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Oxidative Stress Mediates a Reduced Expression of the Activating Receptor NKG2D in NK Cells from End-Stage Renal Disease Patients

Marie-Noëlle Peraldi,1,2,3*‡‡ Jeannig Berrou,2,4*‡‡ Nicolas Dulphy,5,6*‡‡ Alexandre Seidowsky,* Philippe Haas,*‡‡ Nicolas Boissel,*‡‡‡ Fabien Metivier,† Christine Randoux,‖ Niloufar Kossari,‖ Alain Guérin,# Sylvie Geoffroy,** Guy Delavaud,†† Viviana Marin-Esteban,* Denis Glotz,*‡‡‡ Dominique Charron,*‡‡ and Antoine Toubert*‡‡§

To characterize the immune defect of patients with end-stage renal disease (ESRD), we performed NK cell subset analysis in 66 patients with ESRD treated by hemodialysis (n = 59) or peritoneal dialysis (n = 7). Compared with healthy blood donors, patients undergoing chronic dialysis showed a profound decrease in NKG2D+ cells within both the CD8+ T cell (58% vs 67%, p = 0.03) and NK cell (39% vs 56%, p = 0.002) populations. CD56dim cells, which comprise the majority of NK cells in the periphery, were more affected in this regard than were CD56bright cells. Uremic serum could decrease NKG2D expression on NK cells from healthy donors. Among factors that could contribute to the decrease in NKG2D expression in ESRD patients, reactive oxygen species (ROS) play a major role. We found that catalase could reverse the effects of uremic serum on NKG2D expression (p < 0.001) and that ROS down-regulated NKG2D at the mRNA level and at the NK cell surface. Additionally, ESRD patients had both increased membrane-bound MHC class I-related chain A (MICA) on monocytes (p = 0.04) and increased soluble MICA (203 pg/ml vs 110 pg/ml; p < 0.001). Both ROS and uremic serum could significantly increase in vitro the expression of the NKG2D ligand MICA on the renal epithelial cell line HK-2. Taken together, these studies suggest for the first time that both low NKG2D expression and up-regulation of its ligand MICA are related to ROS production and may be involved in the immune deficiency of ESRD patients.

Immune deficiency is one of the many consequences of chronic renal failure. Patients undergoing hemodialysis often suffer from recurrent bacterial infections, have a well-documented decreased response to B hepatitis vaccine, and have a higher incidence of cancer than does the general population (1). During the last 20 years, many studies have focused on the immune deficiency of patients with end-stage renal disease (ESRD)1 (2). Immunoincompetence is observed during the development of chronic renal failure and is even aggravated by dialysis (2, 3). In this regard, both cellular and humoral functions may be altered. Monocytes appear to be activated but have defects in opsonization (4). T lymphocytes show lower mitogen-induced proliferation, although they appear to have an activated Th1 phenotype, possibly due to increased serum IL-12 (5, 6). Despite this activated phenotype, the effector capacity of immune cells seems to be reduced. These abnormalities are not corrected by hemodialysis. Some of these defects are probably mediated, at least in part, by the so-called “uremic toxins” (7, 8), which include a wide variety of molecules such as β2-microglobulin and reactive oxygen species (ROS).

NK cells are known to play an important role in the defense against infections and tumors (9, 10). However, there is currently very little information about these cells in patients with ESRD. NK cell activity is regulated by inhibitory receptors, which bind class I MHC molecules, and activating receptors, which bind ligands present on tumors or virus-infected cells and that include NKp30, NKp44, NKp46, and NKG2D (11, 12). Of these, NKG2D is expressed on NK cells, CD8- TCRαβ T cells, and CD8+ TCRγδ T cells (13), and it is thought to play a role in tumor immune surveillance (14).

Several ligands for NKG2D have been described (15). One of these ligands, the MHC class I chain-related molecule A (MICA) (16), is expressed on the surface of endothelial cells (17), epithelial cells (18), and, to a lesser extent, blood monocytes (17). In these cells, MICA expression can be induced by different types of stress, such as genotoxic stress in tumors, infections (e.g., by cytomegalovirus, Mycobacterium tuberculosis, or Escherichia coli), heat shock, and oxidative stress (19–21). NKG2D mediates activating signals through the adaptor molecule DAP10 in


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4 Abbreviations used in this paper: ESRD, end-stage renal disease; ROS, reactive oxygen species; MICA, MHC class I-related chain A; sMICA, soluble MICA; HD, healthy donors; rMFI, relative mean fluorescence intensity; GO, glucose oxidase.

