analysis of lymphocyte and NK subsets
Whole blood and lymphocyte counts were obtained from haematological laboratory reports (ADVIA haematology analyzer, Bayer). Distinct CD3+, CD3+CD4+, CD3+CD8+ and CD3+CD4+CD8– double negative (DN) T lymphocytes, CD19+ B lymphocytes and CD3–/CD56+/CD16+ NK subsets were characterized by flowcytometry as described [22].

Phenotypic analysis of NK cells was conducted within freshly Ficoll-Paque (Eurobio, Les Ulis, France) isolated peripheral blood mononuclear cells (PBMC). The percentage of CD3–/CD56+/CD16+ NK cells among total lymphocytes and the size of each NK cell subset defined by NK receptor (NKR) expression were determined by flow cytometry. NK cell subsets were defined as percentage of CD3–CD56+ NK cells expressing specific NKR. Mean fluorescence intensity (mfi) of NKR staining was further evaluated on NKR+ CD3–CD56+ NK cells. Antibodies used to define NK cell subsets were obtained from Beckman Coulter unless otherwise specified: FITC-conjugated anti-CD3 (IgG1, UCHT1); PC5-conjugated anti-CD56 (IgG1, N901); PE-conjugated; NKR antibodies: anti-CD158a/h (clone EB6, IgG1 recognizing KIR2DS1/2DL1), anti-CD158b1/b2/j (clone GL183, IgG1 recognizing KIR2DS2/2DL2/2DL3), anti-CD158e1/e2 (clone Z273.7.3, IgG1, recognizing KIR3DL1/KIR3DS1), anti-CD158i (clone FES172, IgG2a, recognizing KIR2DS4), anti-CD85j (LIR-1/ILT-2, clone HP-F1, IgG1), anti-CD94 (clone HP3B1, IgG2a), anti-CD159a (IgG2b, Z199) and anti-NKG2C (IgG1, 134591, R&D Systems); anti-CD25 (IgG2a, B.1.49.9), anti-CD69 (IgG1, FN50, BD-Pharmingen), NKP44 (clone Z231, IgG1), NKP30 (IgG1, Z25), anti-NKp46 (IgG1, BAB281), NKG2D (clone ON72, IgG1), CD161 (NKRP-1, clone 191B8, IgG2a), CD226/DNAM-1 (clone DX11, IgG1, BD Bioscience), CD244 (2B4, clone C1.7, IgG1), CX3CR1 (clone 2A9-1, IgG2b, MBL, Nagoya, Japan), anti-CXCR4 (clone 44717, IgG2b, R&D systems), anti-CCR5 (clone 45531.111, IgG2b, R&D systems), anti-CCR7 (clone 150503, IgG2a, R&D systems), CD62L (PC5, L-Selectine, clone DREG56, IgG1), CD122 (IL-2 βR, clone CF1, IgG1). At least 3000 events were analysed in the NK lymphocyte gate using the FC-500 flow cytometer (Beckman Coulter Corporation).

Assay of NK cell cytotoxic activity
Freshly Ficoll-Paque isolated PBMC were considered for assay when NK blood counts were >50 cells/mm3. NK cell cytotoxic activity against K562 target cells was analysed in triplicate, in a non-radioactive 2 h EUROPIUM release assay as described [22], at effector to target ratios ranging from 100:1 to 3:1.

Flow cytometer analysis of NK activation using the degranulation marker CD107a/LAMP-1 (lysosome-associated membrane protein 1)
CD107a expression was used as described [22] to measure NK cell degranulation induced by K562 stimulation.
PBMC were incubated for 4 h in the following culture mediums: RPMI medium, RPMI with Phorbol Myristate Acetate/Ionomycin (PMA/IONO: 10/500 ng/ml), RPMI with HLA class I-negative K562 target cells at an E:T ratio of 1:1. After adding CD107a-PC5 antibody (IgG1, H4A3) and Monensin (BD/Pharmingen), cells were stained with anti-CD3-FITC (IgG1, UCHT1), CD56-PC7 (IgG1, N901). Flow cytometry CD107a staining of CD3+ gated cells was used to follow NK cell degranulation within PBMC. Additional intracellular staining with interferon-γ-PE antibody was performed with the Intraprep kit as recommended by the manufacturer (Beckman-Coulter). All samples were analysed by means of four-colour flow cytometry and CXP software (Beckman-Coulter).

