Functionally active NKG2A-expressing natural killer cells are elevated in rheumatoid arthritis patients compared to psoriatic arthritis patients and healthy donors

S.T.A. van Bijnen[1,2], M. Cossu[3,4], M.W.H. Roeven[1,2], T.L. Jansen[5], F. Preijers[2], J. Spanholtz[6], H. Dolstra[2], T.R.D.J. Radstake[3]

[1]Department of Hematology, Radboud University Medical Center, Nijmegen, the Netherlands; [2]Department of Laboratory Medicine, Laboratory of Hematology, Radboud University Medical Center, Nijmegen, the Netherlands; [3]Department of Rheumatology, Clinical Immunology and Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, the Netherlands; [4]Referral Center for Systemic Autoimmune Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [5]Department of Rheumatology, Radboud University Medical Center, Nijmegen, the Netherlands; [6]Glycostem Therapeutics, Oss, the Netherlands.

Abstract

Objective

Natural killer cell receptors (NKR) have been implicated in rheumatoid (RA) and psoriatic arthritis (PsA) pathogenesis. To gain more insight into their role, we characterised NKR (co-)expression patterns on NK and T cells and NK cell function in RA and PsA.

Methods

The frequency of NK and T cells expressing killer like immunoglobulin (KIR) and NKG2 receptors and natural cytotoxicity receptors was assessed by 10-colour flow cytometry in peripheral blood of 23 RA, 12 PsA patients and 18 healthy donors (HD). NK cell cytotoxicity and IFN-gamma production was assessed in 8 RA patients and 8 HD.

Results

In RA but not PsA, the frequency of NK cells (median; range) expressing NKG2A (42%; 14-81%) was elevated compared to HD (23%; 9-58%). NKG2A$^+$ NK cells predominantly lack KIR, but display normal cytotoxicity and IFN-γ production. In contrast, RA patients with normal NKG2A$^+$ NK cell frequency have less functional NK cells compared to HD. T cells expressing Fc-gamma receptor CD16 were elevated in RA (median 0.75%) versus HD (0.3%). Furthermore, T cells expressing the KIRs CD158ah in both RA (0.7%) and PsA (0.3%), and CD158e1e2 in RA (1.5%) were elevated compared to HD (0.2% and 0.4%, respectively). In RA, CD4$^+$ T cells expressing the KIRs CD158ah, CD158b1b2j and CD158e1e2 were low (<2%) but significantly elevated compared to HD.

Conclusion

This study demonstrates the presence of an elevated, functionally active NKG2A$^+$ KIR$^-$ NK cell population in RA. Together with an elevated frequency of NKR-expressing T cells, these changes may reflect differential pathogenetic involvement.

Key words

rheumatoid arthritis, psoriatic arthritis, NK cells, T cells, NK cell receptors

*Sandra T.A. van Bijnen, MD, PhD**
*Marta Cossu, MD**
Mieke W.H. Roeven, MD
Tim L. Jansen, MD, PhD
Frank Preijers, PhD
Jan Spanholtz, PhD
*Harry Dolstra, PhD***
*Timothy R.D.J. Radstake, MD, PhD***

**M. Cossu and S.T.A. van Bijnen share first co-authorship;*
*** H. Dolstra and T.R.D.J. Radstake share last co-authorship.*

Please address correspondence to:
T.R.D.J. Radstake, MD, PhD,
Department of Rheumatology and Clinical Immunology,
University Medical Center Utrecht,
Heidelberglaan 100,
3584 CX Utrecht, The Netherlands.
E-mail: T.R.D.J.Radstake@umcutrecht.nl

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Introduction

Natural killer (NK) cell biology is regulated by the balance between activating and inhibitory NK receptors (NKR). Inhibitory receptors, including inhibitory isoforms of killer immunoglobulin receptors (KIRs/CD158 isoforms), generally recognise major histocompatibility complex class I (MHC-I) molecules. Activating receptors, including activating isoforms of KIRs and NKG2 receptors, bind with lower affinity to MHC-I molecules or other ligands. Together, they tune NK cell response upon NKR binding (1). In contrast to NK cells, NKR function on T cells is not fully elucidated. Normally, NKR expressing T cells are infrequent, typically <5% of $CD8^+$ T cells (2). However, in conditions of chronic immune activation such as autoimmune diseases, elevated numbers are found (3).

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are two hallmark chronic inflammatory diseases sharing clinical features such as chronic synovial inflammation leading to destruction of the joints. Accumulating evidence suggests a role of NK cells in both conditions. How NK cells contribute to their pathogenesis and/or the difference in clinical features between RA and PsA is, however, unknown. Studies in PsA showed that the activating KIR genes KIR2DS1 and/or KIR2DS2 increase disease susceptibility, particularly when the HLA ligands for the corresponding inhibitory receptors KIR2DL1 and KIR2DL2/3 are lacking (4-6). In RA, the KIR2DS2 gene was associated with a subgroup of patients with vasculitis (7). In addition, polymorphisms in other NKR genes modify disease risk, including NKG2A, NKG2C, NKG2D and CD244 (8, 9). For PsA, associations with HLA-Cw*0602, a KIR ligand (10), and MIC polymorphisms were reported (11).

Besides genetic associations, immunophenotypical studies revealed a different NKR expression between RA patients and their healthy counterparts. In RA, $CD4^+$ T cells lacking CD28 are increased. This subset partially expresses activating KIRs and NKG2D, whereas $CD4^+CD28^-$ T cells from healthy donors do not (12, 13). Importantly, RA synoviocytes also express the NKG2D ligand MIC (13). A higher frequency of $CD28^-$ T cells expressing the general NK cell marker CD56 was shown in RA patients compared to HD, particularly in those with extra-articular manifestations (14). The role of NK cells in RA pathogenesis is still unclear. Depletion of NK cells in mouse models of collagen-induced arthritis (CIA) has shown both protection and exacerbation of arthritis (15, 16). In RA synovial fluid, $CD56^{bright}$ NK cells are abundant. This subset potently produces TNF-α and IFN-γ, and expresses CD94/NKG2A and low levels of KIRs (17-19).

Altogether, phenotypical, functional and genetic studies suggest a role for NK cells and NKR expressing T cells in RA and PsA pathogenesis. Given the role for NKR, it is tempting to speculate that the fact that RA is considered an autoimmune disease but PsA as an autoinflammatory condition could be determined by a different NK and NKR biology in these conditions. To study this, we investigated the phenotype and function of NK and NKR expressing T cells in RA and PsA. In RA, but not PsA, we observed differences in NK cell phenotype and function compared to healthy individuals. Furthermore, KIR expression by T cells was elevated in both RA and PsA whereas the frequency of $CD16^+$ T cells was significantly increased in RA.