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The study was approved by the Institutional Review Board of the healthy donors (HD) were obtained at the Hôpital Saint-Louis Transfusion blood was also collected at the end of the session. Blood samples from hemodialysis circuit before the hemodialysis session, and for 20 patients Table I. For each patient, blood was collected via the arterial line of the hypertension, reflecting the two main causes of ESRD in Western countries. causes of renal failure in the patients were diabetic nephropathy and hy-

<table>
<thead>
<tr>
<th>ESRD Patients (n = 66)*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (years)</td>
<td>61.4 (31.5–95)</td>
</tr>
<tr>
<td>Sex Male (%)</td>
<td>58</td>
</tr>
<tr>
<td>Female (%)</td>
<td>42</td>
</tr>
<tr>
<td>Dialysis duration in years</td>
<td>4.4 (1.1–12)</td>
</tr>
<tr>
<td>Renal disease (%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>31.1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>27.9</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>18.5</td>
</tr>
<tr>
<td>Other</td>
<td>24.5</td>
</tr>
<tr>
<td>Dialysis technique (%)</td>
<td></td>
</tr>
<tr>
<td>Peritoneal dialysis</td>
<td>11</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>89</td>
</tr>
<tr>
<td>Blood cell count (10⁹/L)</td>
<td></td>
</tr>
<tr>
<td>White blood cell count</td>
<td>6.2 (2.9–12.4)</td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td>1.1 (0.7–1.8)</td>
</tr>
<tr>
<td>Platelet count</td>
<td>204 (125–410)</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>112 (99–139)</td>
</tr>
</tbody>
</table>

* Values expressed are medians. Values in parentheses are ranges.

humans (22). MICA can be cleaved from the membrane of tu-

ner cells by tumor-associated metalloproteinase (23). Soluble forms of MICA can cause the down-regulation of NKGD2 by promoting its internalization and degradation, leading to re-
duced immune responses against tumors (24). Chronic exposure of NK cells to up-regulated NKGD2 ligands may also trigger the down-regulation of NKGD2 (25).

The aim of the current study was to clarify the role of NKGD2 and its ligand, MICA, on NK and T cells in the immune deficit observed in patients with ESRD. We show herein that ESRD pa-
tients have a decreased frequency of NKGD2-expressing cells in the CD8⁺ T cell and NK cell subsets. The down-regulation of NKGD2 on NK cells was reversed by IL-15 or IL-2. Several fac-
tors in uremic serum may contribute to this down-regulation of NKGD2, including high circulating levels of soluble MICA (sMICA) molecules and ROS. These results reveal an unexpected effect of ESRD on NK cells, especially on the expression of the activating receptor NKGD2, which plays an important role in the control of immune responses.

Materials and Methods

Patients

Patients included in the study were on maintenance dialysis at five different dialysis centers. All patients had undergone standard chronic dialysis. In-

clusion criteria were as follows: age older than 18 years and on mainte-
nance dialysis for at least 1 year. Exclusion criteria included a history of malignancy, chronic or acute B or C hepatitis, or HIV infection and pre-

vious renal transplantation. The study included 66 patients with ESRD treated by hemodialysis (n = 59) or peritoneal dialysis (n = 5). The main causes of renal failure in the patients were diabetic nephropathy and hy-

pertension, reflecting the two main causes of ESRD in Western countries. Demographic data for the patients and their blood counts are shown in Table I. For each patient, blood was collected via the arterial line of the hemodialysis circuit before the hemodialysis session, and for 20 patients blood was also collected at the end of the session. Blood samples from healthy donors (HD) were obtained at the Hôpital Saint-Louis Transfusion Center. The study was approved by the Institutional Review Board of the Hôpital Saint-Louis, Paris, France, and all patients gave their informed consent.

Abs and FACS

Lymphocyte immunophenotyping was performed on frozen samples of PBMC's by four-color analysis using a flow cytometer (LSR) from BD Biosciences. Samples were fixed with PBS containing 2% paraformalde-
hyde. The following Abs were used for the analysis: FITC-conjugated anti-

CD16 and PE-conjugated anti-NKp30, anti-NKp44, anti-NKp46, and anti-

NKGD2 from Beckman Coulter, and PerCP-conjugated anti-CD3 and allophycocyanin-conjugated anti-CD56 from BD Biosciences. All flow cy-

tometry analyses were performed using a combination of CD3, CD16, and CD56 Abs along with a fourth Ab to specifically gate either CD3⁺ T lymphocytes or the CD3⁺ CD56dimCD1610⁻ NK cells and CD3⁺ CD56dim CD1610⁺ NK populations. The frequency of positive cells and the level of expression of the fourth marker in each subset were evaluated using the CellQuest software (BD Biosciences). Appropriate isotype controls were conducted on each sample. A PE-conjugated monoclonal anti-MICA Ab (BD Biosciences) was used to analyze MICA expression on monocytes and on a HK-2 cell line (26). The expression was defined by the relative mean fluorescence intensity (mFI), where MICA mFI = MFI MICA/MIF iso-
type control. To monitor the induction of cell death, NK and HK-2 cells were stained with FITC-conjugated annexin V (BD Biosciences) according to the manufacturer's instructions.

PBMC, monocytes, and isolation of NK cells

PBMCs from dialysis patients and HD were isolated from 15 ml of total fresh blood by density gradient centrifugation using lymphocyte separation medium (Eurobio) and stored in liquid nitrogen before phenotyping and functional analysis. Monocytes were isolated from PBMCs using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instruc-
tions. NK cells were then isolated from the CD14⁺ fraction using a NK cell isolation kit II (Miltenyi Biotec) according to the manufacturer’s instructions.

