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ORIGINAL ARTICLE

Reduced immune effector cell NKG2D expression and increased levels of soluble NKG2D ligands in multiple myeloma may not be causally linked

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Abstract
Background There is limited understanding of the dysregulation of the innate immune system in multiple myeloma (MM). We analysed the expression of the activating receptor NKG2D on NK cells and T cells of MM patients and investigated the impact of soluble versus membrane-bound NKG2D ligands on the expression of NKG2D.
Design NKG2D expression on NK cells and CD8+ $\alpha\beta$ T cells from patients with MM or monoclonal gammopathy of uncertain significance and healthy controls was examined flow-cytometrically. Sera from patients and controls were analysed for soluble NKG2D ligands (sNKG2D ligands).
Results Significantly fewer NK cells and CD8+ $\alpha\beta$ T cells from patients expressed NKG2D compared to healthy controls (NK cells: median 54% interquartile range (IQR) 32–68 versus 71% IQR 44–82%, $P = 0.017$, CD8+ $\alpha\beta$ T cells: median 63% IQR 52–81 versus 77% IQR 71–90%, $P = 0.018$). The sNKG2D ligand sMICA was increased in patients [median 175 (IQR 87–295) pg/ml] versus controls [median 80 (IQR 32–129) pg/ml, $P < 0.001$], but levels of sMICA did not correlate with NKG2D expression on effector cells. To elucidate the mechanism of NKG2D down-regulation, we incubated lymphocytes from healthy donors in the presence of sNKG2D ligands or in co-culture with MM cell lines. sNKG2D ligands in clinically relevant concentrations did not down-regulate NKG2D expression, but co-culture of effector cells with myeloma cells with high surface expression of NKG2D ligands reduced NKG2D expression significantly.
Conclusions These results indicate that MM is associated with a significant reduction in NKG2D expression which may be contact-mediated rather than caused by soluble NKG2D ligands.

Keywords Multiple myeloma · Innate immunity · NKG2D · MICA · MGUS · Immune escape

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Introduction

Multiple myeloma (MM) is a malignant plasma cell disease which despite major advances in therapy is as yet incurable. Like many malignancies, MM is associated with a marked deficiency in immune function which allows the tumour to escape and renders any immunotherapeutic approaches inherently difficult [1]. So far, studies investigating MM-induced immunosuppression have focussed on T cell proliferation and function, which is directly impaired

by myeloma cells [2] or indirectly because MM induces impairment of DC function [3]. In addition, an increased frequency of functional regulatory T cells has been reported in MM [4]. The innate immune response has been more or less neglected in the past, though recently, evidence has emerged to demonstrate deficiencies in innate immunity as well [5, 6] although little is known about the underlying mechanism.

The interaction between the activating NK receptor group 2 member D (NKG2D) expressed by NK cells, CD8+ $\alpha\beta$ T cells and $\gamma\delta$ T cells and its ligands MHC-class I related chain A and B (MICA/B) and UL16-binding proteins (ULBP) has been recognised as a crucial part of the innate immune response against several malignancies including multiple myeloma (MM) [7–9]. Recently, a reduction in NKG2D expression on NK cells and CD8+ $\alpha\beta$ T cells has been observed in several malignancies [10, 11]. This may be caused by increased levels of TGFβ [12], though the presence of soluble NKG2D ligands shed by the tumour cells also leads to a substantial down-regulation of NKG2D on effector cells [13]. Tumour-induced down-regulation of NKG2D results in impaired cytotoxic activity, allowing the tumour to escape from immune-surveillance [14]. Soluble NKG2D ligands have been reported in various malignancies including MM [15–17] in the past and recently reduced NKG2D expression has been reported in MM which has been suggested to be mediated by soluble MICA [18].

In this study, we analysed NKG2D expression on the effector cells of patients with MM and MGUS compared to healthy controls. In order to elucidate potential mechanisms of down-regulation, we also determined the presence of soluble NKG2D ligands and the level of TGFβ in the patients' serum. Whilst detecting a raised level of sMICA in patients this was not of a sufficient concentration to cause NKG2D down-regulation. Similarly, levels of TGFβ did not correlate with NKG2D expression and in vitro experiments with patients' serum and culture supernatants did not result in a change in NKG2D expression. On the other hand, co-culture with MICA-expressing tumour cells led to significant, functionally relevant down-regulation of NKG2D on NK cells and CD8+ T cells.

Patients and methods

Following the local ethics committee approval and written informed consent, healthy controls and patients with MGUS or MM were included in the study. Serum samples were centrifuged and stored at −80°C until measurement. The diagnosis of MGUS was based on the presence of a serum monoclonal protein (<30 g/l) and <10% plasma cells in the bone marrow in the absence of hypercalcaemia, bone lesions, anaemia or renal insufficiency. In contrast, MM was diagnosed as soon as >30 g/l serum monoclonal protein or >10% bone marrow plasma cells were present [19]. Symptomatic MM was assumed in cases with organ involvement as described above. For the purpose of this study, patients were grouped into four groups to better reflect the current tumour load: MGUS, newly diagnosed MM (regardless of clinical stage), low disease (comprising of patients with ongoing asymptomatic MM or stable remission after treatment) and progressive disease resulting from asymptomatic MM or relapsing after treatment.