Statistical analysis

Lymphocyte subset counts were analysed in relation to clinical parameters in each group (Table 1). Statistical analysis was conducted using the statistic package for SPSS program (version 10.1). Association between continuous variables was analysed using Pearson or Spearman correlation tests. The median of lymphocyte subset counts in categorical groups was analysed using Student’s t-test, or Mann–Whitney U-test according to the distribution of dependent variables. Multiple linear regression was used to construct models associating lymphocyte subset counts with explanatory variables. Variables relevant to construct the model were selected using stepwise selection, adding variables when $P < 0.05$ and removing variables when $P > 0.1$ in steps until the optimum model is reached. The final model was given in the last step together with its goodness of fit, and significance of the association set as $P < 0.05$.

Results

Percentages and absolute numbers of lymphocyte subsets were retrospectively analysed in 210 HD patients and compared with values observed in 88 healthy controls (HC) and 39 chronic renal failure undialysed patients (UD) (Table 2). Absolute cell counts were retained as informative variables throughout the study. Since age and sex ratio differed between HD and HC, gender and age-matched subgroups were also considered for analysis.

Analysis of T and B lymphocyte subset alterations

White blood cell counts were similar in the three groups. Total lymphocyte counts in HD and UD patients were lower than in HC, a result confirmed by the analysis of gender- and age-matched groups (median, 25th to 75th percentile: HD 1732, 1443–2237 vs HC 2045, 1742–2725, $P < 0.001$).

<table>
<thead>
<tr>
<th>Blood cells/mm$^3$</th>
<th>Haemodialysis patients $n = 210$</th>
<th>Undialysed patients $n = 39$</th>
<th>Healthy controls $n = 88$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Median (25th to 75th percentile)</td>
<td>Median (25th to 75th percentile)</td>
<td>Median (25th to 75th percentile)</td>
</tr>
<tr>
<td>White blood cells</td>
<td>6905 (5640–8425)</td>
<td>7420 (5590–8160)</td>
<td>6660 (5425–8115)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1646 (1409–2167)</td>
<td>1835 (1431–2074)</td>
<td>2045 (1757–2510)</td>
</tr>
<tr>
<td>CD3+</td>
<td>1233 (984–1646)</td>
<td>1208 (1079–1484)</td>
<td>1500 (1232–1879)</td>
</tr>
<tr>
<td>CD3+ CD4+</td>
<td>745 (582–1037)</td>
<td>763 (602–1015)</td>
<td>899 (698–1112)</td>
</tr>
<tr>
<td>CD3+ CD8+</td>
<td>403 (283–589)</td>
<td>401 (328–598)</td>
<td>508 (415–667)</td>
</tr>
<tr>
<td>CD3+ CD56+ CD16+</td>
<td>176 (123–279)</td>
<td>206 (156–309)</td>
<td>226 (163–354)</td>
</tr>
<tr>
<td>CD3+ CD4+ CD8+ (DN)</td>
<td>44 (22–76)</td>
<td>23 (5–47)</td>
<td>65 (32–107)</td>
</tr>
<tr>
<td>CD19+</td>
<td>138 (85–214)</td>
<td>154 (100–197)</td>
<td>242 (193–307)</td>
</tr>
<tr>
<td>CD4+ / CD8+</td>
<td>1.88 (1.33–2.61)</td>
<td>1.91 (1.19–2.57)</td>
<td>1.69 (1.33–2.21)</td>
</tr>
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</table>

