

Altered Natural Killer Cell Subsets in Seropositive Arthralgia and Early Rheumatoid Arthritis Are Associated with Autoantibody Status

Paulina Chalan, Johan Bijzet, Bart-Jan Kroesen, Annemieke M.H. Boots, and Elisabeth Brouwer

ABSTRACT. Objective. The role of natural killer (NK) cells in the immunopathogenesis of rheumatoid arthritis (RA) is unclear. Therefore, numerical and functional alterations of CD56^{dim} and CD56^{bright} NK cells in the early stages of RA development were studied.

Methods. Whole blood samples from newly diagnosed, treatment-naïve, seropositive (SP) and seronegative (SN) patients with RA (SP RA, n = 45 and SN RA, n = 12), patients with SP arthralgia (n = 30), and healthy controls (HC, n = 41) were assessed for numbers and frequencies of T cells, B cells, and NK cells. SP status was defined as positive for anticyclic citrullinated peptide antibodies (anti-CCP) and/or rheumatoid factor (RF). Peripheral blood mononuclear cells were used for further analysis of NK cell phenotype and function.

Results. Total NK cell numbers were decreased in SP RA and SP arthralgia but not in SN RA. Also, NK cells from SP RA showed a decreased potency for interferon- γ (IFN- γ) production. A selective decrease of CD56^{dim}, but not CD56^{bright}, NK cells in SP RA and SP arthralgia was observed. This prompted investigation of CD16 (Fc γ R11a) triggering in NK cell apoptosis and cytokine expression. *In vitro*, CD16 triggering induced apoptosis of CD56^{dim} but not CD56^{bright} NK cells from HC. This apoptosis was augmented by adding interleukin 2 (IL-2). Also, CD16 triggering in the presence of IL-2 stimulated IFN- γ and tumor necrosis factor- α expression by CD56^{dim} NK cells.

Conclusion. The decline of CD56^{dim} NK cells in SP arthralgia and SP RA and the *in vitro* apoptosis of CD56^{dim} NK cells upon CD16 triggering suggest a functional role of immunoglobulin G-containing autoantibody (anti-CCP and/or RF)-immune complexes in this process. Moreover, CD16-triggered cytokine production by CD56^{dim} NK cells may contribute to systemic inflammation as seen in SP arthralgia and SP RA. (J Rheumatol First Release April 1 2016; doi:10.3899/jrheum.150644)

Key Indexing Terms:

ARTHRALGIA
RHEUMATOID ARTHRITIS

AUTOANTIBODIES

NATURAL KILLER CELLS
RHEUMATOID FACTOR

Rheumatoid arthritis (RA) is a chronic autoimmune disease. RA is manifested by inflammation of the synovial membrane mediated by joint-infiltrating immune cells. Increased expression of numerous cytokines and cytokine receptors has been observed early in the disease pathogenesis^{1,2,3,4}. This poses a challenge for understanding the primary events in immune dysregulation involved in RA development. Current

data support a role of interleukin 17 (IL-17) in the early phases of RA^{3,5}. Despite the originally postulated pathogenicity of interferon- γ (IFN- γ), several reports demonstrated its protective role in the development of collagen-induced arthritis (CIA), a mouse model of RA^{6,7,8}. The exact protective mechanism of IFN- γ in RA is currently not fully known⁹. Natural killer (NK) cells are primary IFN- γ producers⁹, by which they connect to the adaptive immune response and favor Th1 cell polarization in the course of an inflammatory response^{10,11,12}. NK cell depletion was found to accelerate CIA onset, which was associated with an impaired IFN- γ -dependent regulation of the Th17 response⁷. Also, NK cells contribute to immune tolerance by killing autoreactive T cells and B cells^{13,14}.