Cell culture

Peripheral blood mononuclear cells and bone marrow cells were sampled from a subgroup of patients and healthy donors and isolated by density gradient centrifugation on a Ficoll (Lymphoprep, Fresenius, Oslo, Norway) cushion. Cells were frozen after isolation and stored at −270°C in liquid nitrogen until measurement. Human myeloma cell lines RPMI-8226, U266B, OPM-2, KMS11, KMS18, JJN3, JIM3, and the leukaemia cell line K562 were purchased from DSMZ, Braunschweig, Germany, or ATCC, Middlesex, UK, and cultured in RPMI1640 (GIBCO, Invitrogen Parsley, UK) supplemented with 10% Foetal Calf Serum and 1% penicillin/streptomycin and 1% L-glutamine (complete medium, CM). Cells for co-culture experiments and effect of soluble ligands were sampled from healthy donors. After density-gradient centrifugation, non-adherent cells were co-cultured with tumour cells at various ratios of tumour cells to lymphocytes for 24 h. To avoid tumour cell growth, cell lines were pretreated with Mitomycin C (Kyowa Hakko UK Ltd, Slough Berkshire, UK; 50 µg/ml for 30 min). For the influence of soluble ligands, non-adherent cells were cultured in the presence of varying concentrations of sMICA (R&D systems, Wiesbaden-Nordenstadt, Germany) or serum/cell culture supernatants, respectively, for 24 h.

ELISA

ELISA-kits for sMICA, sMICB and TGF-β were purchased from R&D systems and used according to the manufacturer's instruction. For detection of sULBP1-3 96-well microtiter plates were coated with 100 µl serum per well. After 2 h incubation at room temperature (RT), PBS/4%BSA was added for blocking and the plates left over night at 4°C. Monoclonal antibodies against ULBP1-3 (R&D systems, Wiesbaden-Nordenstadt, Germany) were added at 1 µg/ml and incubated for 1.5 h at RT, following which anti-IgG_{2a}-Biotin (BD Bioscience, Oxford, UK) was added at 1 µg/ml for 1 h at RT. Streptavidin-HRP (R&D systems, Wiesbaden-Nordenstadt, Germany) was used as conjugate (45 min at RT) and for development substrate reagents

A and B from R&D systems, Wiesbaden-Nordenstadt, Germany, were used. After each incubation step, the plates were washed four times with PBS/0.05% Tween 20 (200 µl/well). The absorbance was measured at 450 nm.

Flow-cytometry

Cells in suspension were stained according to the manufacturer's instructions and analysed on an LSRII, Becton–Dickinson, using antibodies against MICA, MICB, ULBP2, NKG2D (R&D systems, Wiesbaden-Nordenstadt, Germany), CD3, CD8, CD45, CD56 (BD Biosciences), $\gamma\delta$ TCR, $\alpha\beta$ TCR (Coulter Immunotech, Krefeld, Germany). Anti-CD38 ICO-20 was a kind gift from HMDS, Leeds, and plasma cells were identified according to a previously published protocol from HMDS, Leeds [20]. FACSDiva (BD Bioscience, Oxford, UK) and FlowJo Software (Treestar, USA) were used for analysis of data. The percentage of positive cells was determined and used for further statistical analysis.

Cytotoxic activity

For analysis of cytotoxic activity a standard ^{51}Chromium release assay was performed. In brief, tumour cells were labelled with 10 µCi ^{51}Chromium for 2 h, washed twice and co-cultured with effector cells in 150 µl at various effectors to target ratios for 4 h. 50 µl supernatant were taken for determination of radioactivity on a Top Count NXT (Canberra Packard, Rüsselsheim, Germany). Prior to use in the chromium release assay, the effector cells were negatively (NK cells) or positively (CD8+ T cells) selected using an NK cell selection kit with addition of CD138-microbeads or anti-CD8 PE and anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) over appropriate MACS-columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity was determined as >80% for NK cells and >95% for CD8+ T cells.

Cytotoxicity calculation was performed by the following formula:

$$\text{Percent cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release of target cells}}{\text{maximal release} - \text{spontaneous release of target cells}}$$

Statistics

Descriptive statistics (median, interquartile range or mean, standard error of mean) were used as required. To test for differences, student's t test, Mann–Whitney U test, Kruskall–Wallis test, Spearman rank correlation coefficient, χ^2 test and receiver operating characteristic (ROC) curves were used where appropriate with SPSS version 14.0 for windows (Munich, Germany). Significance was assumed if $P < 0.05$.

Results

Patient characteristics

The characteristics of 117 patients and 45 age-matched controls included for analysis of soluble NKG2D ligands are illustrated in Table 1. NKG2D expression was analysed on effector cells from a subgroup of patients and controls (49 patients, 17 controls).

Reduced NKG2D expression on NK and T cells from patients with MM

To test if reduced NKG2D expression might be one of the means of tumour immune escape in MM we analysed effector cells in a subgroup of patients ($n = 49$) and controls ($n = 17$). Importantly, none of the patients received steroid treatment at the time of blood sampling, which has been reported to decrease NKG2D expression [21].

Significantly fewer NK cells from patients with plasma cell disorders expressed NKG2D compared with those from healthy controls (median 54% IQR 32–68 vs. 71% IQR 44-82%, $P = 0.011$, Fig. 1). Similarly, CD8+ $\alpha\beta$ T cells from patients showed a lower expression of NKG2D than controls (median 63%, IQR 52–81% versus 77%, IQR 71–90%, $P = 0.018$, Fig. 1). Number of NKG2D-expressing cells and mean fluorescence intensity (MFI) of NKG2D expression correlated positively in both effector cell populations (NK cells: $r = 0.367$, $P = 0.007$; CD8+ $\alpha\beta$ T cells: $r = 0.523$, $P < 0.001$), but the absolute difference in MFI was too small to reach statistical significance (Fig. 1b). In contrast, $\gamma\delta$ T cells from patients and controls did not significantly differ in their NKG2D expression (median 71%, IQR 61–86% versus 80%, IQR 67–87%, $P > 0.05$).