Matherials and methods

Patients and cell isolation

Blood samples from 31 RA, 12 PsA patients and 26 HD were collected after obtaining written informed consent. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA (20) or the Classification of Psoriatic Arthritis Study Group criteria for PsA (21). This study was approved by the local medical ethics committee and performed in accordance with the Declaration of Helsinki. Clinical characteristics of patients and HD included for phenotypical analysis and *in vitro* cellular assays are shown in Table I and Table II respectively. For functional assays, RA patients were further stratified according to their frequency of $NKG2A^+$ NK cells. NKG2A*high* are patients with a frequency of $NKG2A^+$ NK cells >1 standard deviation (SD) higher than the

Table I. Clinical characteristics of patients and healthy donors included for phenotyping.

	HD	RA	PsA
Number	18	23	12
Age (y; median, range)	44 (25-60)	59 (15-79)	44 (25-78)
M/F (number of patients; ratio)	8/10 (0.80)	10/13 (0.77)	5/7 (0.71)
Disease duration (y; median, range)	NA	2 (0.08-23.1)	2.4 (0-11)
Disease activity:	NA		
DAS28 score (median, range)		3.3 (2.38-4.93)	NA
TJC (median, range)		NA	1 (0-7)
SJC (median, range)		NA	1 (0-7)
ESR (mm; median, range)		9 (2-29)	6 (0-44)
CRP (g/l; median, range)		<5 (5-23)	<5 (<5-22)
Therapy (number of patients):	NA		
None		2	2
MTX		6	4
MTX + anti- TNF-α + HCQ		1	0
MTX + anti- TNF-α		6	1
MTX + rituximab		2	0
MTX + sulfasalazine		2	0
MTX + HCQ		1	0
MTX + prednisolone		1	1
anti- TNF-α		0	3
sulfasalazine		1	1
anti- TNF-α + sulfasalazine		1	0
abatacept		1	0

y: years; NA: not applicable; TJC: tender joint count; SJC: swollen joint count; MTX: methotrexate; HCQ: hydroxychloroquinine.

Table II. Clinical characteristics of patients and healthy donors included for cellular assays.

	HD	RA	NKG2A*norm* RA	NKG2A*high* RA
Number	8	8	5	3
NKG2A (% of positive NK cells; mean, SD)	46 (±10.3)	54.3 (±10.8)	48.1 (±7.4)	64.6 (±6.5)
Age (y; median, range)	47 (44-60)	48 (44-59)	47 (44-59)	48 (44-50)
M/F (number of patients; ratio)	3/5 (0.6)	3/5 (0.6)	1/4 (0.25)	2/1 (2)
Disease duration (y; median, range)	NA	11 (2.1-28)	16 (4-28)	11 (2.1-14)
Disease activity (DAS28; median, range)	NA	2.71 (1.13-3.44)	3.03(1.13-3.44)	2.45 (1.27-2.97)
Therapy (number of patients)	NA:			
none		1	1	0
MTX		2	1	1
MTX + NSAID		3	2	1
MTX + prednisolone		1	1	0
Leflunomide + prednisolone		1	0	1

SD: standard deviation; y: years; NA: not applicable; MTX: methotrexate; NSAID: non-steroidal, anti-inflammatory drugs.

mean frequency of NKG2A+ NK cells in HD; all other patients were defined as NKG2A*norm* and have a frequency of NKG2A+ NK cells similar to HD. Patients receiving biologicals and/or hydroxychloroquine were excluded from the *in vitro* cellular assays. Peripheral blood mononuclear cells (PBMC) were freshly isolated from whole blood by Ficoll Hypaque gradient separation and used for flow cytometry or cellular assays.

Flow cytometry

PBMC were stained with 3 different antibody panels. Antibody panel composition and antibody details are shown in Table III. Samples were measured on a 10-colour, 3-laser Navios flow cytometer (Beckman Coulter, Fullerton, CA, USA). After optimising the PMT settings, spectral overlap was compensated using single antibody stainings. Cells were gated on peripheral blood lymphocytes (PBL) based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity (Supplementary Fig. 1A). Percentages of positive cells were determined within $CD3^+$ (T cells), $CD3^+CD4^+CD8^-$ ($CD4^+$ T cells), $CD3^+CD8^+CD4^-$ ($CD8^+$ T cells) and $CD3^-CD56^+$ (NK) cells. A minimum of 400.000 cells was measured. In each T and NK cell subpopulation, at least 2.500 cells were analysed. Data were analysed with Kaluza software (Beckman Coulter).

Cellular assays

NK cells were enriched from PBMCs by negative selection with an NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The human erythroleukemic target cell line K562 was maintained in IMDM (Invitrogen, Carlsbad, CA, USA) containing penicillin/streptomycin (50 U/50 μg/ml; MP Biomedicals, Solon, OH, USA) and 10% FCS (Integro, Zaandam, The Netherlands). Flow cytometry-based cytotoxicity assays were performed as described previously (22). Briefly, target cells were labelled with 5 μM carboxyfluorescein diacetate succimidyl ester (CFSE; Molecular Probes Invitrogen, Eugene, OR, USA) in a concentration of $1x10^7$/mL for 10 minutes at 37°C. CFSE-labelled target cells were resuspended in IMDM with 2% human serum (PAA Laboratories GmbH, Pasching, Austria) at a final concentration of 0.3–$3x10^5$/ml. NK cells were resuspended at a final concentration of $3x10^5$/ml and co-cultured with target cells at different effector:target (E:T) ratios in 96-wells round-bottom plates. After 18 hours, the number of viable target cells was quantified by flow cytometry based on FSC/SSC and exclusion of dead/apoptotic cells using the plasma membrane integrity marker DRAQ7 (Biostatus Ltd., Shepshed, UK). Samples were acquired on a FC500 flow cytometer (Beckman Coulter). Target cell survival was calculated as follows: % survival = (absolute no. viable $CFSE^+$ $DRAQ7^-$ target cells co-cultured with NK cells / absolute no. viable $CFSE^+$ $DRAQ7^-$ target cells cultured in medium)*100. The percentage of specific killing was 100% survival.

Table III. Antibody panels.