NK cells are divided into 2 major subsets based on the expression of CD56 (neural cell adhesion molecule)¹⁵. CD56^{dim} NK cells, which constitute ~90% of peripheral blood NK cells, are characterized by a potent cytotoxic capacity associated with increased perforin, granzyme, and cytolytic granule expression¹⁶. This suggests a primary role for CD56^{dim} NK cells in the killing of autoreactive cells. CD56^{dim}

From the Department of Rheumatology and Clinical Immunology, and the Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands.

Supported by the Groningen University Institute for Drug Exploration.

P. Chalan, PhD, Department of Rheumatology and Clinical Immunology, University of Groningen; J. Bijzet, BS, Department of Rheumatology and Clinical Immunology, University of Groningen; E. Brouwer, MD, PhD, Department of Rheumatology and Clinical Immunology, University of Groningen; A.M. Boots, Prof., Department of Rheumatology and Clinical Immunology, University of Groningen; B.J. Kroesen, PhD, Department of Laboratory Medicine, University of Groningen.

Address correspondence to Prof. A.M. Boots, University Medical Center Groningen, Department of Rheumatology and Clinical Immunology, Hanzplein 1, AA21 Groningen, 9713 GZ, the Netherlands.

E-mail: m.boots@umcg.nl.

Accepted for publication February 5, 2016.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2016. All rights reserved.

cells are more effective in antibody-dependent cellular cytotoxicity when compared to the CD56^{bright} subset, as a result of higher surface expression of FcγRIIIa (CD16). CD56^{bright} NK cells are the minor subset (~10%) within the circulating NK cell pool. However, in secondary lymphoid organs (e.g., lymph nodes^{17,18}) and at several inflammatory sites (e.g., synovial fluid¹⁹, psoriatic plaques²⁰) CD56^{bright} NK cells have been shown to outnumber CD56^{dim} cells. CD56^{bright} NK cells may also have an immunoregulatory role owing to an increased ability (compared to CD56^{dim} subset) to produce pro- and antiinflammatory cytokines^{15,16,21,22,23,24}.

We aimed to investigate the role of NK cells in early stages of RA development, given their potentially initiating capacity in skewing and regulating the immune response. We studied newly diagnosed, treatment-naïve patients with RA and patients with seropositive (SP) arthralgia. SP arthralgia is characterized by the presence of RA-associated autoantibodies [anticyclic citrullinated peptide antibodies (anti-CCP) and/or rheumatoid factor (RF)] and by tender and painful joints (arthralgia) but no synovitis. Previous studies show that 35% of patients with SP arthralgia develop RA after about 1 year of followup²⁵. Thus, in the present study we investigated numerical and functional alterations of CD56^{dim} and CD56^{bright} NK cell subsets in patients with SP arthralgia and newly diagnosed RA.

MATERIALS AND METHODS

Patients. Thirty patients with SP arthralgia were defined based on seropositivity for RF (serum levels ≥ 15 IU/ml) and/or anti-CCP (serum levels ≥ 10 IU/ml), arthralgia in at least 1 joint, and lack of arthritis. Also, 45 patients with early RA SP for anti-CCP and/or RF, 12 patients with early seronegative (SN) RA (anti-CCP- and RF-), and 41 healthy controls (HC) were included in the study (Table 1). All patients with RA, fulfilling 1987 or 2010 American College of Rheumatology classification criteria for RA, were included in the study at the time of diagnosis, before start of treatment with disease-modifying antirheumatic drugs. Patients with SP arthralgia and RA received nonsteroidal antiinflammatory drugs (NSAID) only. HC were included only if, at the time of blood withdrawal, they had no infections, no recent vaccination, and did not use immunosuppressive drugs. All participants gave their written informed consent and the study was approved by the local medical ethics committee (University Medical Center Groningen, the Netherlands).

Analysis of circulating leukocyte populations. Whole blood was analyzed using the BD MultiTest TruCount method with reagents detecting CD45, CD3, CD4, CD8, CD19, CD16/CD56, according to the manufacturer's instructions (BD Biosciences). Flow cytometry was performed on FACS Canto II and analysis was performed using FACS Canto Clinical Software (BD Biosciences).