Since TGF-β has been reported to cause down-regulation of NKG2D, we evaluated serum levels of patients and

Table 1 Patient characteristics

	Control	MGUS	Low disease	Progressive disease	Newly diagnosed
N	45	14	64	15	16
Age [years, median (range)]	58 (53–70)	73 (50–84)	63 (37–86)	66 (43–77)	62 (34–85)
Type paraprotein *n*/*N* (%)[a]					
IgG		12/13 (86)	40/64 (63)	10/15 (67)	8/16 (50)
IgA		1/13 (7)	14/64 (22)	5/15 (33)	8/16 (50)
Light chains			10/64 (16)		
Current level paraprotein [g/l, median (range)]		8 (0–31)	9 (0–59)	14 (0–61)	21 (0–63)

MGUS monoclonal gammopathy of uncertain significance, *Low Disease* remission after treatment, *Progressive Disease* relapsing or refractory to treatment, *Newly Diagnosed* De novo diagnosis

[a] Unknown in 1 patient with MGUS, 1 newly diagnosed, 1 progressive disease, and 4 low disease. Of 8 patients the disease status is unknown

controls to find no difference in serum levels (Control 5,003 ng/ml (IQR 4,158–5,582), MGUS 3,834 ng/ml (IQR 3,017–4,712), low disease 2,551 ng/ml (IQR 1,496–3,476), progressive disease 2,745 ng/ml (IQR 2,342–3,141), newly diagnosed 2,968 ng/ml (IQR 1,923–3,945, $P = 0.434$). Also, no significant correlation with NKG2D positive NK cells ($r = 0.3$, $P = 0.06$) or CD8+ $\alpha\beta$ T cells ($r = 0.255$, $P = 0.11$) could be detected.

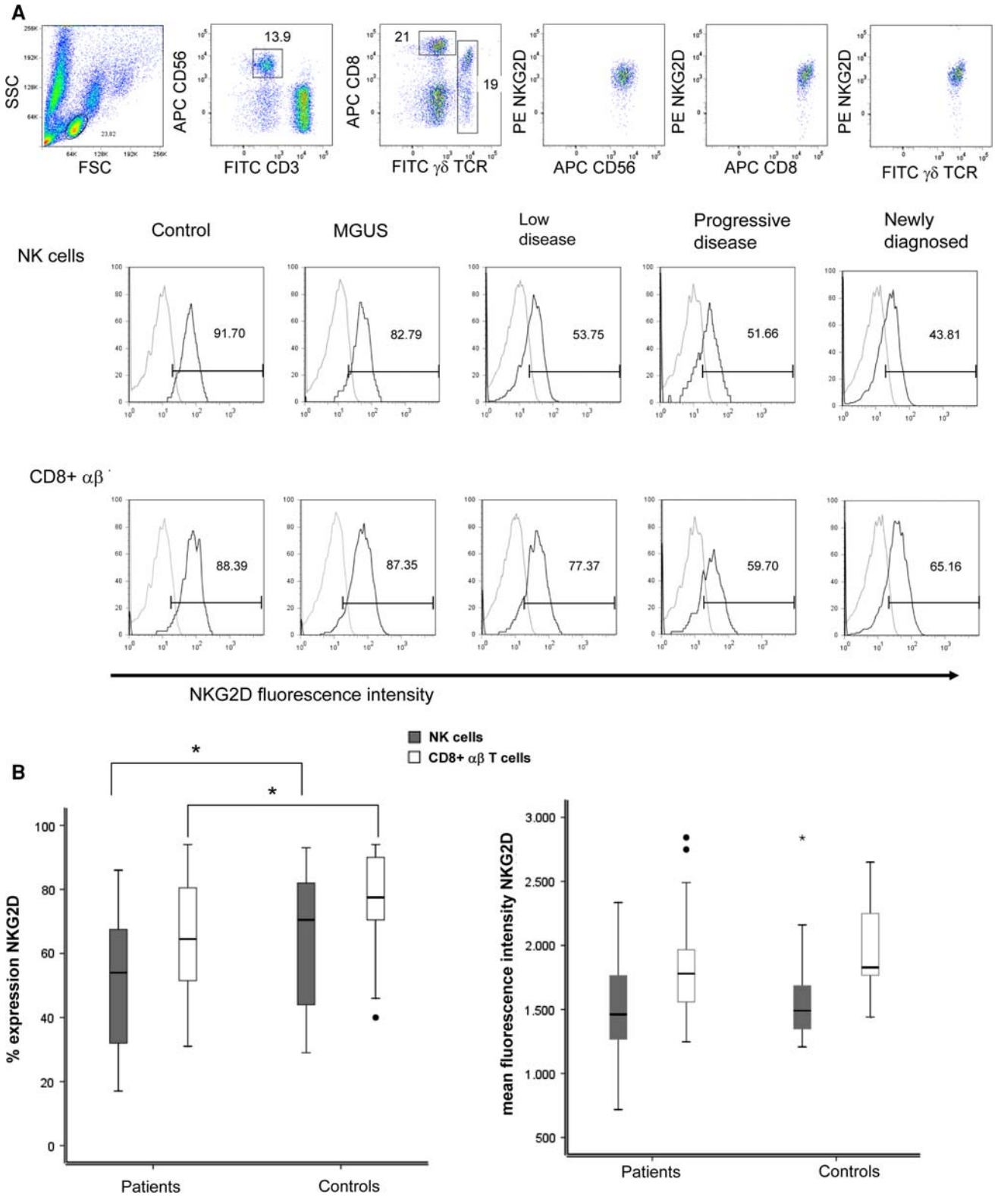
Soluble NKG2D ligands are increased in the serum of Myeloma patients

Patients with Myeloma at various stages of disease manifestation had significantly higher levels of soluble MICA than healthy controls (Fig. 2a) with a median of 175 (IQR 87–295) pg/ml versus 80 (IQR 32–129) pg/ml in controls ($P < 0.001$). In contrast, sMICB and sULBP1-3 were not significantly elevated in patient samples (Fig. 2a, b). The increase of sMICA was particularly pronounced in untreated patients (MGUS: median 107, IQR 84–156 pg/ml, low disease burden: median 189, IQR 96–312 pg/ml, progressive disease: median 121, IQR 26–437 pg/ml, newly diagnosed: median 232, IQR 147–331 pg/ml, $P < 0.001$). No influence of disease duration or treatment on sMICA could be found and there was no correlation with renal function (data not shown). In addition, we analysed the subgroup of newly diagnosed patients, because in this patient population the disease characteristics have not been skewed by prior treatment. Like others [17], we found a significant correlation between the level of sMICA and tumour burden as measured by level of paraprotein ($R = 0.64$, $P = 0.01$) or percentage of bone marrow infiltration ($R = 0.765$, $P = 0.001$).