Channel (antibody conjugate)	Panel 1	Panel 2	Panel 3
FL1 (FITC)	CD4	CD28*	CD16*
FL2 (PE)	CD56*	NKG2C†	CD336 (NKp44)
FL3 (ECD)	CD8	CD8	CD3
FL4 (PE-Cy5.5)	CD158i	CD4	CD337 (NKp30)
FL5 (PE-Cy7)	CD158b1b2j	CD56	CD335 (NKp46)
FL6 (APC)	NKG2D	CD158e1e2	NKG2D
FL7 (APC-Alexa700)	CD158ah	CD244	CD8
FL8 (APC-Alexa750)	CD3	CD3	CD56
FL9 (Pacific Blue)	NKG2A	NKG2A	CD94
FL10 (Krome Orange)	CD45	CD45	CD45

Unless otherwise specified, all antibodies were purchased from or kindly provided by Beckman Coulter Inc. (Marseille, France). *Obtained from BD Biosciences, Franklin Lakes, NJ, USA. †Obtained from R&D systems, Minneapolis, MN, USA.

To determine IFN-γ production by IL-12/IL-18 stimulated NK cells (IL-12 10 ng/ml, IL-18 100 ng/ml; Immunotools, Friesoythe, Germany), NK cells were seeded in a final concentration of $2.5x10^5$/ml in 96-wells round-bottom plates. After 1 hour, GolgiPlug (1 µl/ml, BD Biosciences, San Diego, CA, USA) was added. After overnight incubation at 37°C, cells were stained for cell surface markers (anti-CD56-BV510, clone HCD56; anti-CD158e1-PE, clone DX9; anti-CD158b-PE, clone DX27; anti-CD158a/h/g-PE, clone HP-MA4; all purchased from Biolegend, San Diego, CA, USA; anti-NKG2A-APC, clone Z199; Beckman Coulter, Marseille, France; and the eFluor780 Fixable Viability Dye; eBioscience, San Diego, CA, USA). NK cells expressing at least one among KIR2DL1/KIR2DS1 (CD158 a/h), KIR2DS3, KIR2DS5 (CD158g), KIR2DL2/KIR2DL3 (CD158b) or KIR3DL1 (CD158e) were considered KIR^+; all other cells were considered KIR^-. Subsequently, cells were treated with fixation/permeabilisation buffer (eBioscience), stained for intracellular IFN-γ (FITC-coniugated, clone B27; BD Biosciences) and acquired on a Gallios flow cytometer (Beckman Coulter). Analysis was performed with Kaluza software (Beckman Coulter) using unstimulated cells as control. The gating strategy is shown in Supplementary Figure 2A.

Statistics

Mann-Whitney U-test or Kruskal-Wallis test was used to compare T and NK cell subset frequencies, killing and IFN-γ expression, as appropriate. Statistical significance was accepted for *p*-values below 0.05. Correlations between lymphocyte subset frequencies and clinical characteristics were determined by linear regression analysis.

Results

Increased frequency of NKG2A expressing NK cells in RA but not PsA patients

NK cell frequency (median; range) within PBL was similar in RA (12%; 2–32%), PsA (10%; 2–27%) and healthy donors (HD) (11%; 4–27%). No significant differences were found in the median frequencies of $CD56^{bright}$ NK cells in RA (8.6%, range 1–16%), PsA (6.7%, range 3–25%) and HD (6.1%, range 1–15%), nor in $CD56^{dim}$ NK cells (Supplementary Fig. 1B). In RA patients, the frequency of NK cells expressing NKG2A (median 42%, range 14–81%) was significantly higher than in HD (median 23%, range 9–58%, $p<0.01$, Fig. 1A-B). This also accounted for CD94, the other component of the NKG2A/CD94 heterodimer ($p=0.02$, Fig. 1A-B). The median frequency of $NKG2A^+$ NK cells was higher in pa-

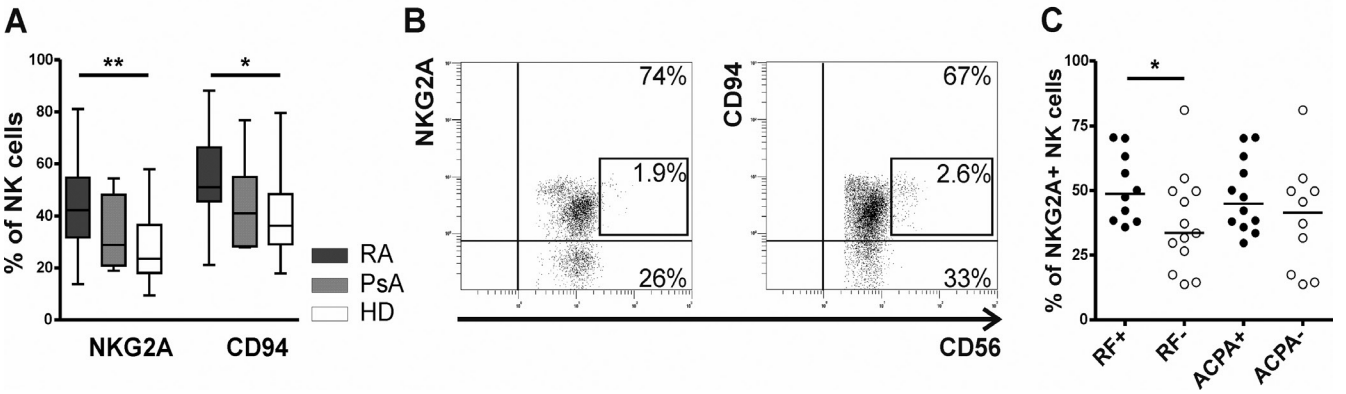

Fig. 1. Increased frequency of NKG2A and CD94 expressing NK cells in RA patients compared to HD.

A. Expression of NKG2A and CD94 within NK cells of RA patients, PsA patients and healthy donors (HD). Cells were gated on peripheral blood lymphocytes based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity. Percentages of positive cells were determined within $CD3^-CD56^+$ (NK) cells. Lines represent the median percentage of positive cells within the $CD56^+CD3^-$ NK cell population, boxes indicate 25th and 75th percentiles, and outer whiskers indicate the most extreme data points of 23 RA patients, 12 PsA patients, and 18 HD.

B. A representative example of a RA patient with $NKG2A^+$ and $CD94^+$ NK cells. Flow cytometry dot plots were gated on NK cells and show log fluorescence intensity of NKG2A/CD94 (y-axis) and CD56 (x-axis), demonstrating that $NKG2A^+$ and $CD94^+$ NK cells are predominantly $CD56^{dim}$. Percentages indicate the percentage of cells within a specific quadrant. Quadrants were set to define $NKG2A^+$ and $CD94^+$ cells, and to distinguish between $CD56^{dim}$ and $CD56^{bright}$ NK cells.