$P$ values: $P_{HD \text{ vs } HS} \text{ vs } P_{HD \text{ vs } UD} \text{ vs } P_{UD \text{ vs } HC}$.
correlated to B-cell counts in HD, none of the other factors influenced B-cell counts. In multivariate regression models adding other lymphocyte subsets as variables, correlation with age was maintained (\(P = 0.001\)) and CD3+CD4 (\(P < 0.0001\)), CD3+CD8 (\(P = 0.041\)) and NK cell counts (\(P = 0.012\)) were positively associated with B-cell counts. In UD and HC, the same multivariate analysis only retained CD3+CD4 cell number as positively linked to B-cell counts (\(P = 0.001\)).

CD3+ cell counts in HD and UD patients were lower than in HC (Table 2), even when HD and HC groups were age- and gender-matched (median, 25th to 75th percentile: HD 1324, 1083–1678 vs HC 1470, 1218–1887, \(P = 0.011\)). When included in a multivariate analysis, CD19+ (\(P = 0.004\)) and NK (\(P < 0.001\)) subsets were positively associated with CD3+ cell counts, while no link for other factors, notably dialysis duration and anterior graft was found.

CD3+CD4+ cell counts in HD and UD patients were lower than in HC (Table 2, Figure 1). Nevertheless, this difference was not confirmed as significant when HD were age- and gender-matched (median, 25th to 75th percentile: HD 852, 579–1041 vs HC 884, 694–1112). Although occurrence of anterior graft was inversely correlated to CD3+CD4+ by univariate analysis (Spearman \(r = -0.20, P = 0.03\)), multivariate regression model only retained CD3+CD8+ and CD19+ lymphocyte subsets (\(P < 0.001\)) as factors positively linked to CD3+CD4+ cell counts.

CD3+CD8+ cell counts in HD and UD patients were also lower than in HC (Table 2, Figure 1), even when HD and HC groups were age- and gender-matched (median, 25th to 75th percentile: HD 415, 337–591 vs HC 488, 412–667, \(P = 0.004\)). Using multivariate analysis, none of the retained factors was linked to CD3+CD8+ cell counts, except other lymphocyte subsets (CD4+, \(P < 0.001\), DN, \(P < 0.001\), CD19+, \(P = 0.04\)) that were positively associated with CD3+ cell counts.

CD3+CD4−CD8− double negative (DN) cell counts in HD and UD patients were lower than in HC (Table 2, Figure 1), but this difference did not reach significance in age- and gender-matched groups (median, 25th to 75th percentile: HD 47, 25–75 vs HC 61, 31–102). Moreover, the CD3+CD4−CD8− cell counts in UD patients were lower than in HD (\(P < 0.001\), Table 2, Figure 1). Using multivariate analysis, CD3+CD4−CD8− cell counts were inversely correlated to age in both HC (\(P < 0.001\)) and HD (\(P < 0.01\)) and positively correlated to diabetes (\(P = 0.015\)) and CD3+CD8+ cell number (\(P < 0.001\)) in HD. We further focus our study on NK subsets.

**Altered distribution of NK cell counts**

CD3−CD56+CD16+ cell counts in HD patients were lowered by 20% when compared with HC (Table 2, Figure 1), and the decrease reached 32% when groups were fully matched for age and gender (median, 25th to 75th percentile HD 157, 114–267 vs 231, 164–361 in controls, \(P = 0.0008\), Figure 2). Although not reaching significance, NK cell counts tended to be lower in UD (median, 25th to 75th percentile UD 200, 156–312), at an intermediate level between HD and HC (Table 2, Figures 1 and 2). Univariate analysis identified age, BMI and male gender as parameters that positively influenced NK cell counts in the HD group, but did not identify an impact of previous transplant, dialysis duration, original kidney disease or statin treatment. Multivariate analysis taking into account associated parameters retained a positive correlation of HD NK cell counts with age (\(P = 0.014\)), male gender (\(P = 0.05\)), CD19+ (\(P = 0.01\)) and CD3+CD4+ (\(P = 0.02\)). Multivariate analysis of the UD group shows that Cockcroft clearance was positively correlated to NK cell counts (Spearman \(r = 0.43, P = 0.009\), Figure 2), while no effect of age, gender or other lymphocyte subsets was noted.