To evaluate sMICA as a tumour marker, we determined the area under the ROC curve (AUC) with 0.685 ($P < 0.001$) for controls versus patients, confirming previous data [22] for the accuracy of sMICA to distinguish between controls and patients with malignancies. In the setting of MM, the more relevant question is to distinguish patients with MGUS from newly diagnosed MM. Here, the AUC was 0.8 ($P = 0.005$) and a cut-off level of 225 pg/ml allowed to distinguish MM from MGUS (specificity 93%, sensitivity 56%, positive predictive value 90%, negative predictive value 65%).

It has previously been suggested that the origin of sMICA may be the malignant plasma cells [18], and to investigate this, we examined the expression of the NKG2D ligands by Myeloma plasma cells. Myeloma cell lines demonstrate a heterogenous expression of MICA (range 0–95%), MICB (range 0–70%) and ULBP2 (range 3–79%) with similar expression heterogeneity being demonstrated by primary plasma cells as illustrated in Table 2. No clear association between low surface expression and high soluble ligand concentration was evident.

Fig. 1 **a** Flow-cytometrical analysis of expression of NKG2D on NK ▶ cells and CD8+ $\alpha\beta$ T cells of healthy controls and patients with MGUS, low disease burden, progressive disease and newly diagnosed disease. The *first line* depicts the gating strategy of NK cells (CD3–CD56+), CD8+ $\alpha\beta$ T cells (CD8++$\gamma\delta$TCR-) and $\gamma\delta$ T cells ($\gamma\delta$TCR+) and representative dot plots of the NKG2D expression of the respective cell populations. The second line depicts representative examples of NK cells and the third line representative examples of CD8+ $\alpha\beta$ T cells of 17 controls, 12 patients with MGUS, 17 patients with low disease burden, 8 patients with progressive disease and 12 newly diagnosed patients. The *grey line* represents the isotype control, the *black line* the stained sample; **b** Median percentage of NKG2D-expressing NK cells and CD8+ $\alpha\beta$ T cells from patients and controls (*left plot*) as well as median mean fluorescence intensity of NKG2D expression of NK cells and CD8+ $\alpha\beta$ T cells from patients and controls. The *line* depicts the median, the *box* the interquartile range and the *whiskers* the 95% confidence interval. Patients: NK cells: percentage NKG2D+ cells median 54% IQR 32–68, MFI median 1,461 IQR 1,268–1,765; CD8+ $\alpha\beta$ T cells: percentage NKG2D+ cells median 63%, IQR 52–81%, MFI median 1,780 IQR 1,559–1,967. Controls: NK cells: percentage NKG2D+ cells median 71% IQR 44–82%, MFI median 1,491 IQR 1,348–1,686; CD8+ $\alpha\beta$ T cells: percentage NKG2D+ cells median 77%, IQR 71–90%, MFI median 1,828 IQR 1,767–2,249. *$P < 0.05$


A
SSC
FSC
APC CD56
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13.9
APC CD8
FITC γδ TCR
21
19
PE NKG2D
APC CD56
PE NKG2D
APC CD8
PE NKG2D
FITC γδ TCR
Control
MGUS
Low disease
Progressive disease
Newly diagnosed
NK cells
91.70
82.79
53.75
51.66
43.81
CD8+ αβ
88.39
87.35
77.37
59.70
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NKG2D fluorescence intensity
B
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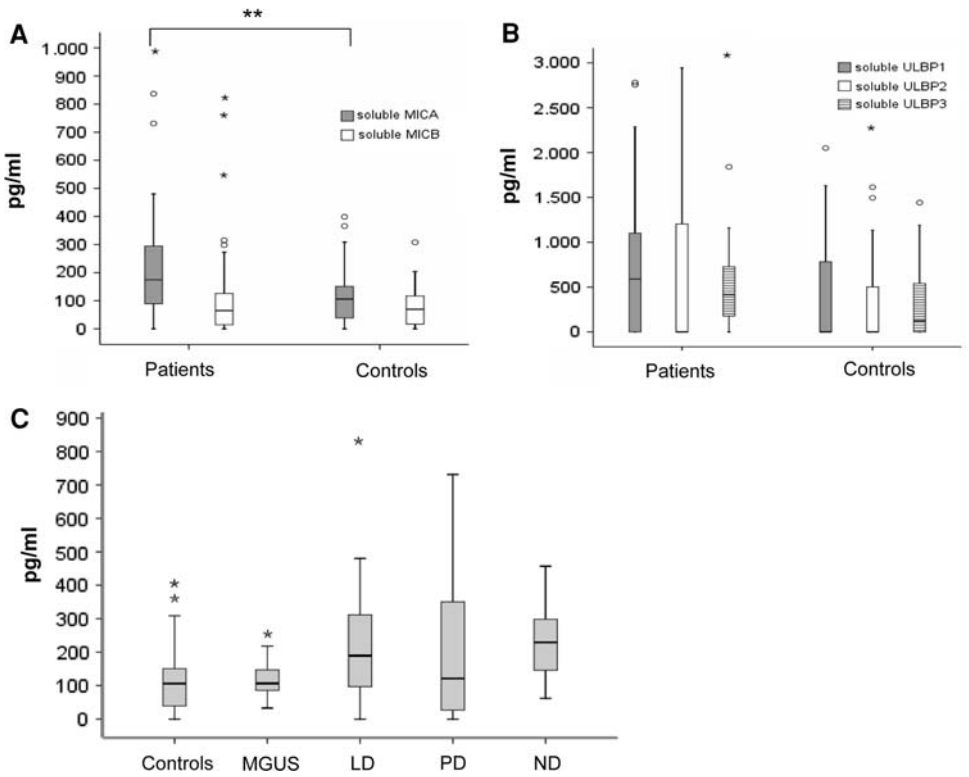