C. Scatter dot plot comparing the frequency of $NKG2A^+$ NK cells in RA patients with or without RF or ACPA. Lines represent median percentages. * $p<0.05$, ** $p<0.01$, Mann-Whitney U-test. RF: rheumatoid factor; ACPA: anti-citrullinated protein antibodies.

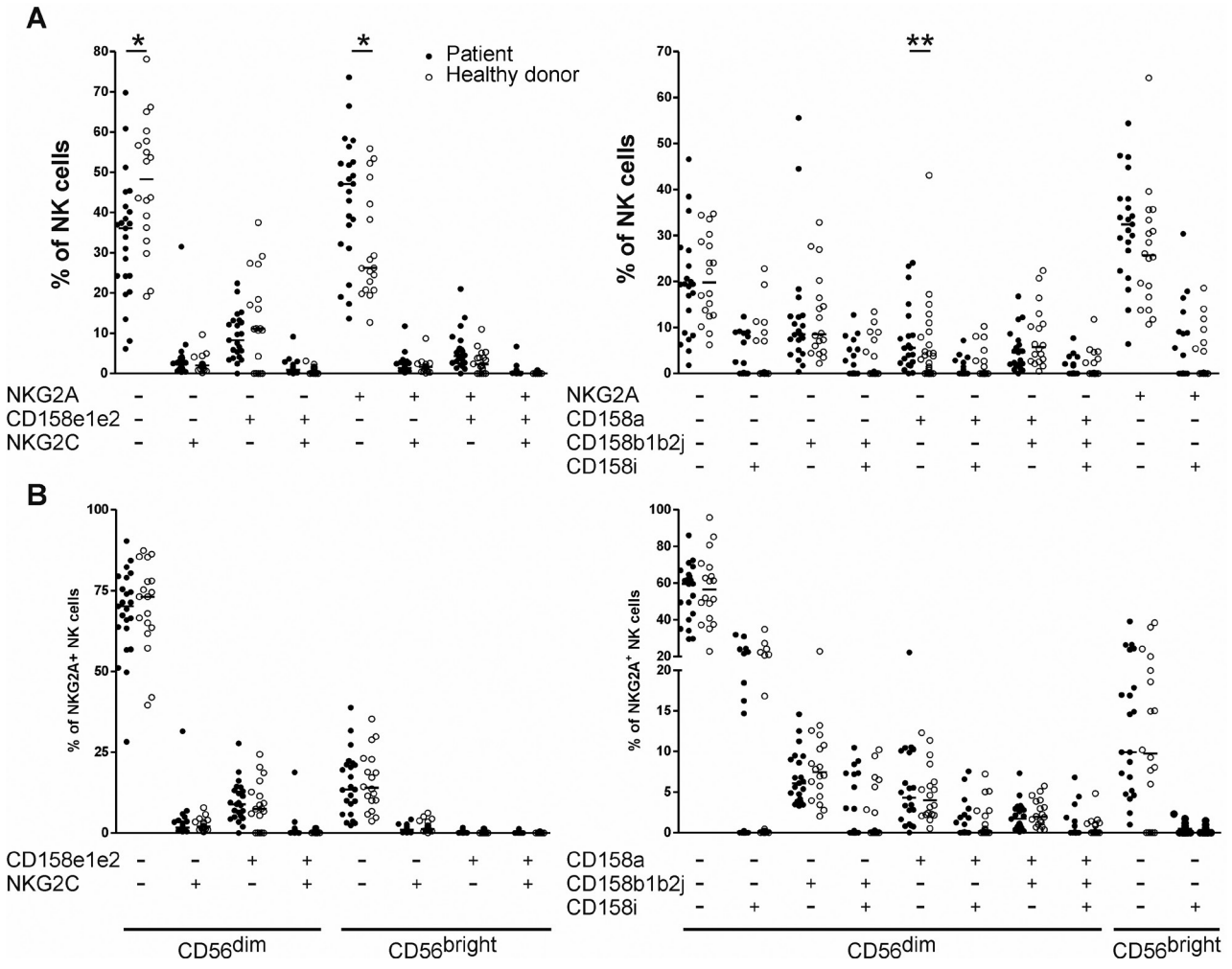

Fig. 2. NKG2A, KIR, NKG2C co-expression patterns on NK cells differ in RA patients and HD.

A. Scatter dot plots comparing NKR co-expression patterns within the total population of NK cells between RA patients (•) and HD (°). Lines represent the median percentage of positive cells within the $CD56^{+}CD3^{-}$ NK cell population. Below each column set of RA patients and HD, the presence or absence of a certain NKR is indicated by + or -. Left panel: co-expression patterns of NKG2A, CD158e1e2 and NKG2C. Right panel: co-expression patterns of NKG2A, CD158ah, CD158b1b2j and CD158i. $NKG2A^{+}$ NK cell subsets with expression of either CD158ah or CD158b1b2j were not shown since their frequency was low (generally <10%) and identical to HD.

B. Scatter dot plots characterising NKR co-expression patterns within $NKG2A^{+}$ NK cells and compared between RA patients (•) and HD (°). Lines represent the median percentage of cells within the $NKG2A^{+}$ NK cell population expressing (+) or lacking (-) the NKR as indicated below the graphs. Left panel: co-expression patterns of CD158e1e2 and NKG2C within $CD56^{dim}$ $NKG2A^{+}$ NK cells (left four columns) and $CD56^{bright}$ $NKG2A^{+}$ NK cells (right 4 columns). Right panel: co-expression patterns of CD158ah, CD158b1b2j and CD158i within $CD56^{dim}$ $NKG2A^{+}$ NK cells (left 8 columns) and $CD56^{bright}$ $NKG2A^{+}$ NK cells (right 2 columns). $CD56^{bright}$ $NKG2A^{+}$ NK cell subsets with expression of either CD158ah, CD158b1b2j or CD158i were omitted from the figure since their frequency was low (generally <10%) and similar to HD. * $p<0.05$, ** $p<0.01$, Mann-Whitney U-test.

tients with rheumatoid factor (RF) (49%, range 36–71%) than in those without (34%, range 14–81%, Fig. 1C). No correlations were found with other clinical parameters such as disease activity (DAS28 score, ESR, CRP, erosiveness), the presence of anti-citrullinated protein antibodies (ACPA), duration of disease, patient age, or type of treatment (data not shown).