NK cell culture

Freshly isolated purified monocytes and NK cells were cultured in RPMI 1640 medium containing 10% FBS. Where indicated, cells were cultured in the presence of various concentrations of cytokines, Glucose oxidase (GO) (Sigma-Aldrich), recombinant soluble MICA (ProSpec), and serum from HD or ESRD patients for 48 or 72 h. Cells were harvested and stained with appropriate Abs for FACS. For experiments in which NK cells were treated with GO, purified normal NK cells were cultured for 72 h in RPMI 1640 medium containing 10% FBS with 50 or 100 ng/ml GO, which continu-
ously converts glucose into H2O2 in the culture medium. For experiments in which NK cells were treated with catalase, 15 min before the addition of serum from HD or from ESRD patients after dialysis, purified normal NK cells were incubated with 2000 U/ml catalase, which converts H2O2 to H2O and O2⁻. The K562 cell line, used as a target in the cytotoxicity assay, was maintained in RPMI 1640 medium containing 10% FBS.

Renal proximal tubular epithelial cells (HK-2) culture

The human kidney-derived HK-2 cell line (transduced with HPV-16) was kindly provided by Dr. L. Baud (Institut National de la Sante´ et de la Recherche Medica`le, Unité 702, Tenon Hospital, Paris). HK-2 cells retain functional characteristics of proximal tubular epithelium such as Na⁺−de-
pendent/phlorizin-sensitive sugar transport and adenylate cyclase respon-
siveness to parathyroid. HK-2 cells are anchorage-dependent, and cell growth is dependent on epidermal growth factor. HK-2 cells can reproduce experimental results obtained with freshly isolated proximal tubule cells (26). The cells were subcultured at 80% confluence in 75-cm² flasks using keratinocyte serum-free medium (Invitrogen) supplemented with 5 ng/ml recombinant epidermal growth factor and 0.04 mg/ml bovine pituitary ex-
tract. HK-2 cells were used at ~80% confluence incubated 8 or 24 h at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% O2.

NK cytotoxicity assay

Effector NK cells were incubated for 48 h at 37°C in RPMI 1640 medium with 10% FBS, and when indicated with 10 ng/ml IL-15 (R&D Systems) or 10 µg/ml anti-NKGD2 Ab (R&D Systems) or the IgG1 isotype control (Sigma-Aldrich). NK cell-mediated cytotoxicity was then measured using a standard chromium-release assay. Briefly, K562 cells, which served as the target cells, were labeled with 50 Ci of [51Cr] (as sodium chromate; Amersham Biosciences) for 1 h and then washed three times. The NK cells and labeled K562 cells were then incubated together at various E:T ratios for 4 h at 37°C in RPMI 1640 medium with 10% FBS. Supernatants were har-
vested, and released 51Cr was measured using a gamma counter. Spontaneous release was determined by measuring 51Cr release by target cells alone. Max-
imum release was obtained by lysing target cells with 4% Triton X-100 (VWR International). The percentage of specific lysis was calculated as follows: % specific lysis = 100 × (experimental release – spontaneous release)/(maxi-
mum release – spontaneous release)
To measure sMICA, TGFβ1, and IL-15 in serum, blood samples were taken from each individual, immediately transferred to hemolysis tubes, incubated at 37°C for 30–60 min, and centrifuged at room temperature for measurements. The resulting serum samples were transferred to polypropylene microtubes and stored at 80°C. Samples were thawed at room temperature, the plates were washed, and the detection mAb BAMO3 (Immatics) was added. The plates were again incubated for 2 h at room temperature and washed, after which HRP-conjugated anti-mouse IgG2a (SouthernBiotech) was added. After a 1-h incubation at room temperature and washed, after which HRP-conjugated anti-mouse IgG2a (SouthernBiotech) was added. After a 1-h incubation at room temperature, the plates were washed, and 100 μl of tetramethylbenzidine (Sigma-Aldrich), a colorimetric substrate for HRP, was added. Color development was stopped by addition of 1 M phosphoric acid (Sigma-Aldrich). The absorbance was measured at 450 nm. The concentration of sMICA in the samples was determined by comparison with a standard curve. The concentration of IL-15 in serum was determined using the Quantikine human IL-15 immunoassay (R&D Systems) according to the manufacturer’s instructions. The lower detectable concentration for this assay is typically <2 pg/ml. The concentration of sMICA in serum was determined using the MICA sandwich ELISA (Immatics) according to the manufacturer’s instructions. Briefly, MaxiSorp ELISA plates (Nunc/Dominique Dutscher) were coated overnight at 4°C with PBS containing 2 μg/ml of the AMO-1 capture mAb against MICA (Immatics), blocked overnight at room temperature with PBS containing 10% FBS, and washed with PBS containing 0.05% Tween 20 (Sigma-Aldrich). Standard recombinant sMICA (Immatics) or 2-fold-diluted serum samples were added to the plates. After a 2-h incubation at room temperature, the plates were washed, and the detection mAb BAMO3 (Immatics) was added. The plates were again incubated for 2 h at room temperature and washed, after which HRP-conjugated anti-mouse IgG2a (SouthernBiotech) was added. After a 1-h incubation at room temperature, the plates were washed, and 100 μl of tetramethylbenzidine (Sigma-Aldrich), a colorimetric substrate for HRP, was added. Color development was stopped by addition of 1 M phosphoric acid (Sigma-Aldrich). The absorbance was measured at 450 nm. The concentration of sMICA in the samples was determined by comparison with a standard curve. The concentration of IL-15 in serum was determined using the Quantikine human IL-15 immunoassay (R&D Systems) according to the manufacturer’s instructions. The lower detectable concentration for this assay is typically <2 pg/ml.