**Analysis of NK cell function and phenotype in HD patients**

In addition to this quantitative alteration, we further evaluated if NK functional activity was altered per se in uraemic patients that did not exhibit a major NK cell count decrease. Functional analysis of NK cells was conducted in a subgroup of 41 HD and 31 HC with similar NK cell counts (median, 25th to 75th percentile: HD 191, 137–269 vs HC 196, 163–235, Table 3). NK function was evaluated using three assays: (i) potential of NK cells to lyse the reference K562 cell target, (ii) CD107a degranulation assay of NK cells before and after K562 stimulation and (iii) NK cell-mediated IFN-γ production. Using these assays, no significant alteration of cytotoxicity, granule exocytose pathways and NK-mediated IFN-γ production could be observed in HD (Table 3). Nevertheless, the NK cytotoxic potential toward K562 was positively correlated to dialysis duration, both at 100:1 and 50:1 effector (E) to target (T) cell ratios (E : T ratio 50 : 1 Spearman \(r = 0.44, P < 0.005\); E : T ratio 100 : 1, Spearman \(r = 0.47, P = 0.0022\)). Patients haemodialysed for >5 years exhibit higher cytotoxic potential than those undergoing dialysis for <5 years, (E : T ratio: 100 : 1, median, 25th to 75th percentile: 30.5, 17.6–84.2% vs 14.65, 5.6–23.1%, \(P = 0.03\)), while comparable NK cell counts were observed (median, 25th to 75th percentile: >5 years: 149, 125–219 vs <5 years: 193, 143–275 cells/mm³).

We then investigated if phenotypic alteration in the NK receptor repertoire could be identified in HD patients. We therefore compared the distribution of CD3−CD56+ NK subsets, defined by phenotypic cell surface expression of 23 NKR markers, in 25 HD and 16 HC (Table 4). No significant changes in the distribution of MHC class I receptors (CD158/KIRs, CD85j/ILT-2, CD94/NKG2A), NK activating receptors (CD337/NKp30, CD335/NKp46, CD314/NKG2D, CD226/DNAM1, CD161/NKRP-1, CD244/2B4) orCCR5, CCR7, CD62L and CD122 receptors were noted. Interestingly, the CD335/NKp44 and CD69 activation markers were more represented in HD than in HC (Table 4, Figure 3). Size of NK cell subsets expressing CD94, the subunit of the CD94/NKG2 heterodimeric receptor to HLA-E, was also enhanced (Table 4, Figure 3). NK cells expressing the CXCR4 5DF1-chemokine receptor were more represented in HD while those expressing the CX3CR1 fractalkine receptor were underrepresented in HD (Table 4, Figure 3).
Fig. 1. Analysis of lymphocyte subsets counts in haemodialysis patients and undialysed renal failure patients in reference to healthy controls. Absolute values of lymphocyte subsets (cells/mm^3) were evaluated by flowcytometry in healthy controls (HC), undialysed (UD) and haemodialysis (HD) patients. Results are expressed as median values and 25th to 75th percentile and P values of significant differences (P < 0.05) are indicated.