Although in both RA and PsA the frequency of KIR, Natural cytotoxicity receptors (NCR; Nkp 30, 44 and 46), NKG2C, NKG2D and CD244 expressing NK cells was similar to HD (data not shown), we found differences in co-expression patterns of combinations of NKR in RA but not PsA (Fig. 2). In RA, the frequency of $NKG2A^{+}$ NK cells negative for CD158e1e2 and NKG2C (median 47%, range 14–74%) was elevated compared to HD (median 26%, range 13–56%, $p<0.05$). Though typically below 10%, the median percentage of $NKG2A^{+}$ $CD158e1e2^{+}$ $NKG2C^{+/-}$ cells was higher than in HD ($p<0.05$, Fig. 2A). In addition, $CD158ah^{+}$ $NKG2A^{-}$ $CD158b1b2j^{-}$ $CD158i^{-}$ NK cells were increased in RA (median 6%, range 0–24%) compared to HD (median 0%, range 0–43%, $p=0.001$, Fig. 2A).

Subsequent characterisation of NKR co-expression within the $NKG2A^{+}$ NK cell population did not reveal differences between RA and HD (Fig. 2B). The majority of $NKG2A^{+}$ NK cells (median; range) in RA is $CD56^{dim}$ and does not express any other receptor tested ($CD158e1e2^{-}NKG2C^{-}$ 70.1%; 28-90%, $CD158a^{-}CD158b1b2j^{-}CD158i^{-}$ 53.9%; 40-87%), apart from CD244 and NKG2D

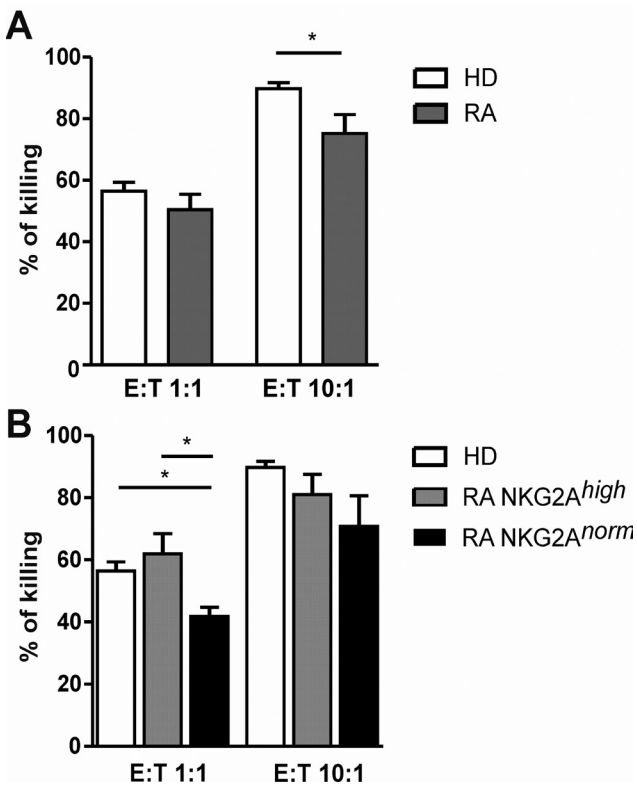

Fig. 3. Normal cytotoxicity by NK cells of NKG2A*high*, but not NKG2A*norm* RA patients.
Cytotoxicity of NK cells isolated from HD and RA patients against K562 target cells. Specific lysis was determined after 18 hours of co-culture in a FCM-based cytotoxicity assay at E:T 1:1 and 10:1 ratios. Data are displayed as means ± standard error of the mean (SEM) of the means of triplicate wells from 8 different experiments.
A. Specific cytotoxicity compared between HD and RA patients overall. * $p<0.05$, Mann-Whitney U-test.
B. Specific cytotoxicity compared between HD, NKG2A*high* and NKG2A*norm* RA patients. * $p<0.05$, Kruskal-Wallis test.

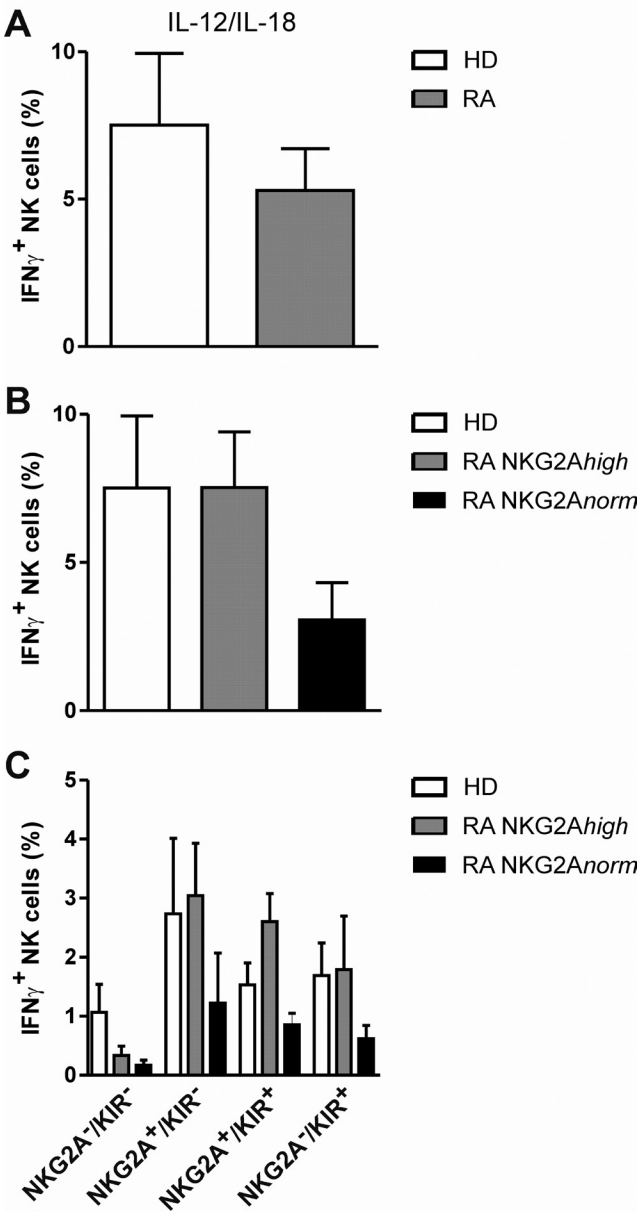

Fig. 4. Normal IFN-γ production in IL-12/IL-18-stimulated NK cell subsets of NKG2A*high*, but not NKG2A*norm* RA patients.
IFN-γ expression in NK cells isolated from HD and RA patients after overnight stimulation with IL-12/IL-18. Data are displayed as means ± SEM of the means of triplicate wells from 8 different experiments. All data are shown after subtracting the baseline level of IFN-γ expression by unstimulated NK cells.
A. IFN-γ expression compared between HD and RA patients overall.
B. IFN-γ expression compared between HD, NKG2A*high* and NKG2A*norm* RA patients.
C. IFN-γ expression compared between HD, NKG2A*high* and NKG2A*norm* RA patients stratified within the different NKG2A/KIR subsets: NKG2A$^-$/KIR, NKG2A$^+$/KIR$^-$, NKG2A$^+$/KIR$^+$, NKG2A$^-$/KIR$^+$.