### Table II. Immunophenotype of T and NK lymphocytes from healthy donors and ESRD patients

<table>
<thead>
<tr>
<th></th>
<th>Healthy Donors (n = 37) (%)</th>
<th>Hemodialysis Patients (n = 58)</th>
<th>Peritoneal Dialysis Patients (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>72 ± 2</td>
<td>75 ± 2 NS</td>
<td>79 ± 2 NS</td>
</tr>
<tr>
<td>CD4⁺CD3⁺</td>
<td>70 ± 2</td>
<td>67 ± 2 NS</td>
<td>55 ± 2 0.030</td>
</tr>
<tr>
<td>CD8⁺CD3⁺</td>
<td>22 ± 2</td>
<td>25 ± 2 NS</td>
<td>34 ± 2 0.018</td>
</tr>
<tr>
<td>NK2G2⁺CD8⁺</td>
<td>67 ± 3</td>
<td>58 ± 3 0.037</td>
<td>75 ± 2 NS</td>
</tr>
<tr>
<td>Total NK cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total NK cells/lymphocytes</td>
<td>7 ± 1</td>
<td>6 ± 1 NS</td>
<td>6 ± 2 NS</td>
</tr>
<tr>
<td>NK2G2⁺/total NK cells</td>
<td>56 ± 4</td>
<td>39 ± 3 &lt;0.001</td>
<td>36 ± 2 0.033</td>
</tr>
<tr>
<td>NKp30⁺/total NK cells</td>
<td>21 ± 2</td>
<td>14 ± 3 0.021</td>
<td>ND ND</td>
</tr>
<tr>
<td>NKp44⁺/total NK cells</td>
<td>4 ± 1</td>
<td>2 ± 0.5 NS</td>
<td>ND ND</td>
</tr>
<tr>
<td>NKp46⁺/total NK cells</td>
<td>51 ± 3</td>
<td>70 ± 5 0.006</td>
<td>ND ND</td>
</tr>
<tr>
<td>CD56⁺dim NK cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56⁺bright NK cells/lymphocytes</td>
<td>1 ± 0.1</td>
<td>2 ± 0.2 &lt;0.001</td>
<td>2 ± 0.2 0.002</td>
</tr>
<tr>
<td>CD56⁺bright NK cells/total NK cells</td>
<td>21 ± 3</td>
<td>37 ± 2 &lt;0.001</td>
<td>39 ± 7 0.015</td>
</tr>
<tr>
<td>NK2G2⁺CD56⁺bright NK cells</td>
<td>78 ± 2</td>
<td>63 ± 3 &lt;0.001</td>
<td>67 ± 5 NS</td>
</tr>
<tr>
<td>CD56⁺dim NK Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56⁺lim NK cells/lymphocytes</td>
<td>6 ± 1</td>
<td>4 ± 1 NS</td>
<td>5 ± 1 NS</td>
</tr>
<tr>
<td>CD56⁺lim NK cells/total NK cells</td>
<td>79 ± 3</td>
<td>63 ± 2 &lt;0.001</td>
<td>61 ± 7 0.015</td>
</tr>
<tr>
<td>NK2G2⁺CD56⁺dim NK cells</td>
<td>49 ± 5</td>
<td>24 ± 3 &lt;0.001</td>
<td>17 ± 3 0.004</td>
</tr>
</tbody>
</table>

a For NKp30, NKp44, and NKp46 phenotyping, n = 11.  
b Comparison with HD.

**Serum preparation and ELISA**

To measure sMICA, TGFβ1, and IL-15 in serum, blood samples were taken from each individual, immediately transferred to hemolysis tubes, incubated at 37°C for 30–60 min, and centrifuged at room temperature for 15 min at 300 × g. The resulting serum samples were transferred to polystyrene microtubes and stored at −80°C. Samples were thawed at room temperature for measurements.

To measure the total TGFβ1 concentration, 0.1 ml of 2.5 N acetic acid/10 M urea was mixed with 0.1 ml of serum and then incubated at room temperature for 10 min to activate the TGFβ1. The resulting serum was neutralized with 2.7 N NaOH/1 M HEPES and diluted 10-fold with diluent reagent (kit component) before analysis. The concentration of TGFβ1 was determined by ELISA using the DuoSet ELISA development kit (R&D Systems) according to the manufacturer’s instructions. The lower detection limit of the TGFβ1 was 30 pg/ml.