Discussion

We revisited the influence of chronic renal failure on quantitative, phenotypic and functional parameters that characterize the NK compartment in HD. A first issue of the study was to provide a quantitative picture of lymphocyte subset counts in HD and UD patients, using multivariate regression models identifying influence of parameters, such as age, gender, dialysis duration, renal function or uraemia as factors potentially influencing these cell counts. As previously reported [23], we confirm a significant lymphopenia in HD, which exhibits a 15% decrease in comparison to HC. A milder 10% decrease characterized lymphopenia in UD. B lymphocytes appeared as the most affected subset, and respectively exhibited a 39% and 36% decrease in HD and UD. This defect may reflect enhanced apoptosis of B lymphocytes reported in UD [23]. Analysis of NK relative to T-lymphocyte subset counts emphasizes a predominant effect of uraemia on the NK cell compartment, and no other lymphocyte subset correlated with renal creatinine clearance. T-cell deficiency was observed in HD, DN count exhibiting a 23% decrease and CD8^+ and CD4^+...
Natural killer cell alterations in uraemic patients

Fig. 2. Analysis of gender and clearance-related variations of NK cell counts. (A) Absolute values of NK cell counts (cells/mm³) were evaluated according to female or male gender in healthy controls (HC), undialysed (UD) and haemodialysis (HD) patients and compared in age- and gender-matched groups of HC and HD (HCm and HDm). Results are expressed as median values and 25th to 75th percentile and *P* values of significant differences (*P* < 0.05) are indicated. (B) NK cell counts were correlated to creatinine clearance (ml/min).

Table 3. NK cell function in hemodialysis patients in reference to healthy controls

<table>
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<th>Haemodialysis patients n = 41</th>
<th>Healthy controls n = 31</th>
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<tbody>
<tr>
<td>NK cell number, CD3⁻CD56⁺/mm³</td>
<td>191 (137–269)</td>
<td>196 (163–235)</td>
</tr>
<tr>
<td>Percentage of K₅₆₂ lytic (E:T ratio = 50)</td>
<td>14.7 (1.9–27.2)</td>
<td>15.39 (5.66–27.2)</td>
</tr>
<tr>
<td>Percentage of K₅₆₂ lytic (E:T ratio = 100)</td>
<td>18.7 (8.1–30.2)</td>
<td>19.5 (5.38–32.03)</td>
</tr>
<tr>
<td>Percentage of CD107a⁺ on CD3⁻CD56⁺ NK cells</td>
<td>13.68 (8.62–28.22)</td>
<td>16.13 (7.32–25.8)</td>
</tr>
<tr>
<td>Percentage IFN-γ⁺ on CD3⁻CD56⁺ NK cells</td>
<td>1.02 (0.14–1.6)</td>
<td>1 (0.26–1.97)</td>
</tr>
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</table>

Cytoxic activity against K₅₆₂ cell targets (effector and target cell ratio (E:T) of 100:1 and 50:1), degranulating potential (CD107a) and IFN-γ production upon K₅₆₂ stimulation were evaluated by flow cytometry after gating on CD3⁻CD56⁺ NK cells. Results were obtained in 41 HD and 31 HC with similar NK cell counts.

Cytotoxic activity against K₅₆₂ cell targets (effector and target cell ratio (E:T) of 100:1 and 50:1), degranulating potential (CD107a) and IFN-γ production upon K₅₆₂ stimulation were evaluated by flow cytometry after gating on CD3⁻CD56⁺ NK cells. Results were obtained in 41 HD and 31 HC with similar NK cell counts.

Altogether, our analysis of HD evidenced that parameters that were not considered in other studies such as age, gender, homeostatic associations between lymphocyte subset counts, occurrence of anterior graft and diabetes influence size of T-and B-lymphocyte subset in HD. When these parameters were taken into account, our data suggest that B, NK and CD8⁺ are the most representative quantitative lymphocyte subset defects in HD.