Analysis of NK cell phenotype and function. Heparin blood was used to isolate peripheral blood mononuclear cells (PBMC) by Lymphoprep (Axis-Shield) density gradient centrifugation, and PBMC were processed for cryopreservation. PBMC from all subjects were thawed at the same time and stained with the following antibodies: CD3 eFluor605NC, CD57 eF450 (eBioscience), CD56 FITC, CD16 Alexa Fluor700, CD94 APC, NKG2D PE-Cy7 (BioLegend), NKG2A PerCP (R&D Systems), and KIR2DL4 (Exbio Praha).

To assess NK cell IFN- γ expression, thawed PBMC were resuspended in RPMI-1640 containing 10% fetal bovine serum (FBS) and 0.6% gentamicin (Life Technologies) at a concentration of 10^6 cells/100 μ l. Cells were incubated with phorbol myristate acetate (PMA) at a final concentration of 50 ng/ml, calcium ionophore at a final concentration of 1.6 μ g/ml (both from Sigma-Aldrich), and BD GolgiPlu (BD Biosciences) diluted 1:1000. After 4 h at 37°C, PBMC were stained with the antibodies against CD3 eFluor605NC and CD56 PE (eBioscience). Cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained with anti-IFN- γ Brilliant Violet 421 antibody (BioLegend).

To assess NK cell degranulation potency, analysis of CD107a was performed. Briefly, thawed PBMC were resuspended in RPMI-1640 with 10% FBS and 0.6% gentamicin at a concentration of 10^6 cells/100 μ l. Cells were stimulated with PMA at the same concentrations as mentioned above in the presence of 0.5 μ g of anti-CD107a Brilliant Violet 421 (BioLegend) antibody. After 1 h, BD GolgiPlug (diluted 1:1000) and BD GolgiStop (diluted 1:1000, both from BD Biosciences) were added and the stimulation was continued for another 5 h. After washing, PBMC were stained with antibodies: CD3 eFluor605NC and CD56 PE (eBioscience). PBMC were analyzed using an LSR II flow cytometer (BD Biosciences). Data analysis was performed with Kaluza analysis software (Beckman Coulter).

NK cell isolation and in vitro culture. For NK cell isolation and culture, blood from healthy volunteers (n = 6) was used and PBMC were isolated by Lymphoprep (Axis-Shield) density gradient centrifugation. PBMC were resuspended in PBS with 2 mM EDTA, 0.5% bovine serum albumin, and incubated with antibodies: CD3 eF450, CD56 PE, and CD19 PE-Cy7 (eBioscience). NK cell subsets CD3-CD19-CD56^{dim} and CD3-CD19-CD56^{bright} were isolated by fluorescence-activated cell sorting using MoFlo Astrios sorter (Beckman Coulter). Sorted CD56^{dim} and CD56^{bright} NK cells were resuspended in RPMI with 0.6% gentamicin and 5% FBS (Lonza) to a concentration of 5×10^5 cells/ml and incubated for 16 h at 37°C in 96-well flat-bottom polystyrene plates (Thermo Fisher Scientific). Culture conditions

Table 1. Demographic and clinical characteristics of the subjects included in the study.

	HC	SP Arthralgia	SP RA	SN RA
N	41	30	45	12
Age, yrs, mean (SD)	50.3 (11.7)	50.7 (14.4)	57.4 (14.0)	64.3 (8.4)
Sex, % female (n)	68.3 (28)	70.0 (21)	80.0 (36)	75.0 (9)
CRP, mg/l, median (range)	ND	5.0 (5.0–29.0)	12.0 (5.0–108.0)	16.5 (5.0–57.0)
ESR, mm/h, median (range)	ND	12.0 (2.0–69.0)	24.0 (2.0–96.0)	38.5 (11.0–88.0)
DAS28, mean (SD)	NA	NA	4.8 (1.4)	5.0 (1.4)
Anti-CCP-positive, % (n)	ND	93.3 (28)	91.1 (41)	0.0 (0)
RF-positive, % (n)	ND	83.3 (25)	91.1 (41)	0.0 (0)
Erosions, % (n)	NA	NA	22.2 (10)	16.7 (2)