Fig. 2 **a** Soluble MICA and MICB in the sera of healthy controls and patients with MGUS or MM detected by ELISA as described in the methods section. The *line* depicts the median, the *box* the interquartile range and the *whiskers* the 95% confidence interval. sMICA: patients median 175 (IQR 87–295) pg/ml, controls median 80 (IQR 32–129) pg/ml (** $P < 0.001$), sMICB: patients median 66 (IQR 15–128) pg/ml, controls 42 (IQR 7–111) pg/ml ($P = 0.8$); **b** Soluble ULBP1, ULBP2 and ULBP3 in the sera of controls and patients as described above. sULBP1: patients median 448 (IQR 0–1,125) pg/ml, controls median 0 (IQR 0–640) pg/ml, sULBP2: patients median 0 (IQR 0–1,202) pg/ml, controls median 0 (IQR 0–501) pg/ml, sULBP3: patients median 265 (IQR 0–552) pg/ml, controls median 56 (IQR 0–473) pg/ml, $P > 0.05$ for all sULBP; **c** Level of soluble MICA depending on current stage of disease. *MGUS* monoclonal gammopathy of uncertain significance, *LD* Low Disease, *PD* Progressive Disease, *ND* Newly Diagnosed. The *line* depicts the median, the *box* the interquartile range and the *whiskers* the 95% confidence interval. MGUS: median 107, IQR 84–156 pg/ml, low disease burden: median 189, IQR 96–312 pg/ml, progressive disease: median 121, IQR 26–437 pg/ml, newly diagnosed: median 232, IQR 147–331 pg/ml, $P < 0.001$ Kruskall–Wallis

Soluble MICA is not significantly associated with NKG2D down-regulation in MM

To test if the raised sMICA level in patients is responsible for the NKG2D down-regulation, we performed a Spearman correlation between sMICA levels and NKG2D expression on NK cells, CD8+ T cells or $\gamma\delta$ T cells. This proved to be non-significant with $r = 0.067$, $P = 0.7$ for Nk cells; $r = 0.158$, $P = 0.3$ for CD8+ T cells and $r = -0.063$, $P = 0.7$ for $\gamma\delta$ T cells. However, in an in vitro model, culturing effector cells in the presence of sMICA we detected a dose-dependent effect on NKG2D down-regulation (Fig. 3a). At the highest concentration of 1 μg/ml, a significant down-regulation of NKG2D was seen with a clear dose–response demonstrated. However, in the presence of a concentration more similar to that noted in the peripheral blood of patients (125 ng/ml), sMICA did not lead to a down-regulation of NKG2D expression. In addition, culturing effector cells in the presence of patient serum did not result in any alteration in surface expression of NKG2D compared to culture in the presence of human serum albumin (Fig. 3b). Similarly, culture in the presence of supernatants from different myeloma cell lines did not result in a down-regulation of NKG2D (Fig. 4a).

Down-regulation of NKG2D is contact-dependent and requires a high level of expression of MICA

Next, we evaluated if there is a contact-dependent effect of NKG2D-ligands on effector cell NKG2D expression. We co-cultured effector cells with tumour cells expressing different levels of NKG2D ligands (Table 2) before analysing them flow-cytometrically. In this set-up, we detected a significant decrease of NKG2D+ NK cells and down-regulation of NKG2D expression as determined by mean fluorescence intensity (MFI) after 24 h and 48 h of co-culture (Fig. 4b, c). CD8+ T cells exhibited a similar, albeit much less marked, effect (Fig. 4b, c). In our hands, the observed

Table 2 Expression of NKG2D ligands on cell lines and plasma cells from healthy donors and patients with MM

	MICA (% expression)	MICB (% expression)	ULBP2 (% expression)	sMICA (pg/ml)	sMICB (pg/ml)	sULBP2 (pg/ml)
RPMI 8226	95	70	79	14	118	302
JJN3	14	9	75	8	43	619
JIM3	0	0	60	3	32	5,334
U266	0	9	65	16	4,394	886
OPM-2	0	0	6	6	0	534
H929	0	0	16	6	0	995
KMS11	0	0	6	0	168	1,227
KMS18	17	0	3	168	625	886
K562	83	54	37	ND	ND	ND
Healthy control 1	0	0	49	ND	ND	ND
Healthy control 2	3	0	48	ND	ND	ND
MM 1	14	0	2	ND	ND	ND
MM 2	3	0	34	ND	ND	ND
MM 3	0	0	9	ND	ND	ND
MM 4	0	0	23	ND	ND	ND
MM 5	4	0	9	ND	ND	ND
MM 6	0	0	0	ND	ND	ND
MM 7	3	2	3	146	0	ND
MM 8	0	0	3	297	59	ND

sNKG2D ligands were measured in the culture-supernatants for cell lines and in the plasma for patients. K562 (leukaemia cell line) served as a positive control