The concentration of sMICA in serum was determined using the MICA sandwich ELISA (Immatics) according to the manufacturer’s instructions. Briefly, MaxiSorp ELISA plates (Nunc/Dominique Dutscher) were coated overnight at 4°C with PBS containing 2 μg/ml of the AMO-1 capture mAb against MICA (Immatics), blocked overnight at room temperature with PBS containing 10% FBS, and washed with PBS containing 0.05% Tween 20 (Sigma-Aldrich). Standard recombinant sMICA (Immatics) or 2-fold-diluted serum samples were added to the plates. After a 2-h incubation at room temperature, the plates were washed, and the detection mAb BAMO3 (Immatics) was added. The plates were again incubated for 2 h at room temperature and washed, after which HRP-conjugated anti-mouse IgG2a (SouthernBiotech) was added. After a 1-h incubation at room temperature, the plates were washed, and 100 μl of tetramethylbenzidine (Sigma-Aldrich), a colorimetric substrate for HRP, was added. Color development was stopped by addition of 1 M phosphoric acid (Sigma-Aldrich). The absorbance was measured at 450 nm. The concentration of sMICA in the samples was determined by comparison with a standard curve.

The concentration of IL-15 in serum was determined using the Quantikine human IL-15 immunoassay (R&D Systems) according to the manufacturer’s instructions. The lower detectable concentration for this assay is typically <2 pg/ml.

### Confocal microscopy

NK and HK-2 cells were plated at 2 × 10⁵ cells/coverslip already coated with poly-l-lysine (Sigma-Aldrich). Cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 10 min at 4°C, washed with PBS 1×, and then first incubated with a mouse anti-human NK2G2Ab (R&D Systems) at 10 μg/ml for NK cells or a mouse anti-human MICA Ab (AMO-1; Bam-OmaB) at 10 μg/ml for HK-2 cells 20 min at 4°C and secondary with an Alexa Fluor 488 goat anti-mouse IgG Ab (Molecular Probes/Invitrogen). The coverslips were mounted on the slides with 10 μl of mounting medium Vectashield containing DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories). Images were acquired by confocal microscopy on a Zeiss LSM
510 META confocal laser microscope with a Plan-Apochromat ×63 N.A.1.4 oil immersion objective using the LSM510 software v4.0 (Zeiss).

Real-time PCR quantification

NK and HK-2 cells were lysed in TRIzol reagent (Sigma-Aldrich) and the total RNA was isolated according to the manufacturer’s instructions. NKG2D mRNA, DAP10 mRNA, MICA mRNA, and the reference GAPDH mRNA were assayed using a fluorescence-based real-time PCR. Reverse transcription was performed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Real-time PCR was performed with an ABI PRISM 7000 sequence detection system with SYBR Green reagents (Applied Biosystems) using 40 cycles of a two-step PCR (15 s at 95°C and 1 min at 60°C) after initial denaturation (95°C for 10 min). Relative expression was calculated according to the ΔΔCₚ method with GAPDH as reference gene and cloned PCR products as calibrators. The primer sequences were: GAPDH forward, 5’-GCC ATC AAT GAC CCC TTC ATT-3’; GAPDH reverse, 5’-TTG ACG GTG CCA TGG AAT TT-3’ (50 nM final concentration); NKG2D forward, 5’-GGT ATG AGA GCC AGG CTT CTG GT-3’; NKG2D reverse, 5’-CTT TAC ACA GTC CTG TGG ATG CA-3’ (100 nM final concentration); DAP10 forward, 5’-CAG GCT CTT GTG CCC GAT GT-3’; DAP10 reverse, 5’-CCT GCC TGG CAT GTT GAT G-3’ (300 nM final concentration); MICA forward, 5’-ACA ATG CCC CAG TCC TTC AGA-3’; and MICA reverse, 5’-ATT TTA GAT ATC GCC GTA GTT CCT-3’ (300 nM final concentration).

Statistical analysis

Results are shown as the means ± SE. Comparisons between groups were made using a paired t test or a Mann-Whitney U test for unpaired variables. Correlations were assessed using Pearson’s correlation test. All tests were two-sided, and p < 0.05 was considered as indicating a significant difference.

Results

NKG2D expression is decreased in ESRD patients

The aim of the current study was to clarify the role of NKG2D on NK and T cells and one of its ligands, MICA, in the immune deficit observed in patients with ESRD. We first determined the immunophenotypes of peripheral blood lymphocytes just before dialysis sessions. The following markers were examined: CD3, CD4, CD8, CD16, CD56, NKG2D, NKp30, NKp44, and...
NKp46. As shown in Table II, there was no significant difference in the frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and total NK cells between ESRD patients and healthy blood donors. The frequency of NKG2D-expressing cells and NKG2D surface expression, both in CD8<sup>+</sup> T cells and NK cells, were, however, significantly lower in ESRD patients than in HD (MFI ESRD of 19 vs MFI HD of 26; \( p < 0.002 \)) (Table II and Fig. 1). The decreased frequency of NK2D<sup>+</sup> subsets was more pronounced in NK cells (56 ± 4% vs 39 ± 3%; \( p = 0.001 \)) than in CD8<sup>+</sup> T cells (67 ± 3% vs 58 ± 3%; \( p = 0.028 \)). According to their phenotypes and functional capacities, we defined two NK subpopulations as CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells, respectively (27). CD56<sup>bright</sup> NK cells are more prone to produce cytokines, and CD56<sup>dim</sup> cells, the major subset at the periphery, are more prone to cytolytic activity. The percentage of CD56<sup>bright</sup> NK cells was significantly higher in ESRD patients than in HD. However, as NKG2D expression was higher in the CD56<sup>bright</sup> than in the CD56<sup>dim</sup> subset both in HD and in ESRD patients, the imbalance between NK subsets could not account for the decrease in NKG2D expression in the whole NK population. Actually, NKG2D was significantly decreased compared with HD in both subsets, although the effect was greater in the CD56<sup>dim</sup> subset (Table II).