HC: healthy controls; SP: seropositive; SP RA: seropositive rheumatoid arthritis; SN RA: seronegative rheumatoid arthritis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: 28-joint Disease Activity Score; anti-CCP: anticyclic citrullinated peptide antibodies; RF: rheumatoid factor; ND: not defined; NA: not applicable.

included 1000 U/ml human recombinant IL-2 (PeproTech), heat-aggregated rabbit immunoglobulin G (IgG; RAGG; Sigma-Aldrich) at a final concentration of 100 μ g/ml, anti-CD16 antibody (clone 3G8; BioLegend) at a final concentration of 1 μ g/ml, both IL-2 and RAGG, both IL-2 and anti-CD16, or medium alone. RAGG was prepared as described²⁶.

Analysis of CD56^{dim} and CD56^{bright} NK cell apoptosis in vitro. After 16 h incubation, cell suspensions of sorted CD56^{dim} and CD56^{bright} NK cells were centrifuged, and supernatant was collected and stored at -20°C until analysis. Cell pellets were washed with PBS and resuspended with 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3); Life Technologies) at a final concentration of 40 nM. After 15 min incubation at 37°C, cells were washed with PBS and analyzed immediately using an LSR II flow cytometer. Data analysis was performed with Kaluza analysis software.

Detection of cytokines in supernatants from cultured CD56^{dim} and CD56^{bright} NK cells. Levels of IFN- γ , tumor necrosis factor (TNF- α), IL-12, IL-4, IL-5, and IL-6 in the culture supernatants were quantified using Human Th1/Th2 Essential 6-plex Luminex assay (eBioscience) according to the manufacturer's instructions. Data analysis was performed using StarStation software, version 2.3 (Applied Cytometry).

Statistical analysis. Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software) and IBM SPSS Statistics 20 (SPSS). Independent and dependent normally distributed variables were analyzed using ANOVA or repeated measures ANOVA, respectively, and Dunnett's multiple comparison test (comparison against control). For non-normal, dependent samples a nonparametric Friedman test was performed, followed by Dunn's posthoc test (comparison against control). P < 0.05 was considered statistically significant.

RESULTS

Seropositive patients, but not SN RA, are characterized by a decline in circulating NK cells. We aimed to identify peripheral immune alterations putatively involved in the early stages of RA pathogenesis and therefore compared the composition of the circulating lymphocyte pool (CD4+ T cells, CD8+ T cells, B cells, and NK cells) between patients (SP arthralgia, early SP RA, and early SN RA) and HC (Figure 1; Supplementary Figures 1 and 2, available online at jrheum.org). The number of total NK cells was significantly decreased in SP arthralgia and SP RA compared to HC (median 0.21 in SP arthralgia and 0.19 in SP RA vs 0.30 $\times 10^6$ NK cells/ml in HC; Figure 1B). A similar decrease was observed for the proportions of NK cells within the total CD45+ pool (median 10.15% in SP arthralgia and 10.90% in SP RA vs 13.64% in HC; Supplementary Figure 1A, available online at jrheum.org). In contrast, the absolute numbers and the proportions of NK cells were not altered in patients with early SN RA (median 0.33 $\times 10^6$ NK cells/ml and 19.31%, respectively). No significant alterations in the numbers of other circulating lymphocyte subsets were observed between patients and HC (Supplementary Figure 2, available online at jrheum.org).