ND not done

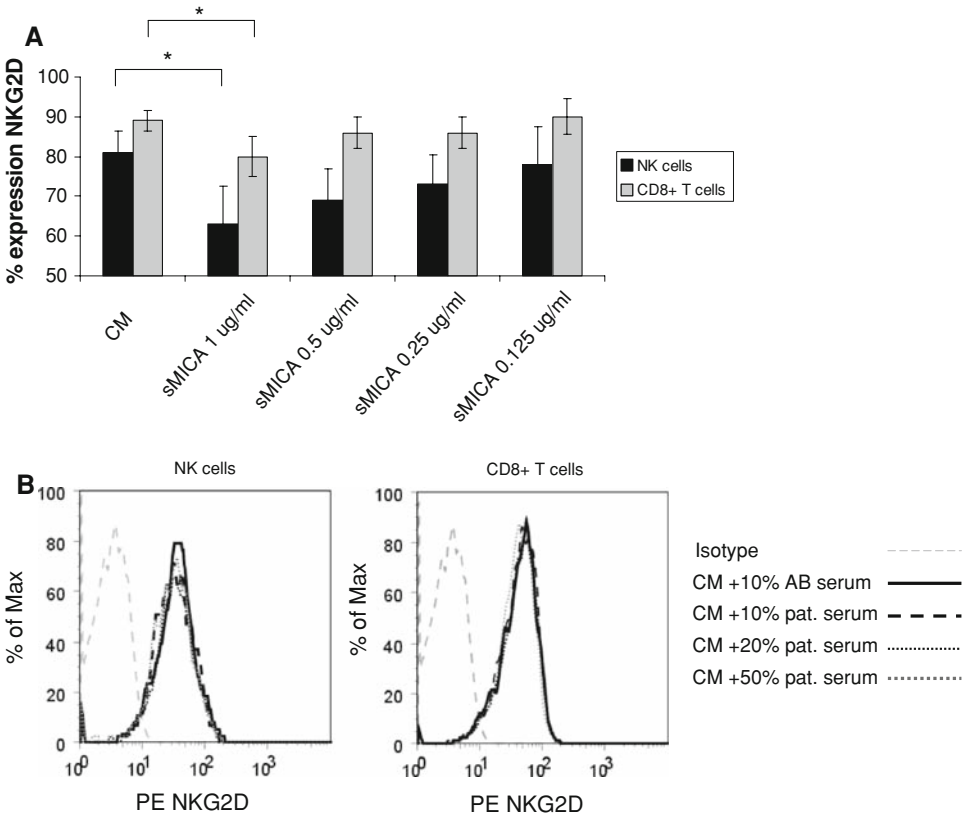

Fig. 3 **a** Concentration-dependent NKG2D down-regulation by sMICA after culture and flow-cytometrical analysis as described in the methods section. *CM* complete medium. Mean ± standard error of the mean of four independent experiments with effector cells from four different healthy donors is depicted. *$P < 0.05$; **b** No effect on NKG2D-expression of NK cells and CD8+ T cells was seen when effector cells from healthy donors where incubated with varying concentrations of patient serum. Here, a representative flow-cytometrical analysis of lymphocytes from a healthy donor cultured in the presence of varying concentrations of patient serum as described in the methods section is shown. In this example, the sMICA concentration in the serum was 317 pg/ml

down-regulation was more marked when the co-cultured tumour cells had a high surface expression of NKG2D ligands as is the case with RPMI 8226 for example. With this myeloma cell line, NK cells expressing NKG2D decreased from a mean of 94 ± 1.2% to a mean of 79 ± 5% at 1:1 lymphocytes : myeloma cells and to 70 ± 6% at 1:3