which were present on virtually all NK cells (data not shown). Collectively, these data indicate that in a subset of the RA patients, especially those with elevated RF, NKG2A$^+$KIR$^-$ CD56^{dim} NK cells are elevated compared to HD.

NK cells of RA patients with higher frequency of NKG2A$^+$ NK cells (NKG2Ahigh) have normal functionality

To assess whether the higher frequency of NKG2A$^+$ NK cells in RA patients is associated with altered functionality, we first tested cytotoxicity of NK cells against K562 target cells in RA patients and HD. The killing capacity of unstimulated NK cells in RA patients (mean at E:T 10:1 ratio 75%, range 42–92%) was lower than in HD (mean 90%,

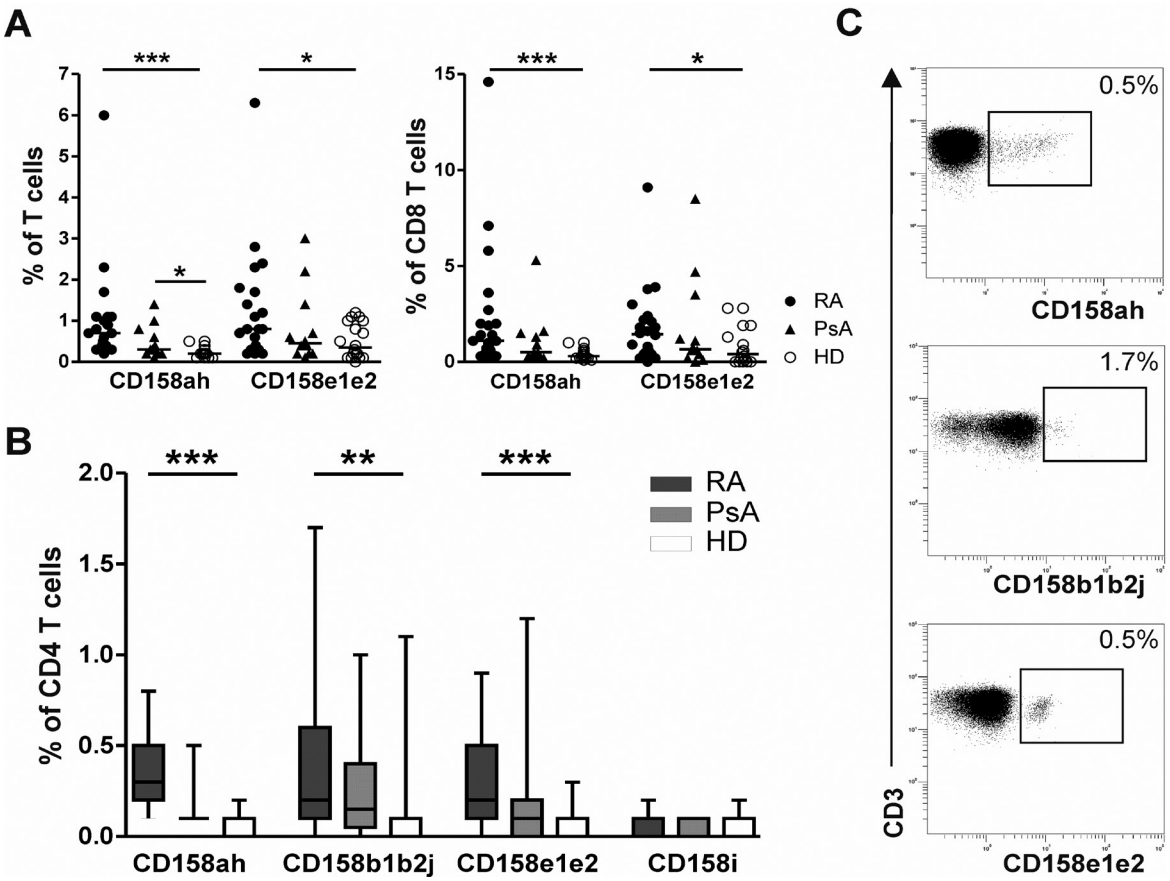

Fig. 5. Increased frequency of KIR expressing T cells in RA and PsA.
KIR expression on T cells in patients with RA and PsA and in HD. Cells were gated on peripheral blood lymphocytes based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity. Percentages of positive cells were determined within $CD3^+$ (T cells), $CD3^+CD4^+CD8^-$ ($CD4^+$ T cells), $CD3^+CD8^+CD4^-$ ($CD8^+$ T cells).
A. Frequency of T cells (left panel) and $CD8^+$ T cells (right panel) expressing CD158ah and CD158e1e2 in patients with RA (•), PsA (▲), and HD (°). Bars represent median percentages.
B. Frequency of $CD4^+$ T cells expressing the KIRs CD158ah, CD158b1b2j, CD158e1e2 and CD158i. Lines represent the median percentage of positive cells within the $CD4^+$ T cell population, boxes indicate 25th and 75th percentiles, and outer whiskers indicate the most extreme data points of 23 RA patients, 12 PsA patients, and 18 HD.
C. Flow cytometry dot plots of RA patients with high proportions of $CD4^+$ T cells expressing KIR. Plots were gated on $CD4^+$ T cells and show log fluorescence intensity for CD3 (y-axis) and CD158ah (top panel), CD158b1b2j (middle panel) and CD158e1e2 (lower panel). Percentage within the plot indicates the proportion of $CD4^+$ T cells positive for the NKR indicated on the X-axis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Mann-Whitney U-test.