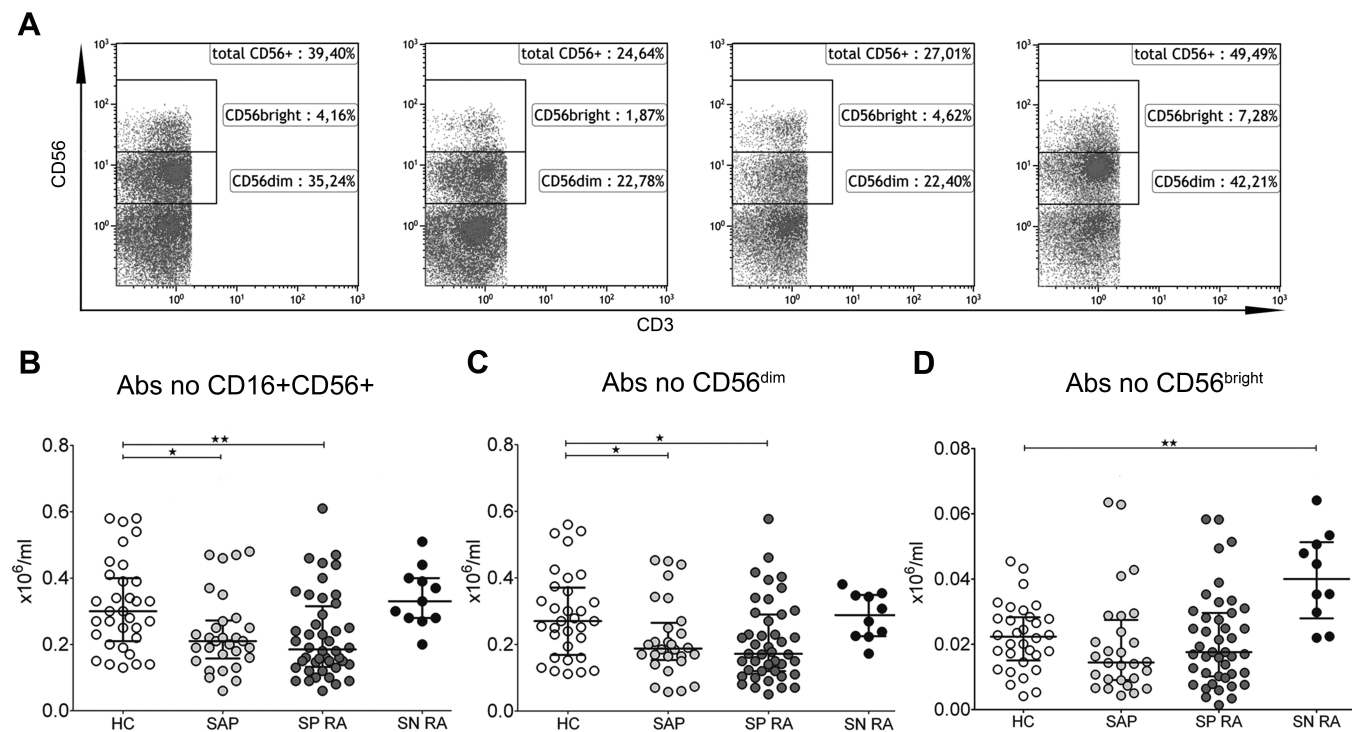


Figure 1. Absolute numbers of CD56^{dim} natural killer (NK) cells are decreased in seropositive (SP) arthralgia and SP rheumatoid arthritis (RA). A. Representative dot plots from a HC, an SP arthralgia patient, an SP RA patient, and an SN RA patient, showing the gating strategy to analyze CD56^{dim} and CD56^{bright} NK cell subsets within the total CD3-CD56+ NK cell population. B. Absolute numbers of total CD56+CD16+ NK cells in the blood of HC (n = 33), SP arthralgia patients (n = 30), early SP RA patients (n = 44), and SN RA patients (n = 11). C. The absolute numbers of CD56^{dim} and (D) CD56^{bright} NK cells were assessed using PBMC from HC (n = 32), SP arthralgia patients (n = 28), early SP RA patients (n = 43), and early SN RA patients (n = 10). The horizontal line in the scatter dot plots indicates the median; error bars indicate interquartile ranges. Statistical significance: * p < 0.05, ** p < 0.01. SN: seronegative; SAP: seropositive arthralgia patients; HC: healthy control; PBMC: peripheral blood mononuclear cells.