Considering the predominant decrease of NK cells observed in HD patients, our aim was to further evaluate the functional status of NK cells, within a representative cohort of HD under current dialysis practice. Age and severe associated diseases, that characterize most haemodialysed patients, are thought to affect immunity. To alleviate these biases, and focus our analysis, as much as possible, on NK cell immunity in relation to the renal defect, the cohort recruited was restricted to a group of HD patients awaiting a kidney graft. Furthermore, groups were matched for gender and multivariate analysis models were designed to identify whether age and other parameters influence lymphocyte counts in HD. The NK cell compartment exhibited a selective decrease of 32% in HD patients analysed in reference to an age- and gender-matched HC group. Although a 20–32% decrease in NK cell counts may per se appear of limited short-term clinical relevance, such a similar moderate lymphocyte decrease was correlated to greater risk of mortality and hospitalization in haemodialysis patients. Furthermore, this decrease in NK should have greater impact towards pathological challenge when associated with other immune disorders observed in uraemic patients. The clinical impact of lowered NK cell counts in patients that are treated by haemodialysis for many years thus needs to be evaluated in further long-term follow-up of patient evolution, in particular in older patients. Indeed, the only immune parameter correlated to history of severe infections or death due to infections in elderly subjects was shown to
be absolute NK cell cytotoxicity [25], supporting the view that NK cells may also be critical to control infections in uraemic patients that exhibit similar immune alterations.

To our knowledge, our study is the first to report a significant influence of age and gender on NK cell counts in HD. Higher representation of aged men in cohorts of HD patients may in part account for contradictory observations. Indeed, creatinine clearance was the only factor influencing NK cell counts in UD patients, even when other confounding factors were considered in multivariate analysis. This multivariate analysis of UD thus suggests that the uraemic status per se selectively affects NK cell counts and prompts further investigation of the impact of uraemic toxins on this compartment.

In addition to the quantitative aspects, functional activity of NK cells was either reported to be lowered nor unchanged in HD. The effect of uraemic serum on 12 samples of healthy donor NK cells suggested that uraemic factors might decrease NK cell cytotoxic activity [27]. Nevertheless, no study addressed the question of whether lowered NK cytotoxic activity was due to lowered cell counts or to intrinsic dysfunction of NK cells. To resolve this issue, we conducted a comparative study of NK functions in HD and HC after normalizing NK cell counts. Cytolytic activity toward K562, NK degranulating potential and NK cell-mediated interferon production resulting from K562-stimulation were observed to be comparable in HD and controls exhibiting similar NK cell counts. Thus, our different read outs are in accordance with a preserved intrinsic function of NK cells, identifying the NK defect in HD as quantitative rather than qualitative.

In contrast to NK cell counts, dialysis duration was correlated to enhanced NK cytotoxic activity. This is in line with reports of higher NK activity in patients haemodialysed for > 10 years in comparison to patients with < 3 years dialysis duration [28], but NK cell counts were not evaluated in this report. Altogether, long-term maintenance in haemodialysis seems to favour intrinsic activation of NK cell lytic potential. The basis of this NK cell activation in long-term haemodialysed patients remains unclear. It may be related to long-term exposure to dialysis. Alternatively, enhanced NK cytotoxicity observed in long-term HD patients may correspond to a higher occurrence of immunized patients (high PRA levels) in our cohort of patients awaiting kidney transplant, but our group was too small to test this hypothesis.

As statins were reported to decrease NK cytotoxic potential [21], both associated with atherosclerosis diseases, we investigated their potential influence on NK cells. In accordance with a report in transplanted patients, no correlation could be established between statin treatment and NK cell counts nor cytotoxic potential in the studied HD patients.

Our phenotypic analysis of the NK cell repertoire in HD evidenced an enhanced expression of NKP44 and CD69 NK activation markers. These results are in accordance with data reported for other lymphocyte subsets that are lowered in number but activated by haemodialysis. It remains to be established whether activation of cell function at the level of NK cells is related to the enhanced cell surface expression of CD69 and NKP44 activation markers. Indeed, although CD69 expression on activated NK cells was shown to be induced by various stimuli (including IL2, PMA, IFN-γ, anti-CD16 triggering), its impact on NK cell cytotoxic function was also shown to be dependent of interactions with other receptors regulating NK cell function. Previous
Fig. 3. Phenotypic distribution of NKR$^+$ NK cell subsets in haemodialysis patients. The percentage of cell surface staining with anti CD69, Nkp44, CD94, CXCR4 and CX3CR1 antibodies was evaluated by flow cytometry after gating on CD3$^-$ CD56$^+$ NK cells. Percentage of NKR$^+$ NK cells are expressed as median values and 25th to 75th percentile. $P$ values of significant differences ($P < 0.05$) observed between HC and HD are indicated.