range 85–98%, $p<0.05$; Fig. 3A). Given the variability in the NKG2A$^+$ NK cell frequency within RA patients, we next discriminated between the killing potential of NKG2A*norm* and NKG2A*high* RA patients. Only NKG2A*norm* patients displayed lower lysis capability compared to HD and NKG2A*high* RA patients (both $p<0.05$, Fig. 3B).
NK IFN-γ response to IL-12/IL-18 was similar between RA patients and HD (Fig. 4A). Upon stratification for NKG2A*norm* and NKG2A*high* patients we observed a lower but non-significant difference in IFN-γ expression in NKG2A*norm* patients compared to NKG2A*high* patients as well as healthy controls (Fig. 4B). Further analysis of NK cell subsets (NKG2A$^-$/KIR$^-$, NKG2A$^+$/KIR$^-$, NKG2A$^+$/ KIR$^+$, NKG2A$^-$/KIR$^+$) showed a similar distribution within NKG2A*norm*, NKG2A*high* RA patients and HD (Supplementary Fig. 2B-C); however, a trend towards an increase of the NKG2A$^+$/KIR$^-$ NK cell subset in the NKG2A*high* subset of RA patients is visible and consistent with our phenotypic characterisation. NK cell subsets in the NKG2A*norm* RA patients, which have a similar distribution compared to HD, appear less capable of IFN-γ production (Fig. 4C). In contrast, the different NK cell subsets in NKG2A*high* RA patients produce IFNγ equally efficient as HD. These data indicate that the elevated NKG2A$^+$ NK cell population found in part of RA patients is functionally responsive to IL-12/IL-18 stimulation.

Increased frequency of KIR and CD16 expressing T cells in patients with RA but not PsA

As NKR expressing T cells have been implicated in pathology, we determined their frequency in RA and PsA patients. No differences were found in the relative frequencies of $CD4^+CD8^-$, $CD8^+CD4^-$, CD4CD8 double negative (DN) and CD4CD8 double positive (DP) T cells (data not shown). The frequencies of CD56, NKG2, NCR, CD16 and CD244 expressing T cells were similar between RA, PsA and HD (data

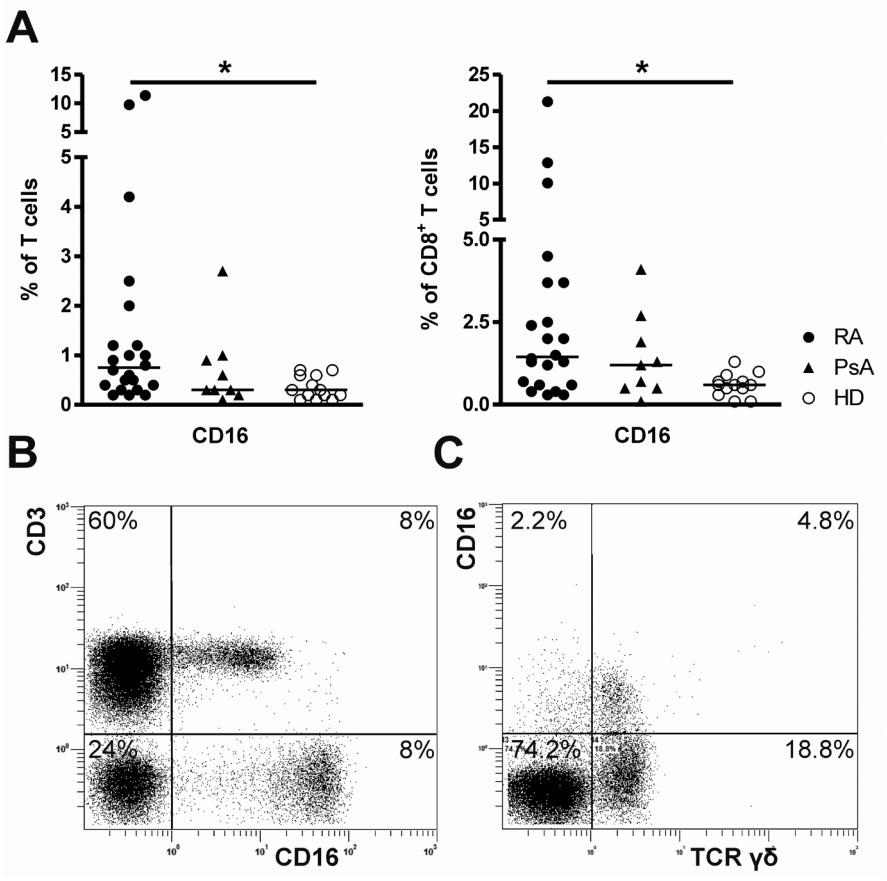

Fig. 6. Increased frequency of T cells expressing CD16 in RA.
CD16 expression on T cells in RA. Cells were gated on peripheral blood lymphocytes based on characteristic forward scatter (FSC) and sideward scatter (SSC) properties and CD45 positivity. The percentage of positive cells was determined within $CD3^+$ (T cells).
A. Frequency of T cells (left panel) and $CD8^+$ T cells (right panel) expressing CD16 in patients with RA (•), PsA (▲), and HD (∘). Lines represent median percentages.
B. Flow cytometry dot plot of a RA patient with a high proportion of $CD16^+$ T cells. The plot was gated on $CD45^+$ lymphocytes and shows log fluorescence intensity of CD3 (y-axis) and CD16 (x-axis).
C. Flow cytometry dot plot gated on $CD3^+$ T cells of a RA patient showing CD16 (y-axis) and TCR γδ (x-axis). * $p<0.05$, Mann-Whitney U-test.

not shown). Moreover, the frequency of $CD4^+CD28^-$ T cells was equal to HD (data not shown). In contrast, KIR expression on T cells in RA was different as CD158ah expressing T cells were more frequent in both RA (median 0.7%, range 0.2–6%) and PsA (median 0.3%, range 0.1–1.4%) compared to HD (median 0.2%, range 0.1–0.5%) ($p<0.0001$ and $p=0.05$ respectively, Fig. 5A). In RA patients, the median frequency of CD158e1e2 expressing cells within the total T cell population (1.5 *vs*. 0.4%, range 0–9.1% *vs*. 0–2.8%) and within $CD8^+$ T cells (1.5%, range 0–9.1%) was increased compared to HD (0.35/1.5%, range 0–1.2/0–2.8% respectively) ($p=0.02$ and $p=0.04$, Fig. 5A). The frequencies of KIR^+CD4^+ T cells were generally below 1% in both patients and controls. Nevertheless, in RA patients the median frequency of $CD158ah^+$ (0.3%, range 0.1–0.8%, $p<0.0001$), $CD158b1b2j^+$ (0.2%, range 0–1.7%, $p=0.001$) and $CD158e1e2^+$ (0.1%, range 0–0.1%, $p=0.0001$) $CD4^+$ T cells was significantly higher than in HD (medians 0.1/0.1/0%, range 0–0.2 / 0–1.1/0–0.3%, respectively) (Fig. 5B-C). Neither in RA nor PsA, elevated NKR expressing T cell numbers correlated to clinical parameters such as disease activity (DAS28 score or tender and swollen joint counts, ESR, CRP, erosiveness), the presence of ACPA or RF, duration of disease, patient age, or type of treatment (data not shown).