The percentage of NKp46<sup>+</sup> NK cells was higher (70 ± 5% vs 51 ± 5%; \( p = 0.005 \)) and NKp30<sup>+</sup> NK cells slightly lower (14 ± 3% vs 21 ± 2%; \( p = 0.011 \)) in ESRD patients than in HD. In five ESRD patients, we examined the immunophenotype three times at 3-mo intervals. In all cases, the reduction in NKG2D<sup>+</sup> cells was constant over time (data not shown).

To determine whether the reduction in NKG2D expression was linked to chronic renal failure or to the hemodialysis procedure itself, we evaluated seven patients with ESRD that were treated with peritoneal dialysis. NKG2D expression on NK cells was significantly decreased in these patients as compared with HD (Table II). This indicated that the reduction in NKG2D expression was present in ESRD patients independently of the dialysis procedure.

**FIGURE 3.** Effects of serum from ESRD patients on NKG2D<sup>+</sup> NK cells from HD. NK cells from HD were incubated for 48 h with serum from either HD or ESRD patients. NKG2D expression was assessed by FACS analysis. A. Results obtained from 16 ESRD serum samples independently tested. Uremic serum is able to significantly reduce NKG2D expression on normal NK cells. B. Uremic serum reduces both CD56<sup>bright</sup> and CD56<sup>dim</sup> NK populations in a representative FACS analysis.

**FIGURE 4.** Quantification of soluble and membrane-bound MICA. A. Levels of sMICA were measured by ELISA in sera from 21 HD and 55 ESRD patients. Values are means of duplicate determinations. ESRD patients have significantly higher serum levels of sMICA. B, MICA levels on blood peripheral monocytes from 9 HD and 29 ESRD patients were assessed by FACS analysis. MICA rMFI (relative to the isotype control) is significantly higher in ESRD than in HD (\( p = 0.008 \)). C, FACS analysis from a typical experiment shows the increased expression of membrane-bound MICA at the ESRD monocyte surface.
NKG2D is functional in ESRD patients’ NK cells and can be up-regulated by IL-15

We next designed experiments to examine the functional properties of the NKG2D+ cells in ESRD patients and to identify potential mediators of the NKG2D down-regulation. Because IL-15 is a key cytokine for NK differentiation and maturation (28) and is known to increase NKG2D surface expression (29), we assessed the ability of freshly isolated NK cells from ESRD patients to respond to IL-2 and IL-15. As shown in Fig. 2A, IL-15 (10 ng/ml) induced a 2.5-fold increase in the number of NKG2D+ cells. This effect was greater than that following incubation with IL-2 (100 U/ml). Both the CD56bright and the CD56dim subsets were responsive to IL-15 (Fig. 2B). K562 cells, which do not express HLA class I but express the NKG2D ligands MICA, MICB, UL16-binding protein (ULBP)-1, and ULBP-2, were used as target cells (29, 30). Using NK cells from ESRD patients, we showed that their cytolytic activity was decreased in the presence of blocking anti-NKG2D Ab (Fig. 2C), suggesting that NK-mediated lysis is at

FIGURE 5. Oxidative stress and NKG2D expression. A, Normal NK cells were cultured in the presence of catalase (2000 U/ml) for 15 min and then incubated for 48 h with either serum from HD (n = 14) or ESRD patients (n = 14) tested individually. Catalase enhanced the expression of NKG2D only when NK cells were incubated with ESRD serum. B, NK cells from HD were incubated for 72 h in the presence or absence of 100 ng/ml GO, and NKG2D expression was assessed by FACS analysis. The addition of GO induces a significant reduction in NKG2D expression (p < 0.01). Further addition of 100 ng/ml recombinant soluble MICA does not affect the GO-induced effect. C, Surface NKG2D expression was labeled with anti-NKG2D Ab followed by an Alexa Fluor 488 goat anti-mouse IgG Ab, and it was analyzed by confocal microscopy. NKG2D is visualized in green. Nuclei appear blue. In the presence of GO (lower panel), NKG2D expression is reduced. D, The amount of NKG2D and DAP10 mRNAs were determined by quantitative RT-PCR in normal NK cells in basal conditions (left) and after GO exposure (right). GO addition induces a highly significant down-regulation of both mRNAs.
least partly NKG2D driven both in basal conditions and after IL-15 stimulation.

Reduced NKG2D expression is mediated by uremic serum

To identify potential mediators of NKG2D down-regulation during ESRD, we incubated freshly isolated NK cells obtained from HD with either normal serum or serum from ESRD patients. As shown in Fig. 3, the surface expression of NKG2D on normal NK cells was lower in cells incubated with uremic sera (n = 16) than in cells incubated with normal sera (n = 8) (median decrease of 25%; p < 0.001). This was shown in both CD56bright and CD56dim NK cell subsets. This suggested that a soluble factor should be involved in the reduced expression of NKG2D in patients.