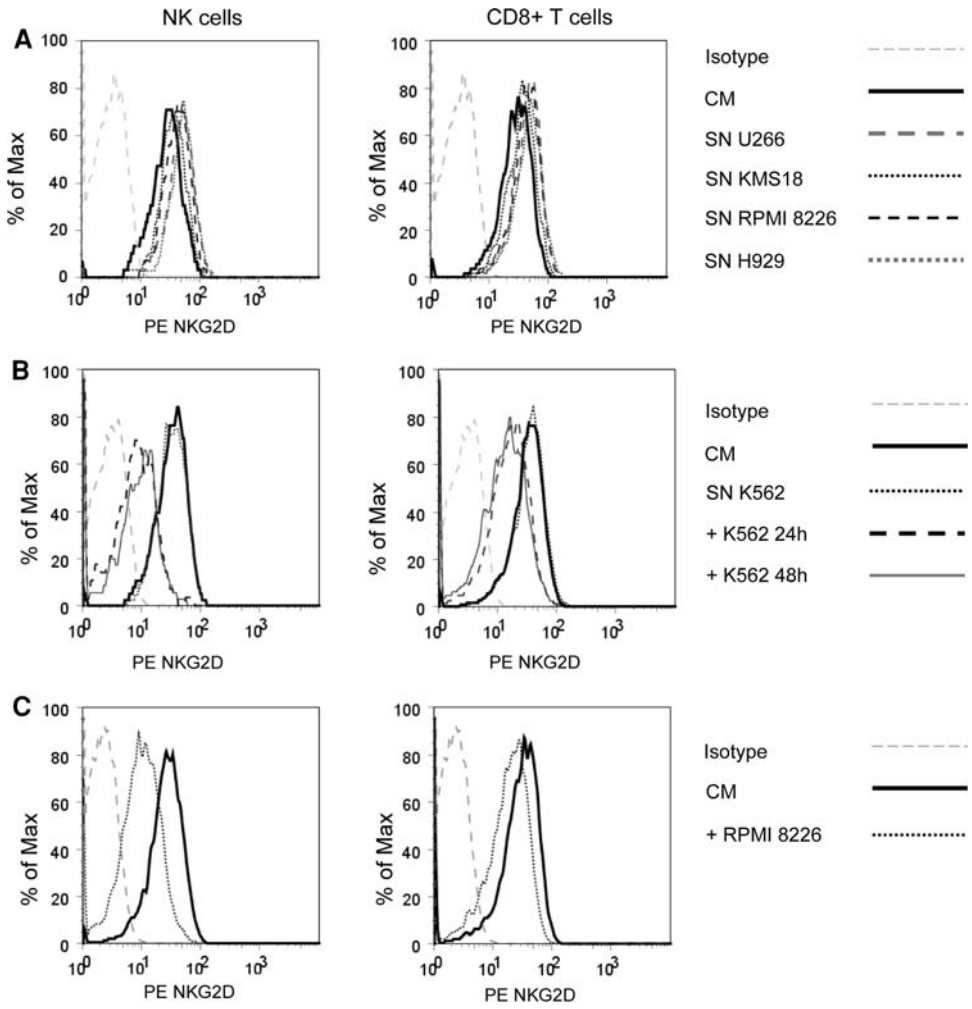

Fig. 4 **a** Culture of effector cells in the presence of supernatants with varying concentrations of sNKG2D ligands as described in the method section does not change NKG2D expression significantly. *CM* complete medium, *SN* supernatant. One representative example showing flow-cytometrical analysis of NKG2D-expression on NK cells and CD8+ $\alpha\beta$ T cells of 4 independent experiments is shown; **b** Co-culture with leukaemia cells (K562) leads to down-regulation of NKG2D on NK cells and CD8+ $\alpha\beta$ T cells whereas the presence of the supernatant does not change NKG2D expression. *SN* supernatant. One representative example of 6 independent flow-cytometrical analyses is shown; **c** Co-culture with myeloma cells (RPMI 8226) as described in the "Patients and methods" leads to down-regulation of NKG2D on NK cells and CD8+ $\alpha\beta$ T cells. One representative example of eight independent flow-cytometrical analyses is shown

lymphocytes : myeloma cells ($P = 0.03$ and $P = 0.007$, respectively) after 24 h co-culture (Fig. 5a). Also, the intensity of NKG2D expression on NK cells was reduced from a mean MFI of 855 ± 40 to 530 ± 33 at 1:1 and 460 ± 26 at 1:3 lymphocytes:myeloma cells (both $P < 0.001$). In contrast, co-culture with H929 and U266 only decreased NKG2D+ NK cells and NKG2D expression at a ratio of 1:3 lymphocytes:myeloma cells ($P = 0.02$ and $P = 0.04$, respectively).

Effector cell function was tested in a chromium release cytotoxicity assay against K562, a cell line known to be killed via NKG2D by NK cells [10, 18, 23] (Fig. 5b). Here, we found a significant impairment of cytotoxic activity. Similarly, IL-2 stimulated CD8+ cells exhibited reduced activity against myeloma cells, if they had been co-cultured with RPMI-8226 beforehand (data not shown). Addition of antibodies against NKG2D ligands almost abrogated co-culture-induced NKG2D down-regulation as depicted in Fig. 5c: here, addition of antibodies against MICA, MICB and ULBP2 into the co-culture set-up prevented decrease of NKG2D+ NK cells and CD8+ T cells as well as down-regulation of NKG2D expression as determined by MFI. In contrast, the isotype control showed no effect.

Discussion

The presence of raised levels of sMICA has been reported for a variety of cancer entities including Multiple Myeloma [17, 18] and has been suggested to be associated with poor prognosis. We confirm these findings in the largest patient population to date with an increase of sMICA to a median of 175 pg/ml in patients compared to 80 pg/ml in healthy controls. The level of sMICA we found compares well with levels found by others in different malignancies [17, 22]. In addition, we address the question if sMICA can distinguish between MGUS and MM in the clinical setting which has not been investigated yet. Our results show that a level of sMICA >225 pg/ml distinguishes between MGUS and MM with a high sensitivity and positive predictive value. This can be a helpful tool in the sometimes difficult

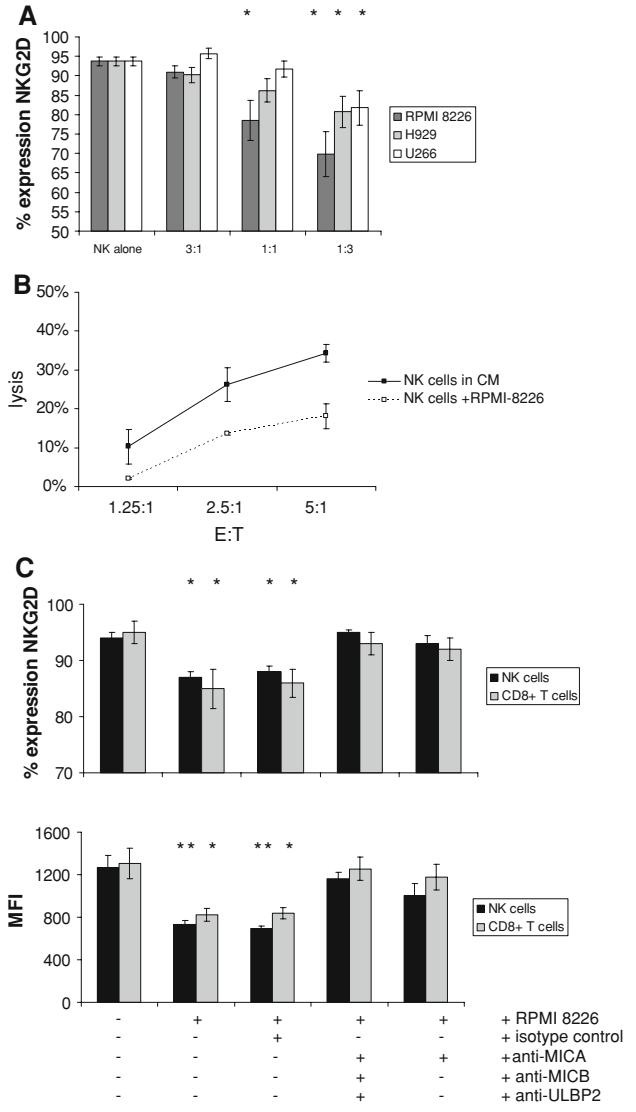

Fig. 5 **a** Differential effect of coculturing effector cells with different tumour cell lines in different tumour cell: NK cell ratios is analysed flow-cytometrically. *CM* complete medium. Here, the mean ± standard error of the mean of four independent experiments is shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to CM; **b** Cytotoxic activity against K562. Purified NK cells cells after 24 h co-culture with RPMI 8226 at 1:1 were evaluated in a standard chromium release assay at various E:T. Representative example (triplicates ± standard deviation) of two independent experiments; **c** Antibodies against NKG2D ligands inhibit down-regulation of NKG2D. *CM* complete medium. Mean ± standard error of the mean of four independent flow-cytometrical analyses is depicted. $^{*}P < 0.05$ $^{**}P < 0.01$ compared to CM

setting of newly diagnosed patients with monoclonal gammopathy.