Finally, we investigated CD16 expression on T cells. $CD16^+$ T cells were significantly higher in RA patients (median 0.75%, range 0.2–11.4%) than in HD (median 0.3%, 0.1–0.7%) ($p=0.004$, Fig. 6A-B). Also among $CD8^+$ T cells, the percentage of $CD16^+$ T cells was increased (median 1.5%, range 0.3–21.3%) compared to HD (median 0.6%, range 0.1–0.3%) ($p=0.005$, Fig. 6A-B). The majority of $CD16^+$ T cells (69%) were γδ T cells, as was shown in one RA patient with a high frequency of $CD16^+$ T cells (11.7%, Fig. 6C). We did not find significant correlations between $CD16^+$ T cell numbers and disease activity (DAS28 score, ESR, CRP, erosiveness), the presence of ACPA or RF, duration of disease, patient age, or type of treatment (data not shown). Taken together, these data indicate that several T cell populations with expression of NKR are elevated in RA patients.

Discussion

As solid evidence points towards the role of NK cells and NKR-expressing T cells in autoimmune disease (3, 23) we set forth to determine potential differences in the phenotype and/or behavior of these cells in two archetypical chronic inflammatory diseases, RA and PsA. We demonstrated that the NK cell phenotype in peripheral blood from RA but not PsA patients differs from healthy individuals in that $NKG2A^+$ NK cells in RA patients are elevated and have a relatively immature status with impaired cytotoxicity. De Matos *et al*. previously described the presence of IFN-γ-producing $NKG2A^+KIR^-CD56^{bright}$ NK cells in the synovium in different types of arthritis (17). In addition, they showed that this synovial NK cell population expressed specific chemokine receptors suggestive of preferential recruitment from peripheral blood, rather than local maturation (19). The fact that NKG2A*norm* RA patients seem to have NK cells with impaired cytotoxic potential and cytokine production, could reflect the results of Aramaki *et al*., who found decreased NK cell cytotoxicity in RA patients (24).

Pathogenic implications as opposed to protective effects of elevated functionally active $NKG2A^+$ NK cells remain to be uncovered. Both *in vitro*

and *in vivo* studies suggest that blocking NKG2A may be beneficial in RA. Leavenworth *et al*. showed in murine CIA that NK cells are capable of killing pathogenic T_{helper} (T_h) subsets and that anti-NKG2A blocking increased joint infiltration by NK cells, decreased pathogenic T_h cell numbers, and importantly, improved arthritis (25). *In vitro* blocking of NKG2A enhanced NK cell degranulation against autologous activated $CD4^+$ T cells and RA fibroblast like synoviocytes (FLS) (26;27). Other than RF positivity, we did not find other correlations between $NKG2A^+$ NK cell frequency and clinical characteristics, such as disease activity score (DAS28), presence of erosions, elevated inflammatory markers or length of disease.

Our study revealed several novel observations. First, increased KIR expression on T cells in both RA and PsA was observed. Assuming these receptors are activating, as shown for KIR expressing $CD4^+$ T cells in RA (12), these T cells may represent autoimmune effectors. Secondly, we observed a markedly increased frequency of $CD16^+$ ($CD8^+$) T cells in RA but not PsA patients. CD16 is a low-affinity Fc receptor for IgG (Fcγ receptor IIIa) involved in antibody mediated cellular cytotoxicity (ADCC) in NK cells. Its role on T cells is less well characterised. It is found on the majority of healthy donor TCR γδ T cells (28), as well as a small proportion of TCR αβ T cells (29). Our finding of elevated $CD16^+$ ($CD8^+$) T cells in RA patients builds further on the study of Bodman-Smith et al. who reported a trend towards increased frequency of $CD16^+$ TCR γδ T cells in RA patient peripheral blood (30). $CD16^+$ γδ T cells are capable of phagocytosis and antigen presentation via MHC class II (31). $CD16^+$ TCR αβ T cells are predominantly $CD8^+$, $perforin^+$ effector memory T cells, present at increased frequency in patients with chronic hepatitis C infections and other causes of reactive lymphocytosis (29, 32). Triggering of CD16 on TCR αβ T cells results in production of effector molecules such as perforin, granzyme B, IFN-γ and TNF-α (32, 33). The increased frequency of $CD16^+$ T cells reported here, together with the effector phenotype and functional properties reported elsewhere, might very well fit a pathogenetic role in autoimmune disease. Therefore, in our view, this cell population requires functional characterisation in RA.

Our study does not confirm the previously reported increased frequencies of T cells expressing the general NK cell marker CD56 in either RA or PsA patients (14) nor could we confirm an expansion of $CD4^+CD28^-$ T cells in RA patients as initially reported by Schmidt *et al*., particularly in those with extra-articular manifestations (34, 35). This may be explained by differences in treatment and the absence of extra-articular complications in our cohort. Our study included patients on different therapies and only a few drug-naïve individuals. Therefore, it cannot be excluded that the type of treatment affects NK cell phenotype or function. However, the most common treatment modalities in our cohort, *i.e*. methotrexate and anti-TNF-α, rather seem to enhance NK cell cytotoxicity and IFN-γ production (36, 37) and no correlation could be found with the different therapeutic regimen. Furthermore, there were no substantial differences in therapeutic regimen between NKG2A*norm* and *high* patients that could explain the observed functional differences.

In summary, this study demonstrates the presence of an elevated, functionally active $NKG2A^+$ KIR^- NK cell population in RA but not PsA, in addition to a different subset of RA patients with decreased NK cell functionality. Furthermore, we demonstrated increased KIR expression by T cells in both RA and PsA and a higher frequency of $CD16^+$ T cells in RA. These changes in NK and T cell phenotype and function may reflect differential pathogenetic involvement; however, their precise role remains to be determined.

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