Cytokines present in the serum can modulate NKG2D expression on NK cells. For example, IL-15 can increase NKG2D expression on lymphocytes, whereas TGFβ1 reduces it (31). We therefore measured IL-15 and TGFβ1 plasma levels in our cohort of patients and HD. Although the IL-15 concentrations were identical in the two groups (4 ± 1.2 vs 4 ± 0.2 ng/ml; no significant difference), the TGFβ1 levels were lower in ESRD patients than in HD (25 ± 2 vs 29 ± 1 ng/ml; p = 0.026), demonstrating that these two cytokines do not account for the reduced expression of NKG2D in NK cells from ESRD patients.

Soluble and membrane-bound MICA are increased in ESRD patients

The soluble form of MICA, sMICA, is known to be associated with the down-regulation of NKG2D (32). We therefore measured the serum levels of sMICA in 55 of the ESRD patients (48 receiving hemodialysis and 7 receiving peritoneal dialysis). As shown in Fig. 4A, patients with ESRD had higher serum levels of sMICA than did HD (203 ± 12 vs 110 ± 15 pg/ml; p < 0.001). We also measured the levels of sMICA before and after dialysis for 20 patients. We found that the level of sMICA in these patients was not modified by dialysis (data not shown). Furthermore, measurements at different time points in six ESRD patients showed that the sMICA level was stable over time (data not shown). ESRD patients with the highest sMICA levels (>200 pg/ml) had a significantly lower expression of NKG2D on their NK cells (p = 0.04, data not shown). As a potential source of sMICA, we analyzed the expression of MICA on peripheral blood monocytes, a cell type that can express MICA (17). MICA cell-surface expression was very low on CD14+ monocytes from HD and was significantly increased in ESRD patients without any culture step (ΔMFI = 2 ± 0.1 vs 3 ± 0.2; p = 0.04) (Fig. 4, B and C). These results show that ESRD patients have both high circulating sMICA levels and a high

FIGURE 6. Oxidative stress and MICA expression on HK-2 cells. A, HK-2 cells were incubated for 24 h in the presence or absence of 100 ng/ml GO, and MICA expression was assessed by FACS analysis. GO induces a significant increase in MICA expression (p < 0.05). FACS analysis from a typical experiment shows the increased expression of membrane-bound MICA at the HK-2 surface. B, Surface MICA was labeled with anti-MICA Ab followed by an Alexa Fluor 488 goat anti-mouse IgG Ab, and it was analyzed by confocal microscopy. MICA is visualized in green. In the presence of GO (right), MICA expression is increased. C, HK-2 cells were cultured for 24 h in the presence of either normal serum or ESRD serum (5%). ESRD serum induced a significant increase of MICA surface expression (p < 0.05).

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level of membrane-associated MICA expression on their monocytes. However, addition of a MICA-specific mAb did not block the effect of ESRD serum (data not shown), supporting the idea that factors other than MICA may help mediate the down-regulation of NKG2D in ESRD patients.

**ROS are major effectors responsible for decreased NKG2D expression**

Oxidative stress is a common feature of chronic renal failure (33). ROS trigger the up-regulation of MICA (34, 35) and have been recently implicated in the down-regulation of NKG2D in NK cells from healthy individuals (36). To directly evaluate the role of ROS in the down-regulation of NKG2D on NK cells from ESRD patients, we incubated NK cells from HD with serum from ESRD patients in the presence or absence of catalase (2000 U/ml), an enzyme that breaks down H₂O₂. Catalase had no effect on NK cells incubated with control serum, whereas this enzyme reversed partially but significantly the ability of serum from ESRD patients to reduce NKG2D expression on NK cells (Fig. 5A).

To further study the role of ROS and the potential additional effect of sMICA, normal NK cells were incubated in the presence of GO alone or GO and sMICA. GO is a H₂O₂-generating enzyme that can be used to continuously produce H₂O₂ in the cell culture environment (37). We found that a 72-h incubation of normal NK cells in the presence of GO (100 ng/ml) significantly reduced NKG2D surface expression (p < 0.01) but sMICA alone (data not shown) or in association with glucose oxidase had no effect on NK cells incubated with control serum, whereas this enzyme reversed partially but significantly the ability of serum from ESRD patients to reduce NKG2D expression on NK cells (Fig. 5A).

To specify mechanisms of ROS-induced NKG2D down-regulation, we quantified by quantitative real-time PCR both NKG2D and its signaling adapter DAP10 mRNAs in basal conditions and after treatment of normal NK cells with GO (Fig. 5D). GO incubation induced a significant decrease in NKG2D and DAP10 mRNAs, suggesting that ROS interfere with the transcriptional regulation of the NKG2D expression.