Besides raised levels of sMICA we detected a significant down-regulation of NKG2D-expressing effector cells in patients with MM comparing well with other reports showing a reduced number of NKG2D-expressing cells in MM as well as in other malignancies [10, 11, 18, 24]. In our patient population, down-regulation of NKG2D when measured as MFI was modest and not statistically significant (from 1,491 to 1,461 in NK cells and from 1,828 to 1,780 in CD8+ $\alpha\beta$ T cells). Since MFI and number of NKG2D-expressing cells show a strong correlation and there is a clear difference in number of NKG2D-expressing cells, we believe this to be a sample size problem—our group is probably not large enough to confirm the effect on NKG2D MFI in vivo.

Several soluble factors like TGFβ have been reported to cause NKG2D down-regulation [12, 25, 26]. In our patient population, the level of TGFβ did not correlate with the expression of NKG2D, making this possibility unlikely. At first sight, the most interesting soluble factor would be shedded NKG2D ligands, which are well known to down-regulate NKG2D in vitro [13] and to be present in a large variety of clinical conditions [27]. This has also recently been suggested as a potential mechanism in MM [18]. However, if the higher levels of sMICA found in the patients' serum caused the lower expression of NKG2D this should lead to a statistically significant correlation between clinical parameters and should be reproducible in vitro. Thus, we sought to determine if a negative correlation of sMICA-levels and NKG2D expression on effector cells was identifiable but we were unable to find such a correlation with either NK cell, CD8+ T cell or $\gamma\delta$ T cell surface expression. To prove the concept of sNKG2D ligand-induced down-regulation, we directly examined the in vitro effect of sMICA on effector cell NKG2D expression in a co-culture system. For titration of concentration-dependent down-regulation we used commercially available MICA, which is a recombinant MICA/Fc chimera. This might be seen as impediment to the activity on the receptor, which is why the experiments need confirmation with MICA from a more natural source like serum or culture-supernatant. In principle, the commercially available MICA is active in the same way sMICA from patients has been reported to be as we could detect a marked down-regulation in our culture system with a clear dose–response demonstrated. However, this effect required a high concentration of 1 μg/ml whereas lower concentrations or the presence of patient serum or culture supernatant revealed no effect on NKG2D expression. We thus conclude that the NKG2D down-regulation seen in the clinical setting of MM is not induced by soluble ligands.

It has previously been suggested that down-regulation of NKG2D could result from persistent contact with NKG2D-ligand expressing cells [28, 29]. To address this question, we co-cultured effector cells with tumour cells expressing different levels of NKG2D ligands (Table 2) before analysing them flow-cytometrically. In this set-up, we detected a significant decrease of NKG2D+ NK cells and down-regulation of NKG2D expression which was more marked

when the co-cultured tumour cells had a high surface expression of MICA as is the case with RPMI 8226 for example. Similar results were obtained for CD8+ T cells, which compares well with results by Osaki et al., who found that cell-bound MICA rather than sMICA led to decreased NKG2D expression on CD8+ T cells in gastric cancer [11]. In contrast, co-culture with myeloma cells with low level expression of MICA only decreased NKG2D+ NK cells and NKG2D expression at a ratio of 1:3 lymphocytes : myeloma cells ($P = 0.02$ and $P = 0.04$, respectively). This might be explained by the lack of MICA-expression whilst expressing a low level of other NKG2D ligands.

In the literature, not only tumour cells but also activated monocytes, dendritic cells or CD4+ T cells are known to express NKG2D ligands. These have been shown to be similarly capable of down-regulating NKG2D in a contact dependent fashion, if MICA is believed to be the most important factor [30]. Interestingly, other NKG2D ligands seem to exert a less pronounced effect if they are cell-bound since Cerboni et al. found that sMICB derived from CD4+ T cells in unselected PBMC was more potent at down-regulating NKG2D than cell-bound MICB [31]. In any case, the observation of potentially suppressive effects of upregulated NKG2D ligand expression leads to a cautious interpretation of enthusiastic reports on the induction of NKG2D ligand expression by various drugs [32, 33].

It has been shown extensively, that NKG2D down-regulation leads to impaired function [28] and we also found that effector cells thus co-cultured showed a significant impairment of cytotoxic activity against K562, a cell line known to be killed mainly via NKG2D. Similar results were obtained for IL-2 stimulated CD8+ cells, which, when co-cultured, exhibited reduced activity against myeloma cells. Co-culture-induced NKG2D down-regulation could be almost abrogated by addition of antibodies against NKG2D ligands, leading us to the conclusion that NKG2D ligands play a vital role in the down-regulation of NKG2D expression.

Our results support the value of sMICA as a potentially useful clinical marker in the setting of MGUS and MM. We also found a reduced number of effector cells expressing NKG2D in patients with MM, which adds to the immunosuppression found in patients with this disease. However, we were unable to determine a significant correlation between these two findings, neither in vivo nor in vitro. We thus postulate that NKG2D down-regulation in MM might be caused by other factors than soluble NKG2D ligands and that it might be a contact-dependent process.

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