### Table 5. Comparison of reported studies analysing NK cell counts and functions in haemodialysis patients

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<tbody>
<tr>
<td>Patients number (HD/HC)</td>
<td>20/26</td>
<td>129/71</td>
<td>35/15</td>
<td>34/45</td>
<td>210/88</td>
<td>41/31</td>
</tr>
<tr>
<td>$\Delta$ NK cell counts</td>
<td>$-60.6%$</td>
<td>$-31.5%$</td>
<td>$-22.6%$</td>
<td>$33.5%$</td>
<td>$-22.1%$</td>
<td>$-32%$</td>
</tr>
<tr>
<td>$\Delta$ % K562 lysis (E:T ratio = 25)</td>
<td>$-48.4%$</td>
<td>nd</td>
<td>$-32.7%$</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>$\Delta$ % K562 lysis (E:T ratio = 50)</td>
<td>$-59.3%$</td>
<td>nd</td>
<td>$-24.0%$</td>
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<tr>
<td>$\Delta$ % K562 lysis (E:T ratio = 100)</td>
<td>$-71.1%$</td>
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NK cells count and K562 lysis were expressed as percentage decrease ($\Delta$) of values observed in haemodialysis patients (HD) in reference to healthy controls (HC).

The study has suggested CD69-mediated NK cytotoxicity can be abrogated by CD94 stimulation, suggesting that CD94 regulates cytotoxic events initiated by stimulatory receptors [29]. As CD94 is also enhanced in HD, we can suggest that CD69 expression may represent a trace of NK cell activation in HD that, due to alterations of other markers such as CD94, may not result in enhanced NK cell function in HD patients [30]. Alternatively, an increased number of early activated CD69$^+$ T cells observed in chronic HD patients was associated with their increased susceptibility of T-cell apoptosis [31], a phenomenon that may also apply to CD69$^+$ NK cells.

In accordance with their preserved intrinsic cytolytic activity, expression of receptors controlling NK cell function (NCR, NKG2D, MHC class I NK cell receptors) was not significantly altered in HD. In contrast, significant modifications in the size of blood NK cells expressing CXCR4 and CX3CR1 chemokines receptors were observed in HD. Interaction of these receptors with their SDF1 and fractalkine ligands controls recruitment of NK cells in tissues [32].
addition to its role in leukocyte trafficking, fractalkine promotes NK-mediated IFN-γ production and enhances their cytotoxic potential towards endothelial or tumour cell targets [32]. Various studies suggested a role for CX3CR1–fractalkine interactions in directing mononuclear cell infiltration during renal inflammation [33]. Our observation that distribution of blood NK subsets expressing these chemokine receptors is altered in HD, suggests that tissue recruitment of blood NK cells by SDF1/CXCL12 and fractalkine may be affected by uremia.

In conclusion, our extensive analysis of NK cells in a large group of HD patients shows that lower NK cell counts, rather than NK cell dysfunction per se, characterize their alteration in haemodialysed patients. We also show that haemodialysis modulates phenotypic, cytotoxic and migratory features of NK cells. Furthermore, this study is the first to associate decrease in renal function with the fading of NK cell number. Considering the role of NK cells in the immune control of diseases that impair the uremic patient’s survival, our work prompts future studies evaluating the precise impact of uremic toxins and dialysis conditions in modulating NK cell activity and the associated consequences with regard to incidence of cardiovascular, cancer, infectious diseases and pregnancy disorders in uremic patients.

Conflict of interest statement. None declared.

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