**ROS and uremic serum increase cell surface MICA expression in renal epithelial cells**

ROS are one of the stress factors able to induce MICA expression (34, 35). As renal epithelial cells are a target of oxidative stress in kidney diseases (38), we used the well-characterized renal epithelial cell line HK-2 (26) to study the in vitro ROS effects. In the presence of GO, the constitutive cell-surface expression of MICA was significantly increased (p = 0.03; Fig. 6A). Confocal microscopy further documented the increased membrane-bound MICA on HK-2 cells in the presence of GO (Fig. 6B). PCR experiments did not show a significant change in MICA expression in the presence of GO (data not shown). Finally, ESRD serum reproduced the GO-induced effect on MICA expression. As shown in Fig. 6C, HK2 cells incubated in the presence of 5% ESRD serum have a higher level of surface MICA expression.

**Discussion**

In the present study we examined the role of NKG2D expression and function in NK cells of ESRD patients. There is currently little information on NK cells in ESRD patients, and the available data mainly show a quantitative decrease in these cells in a context of global lymphopenia (2, 39, 40). To our knowledge, this is the first comprehensive analysis of NK lymphocyte subsets and activating receptors in ESRD patients. We found a striking decrease in levels of NKG2D expression in CD8⁺ T cells as well as in NK cells. This could lead to impaired immune functions, consistent with previous studies showing an increased incidence of viral or bacterial infections (3, 40) and of malignancies in these patients (41–43). The different subsets of NK cells did not show the same extent of reduction in NKG2D expression. CD56dim NK cells, the dominant subset at the periphery (27), showed a more important NKG2D decrease than did CD56bright cells.

We also showed that uremic serum can reduce NKG2D levels on the NK cell surface. Uremic serum contains a large number of uremic toxins, only some of which have been identified (7, 8). We tested several hypotheses to identify agents that might mediate the reduction of NKG2D levels on the surface of NK cells. First, we examined the possibility that this is mediated by TGFβ1, which has been implicated in the down-regulation of NKG2D in cancer (31). However, in agreement with a recent study (44), the level of circulating active TGFβ1 was not increased in this cohort of hemodialysis patients. We also ruled out that low plasma levels of IL-15 could be involved in the reduction of NKG2D levels, as they were similar in HD and in ESRD patients. This left two nonexclusive possibilities. First, a significant increase in sMICA levels in the serum and in MICA cell-surface expression on monocytes or other cell types such as epithelial cells could decrease NKG2D⁺ cells by promoting the internalization and degradation of NKG2D (45). Second, ROS, which are abundant in uremic serum, may reduce NKG2D expression.

We describe herein a major role of ROS in the context of ESRD in decreasing NKG2D expression levels on NK cells. We have no evidence that the increased expression of MICA may be directly responsible for the NKG2D down-regulation because sMICA in vitro could not induce or potentiate NKG2D down-regulation and MICA-specific Abs could not block the effect of ESRD patients’ sera on NKG2D.

Oxidative stress is one of the most important consequences of chronic renal failure (33, 46). It also plays a major role in the pathogenesis of inflammation, atherosclerosis, and cardiovascular disease, which are very common in the ESRD population (47, 48). This process is mediated, for example, by oxidative changes in low-density lipoproteins, which then become substrates for uptake by macrophages. Furthermore, oxidative stress causes endothelial dysfunction, leading to adhesion and attraction of monocytes and endothelial cells (49–51). NK cells appear to be sensitive to ROS-induced apoptosis and functional inhibition, but CD56bright cells possess antioxidant defense mechanisms that protect them from oxygen radical-induced inactivation (52). The relatively higher frequency of CD56bright than CD56dim NK cells and the smaller reduction of NKG2D in CD56bright cells in ESRD patients are consistent with a lower susceptibility of CD56bright cells to oxidative stress in vivo. The generation of ROS is known to be increased in ESRD due to the production of high levels of malondialdehyde by granulocytes and monocytes, which lead to the generation of superoxide and H₂O₂ (53). In the present study we found that treatment of normal NK cells with GO, which increases ROS production, reduces the expression of NKG2D. Moreover, catalase, which breaks down H₂O₂, reversed the reduction of NKG2D expression by serum from ESRD patients. Confocal microscopy analysis confirmed the direct effect of ROS on NKG2D surface expression. ROS act through a transcriptional inhibitory effect, since both NKG2D mRNA and the adaptor DAP10 mRNA were significantly down-regulated in the presence of GO. Furthermore, ROS seem to be also implicated in MICA overexpression. Since we observed that blood monocytes increased their expression of MICA, we reasoned that ROS and the ESRD environment may also induce...
MICA expression on other cell types such as renal epithelial cells, which constitute a major actor in ESRD. We studied MICA expression in the renal epithelial cell line HK-2 and could demonstrate that both GO and ESRD serum increased MICA surface expression. We did not find evidence that GO-induced MICA cell-surface up-regulation could be due to a transcriptional mechanism. These data are in agreement with studies in bronchial epithelial cells (35). In these cells, NKG2D ligand surface expression was increased in the presence of H2O2, whereas MICA mRNA was unaffected. Additional experiments are needed to specify the cellular mechanism responsible for increased membrane-bound MICA in ESRD. The recent report that microRNAs are crucial in the cellular mechanism responsible for increased membrane-bound MICA in ESRD. The recent report that microRNAs are crucial in the cellular mechanism responsible for increased membrane-bound MICA in ESRD. 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