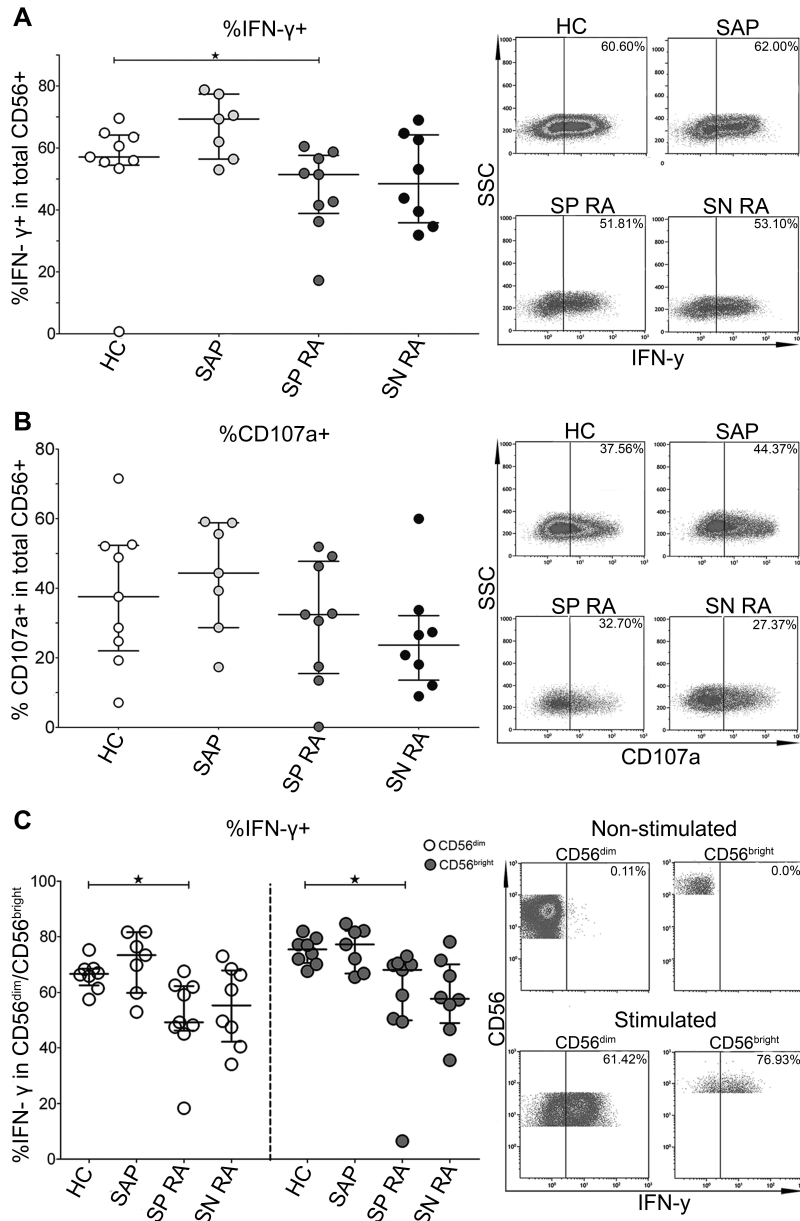


Figure 2. SP RA show decreased frequencies of IFN- γ + NK cells. PBMC from HC (n = 8), SP arthralgia (n = 7), early SP RA (n = 9), and SN RA (n = 8) were stimulated for 4 h with PMA (50 ng/ml) and calcium ionophore (1.6 μ g/ml). The frequency of (A) IFN- γ + and (B) CD107a+ cells within total CD3-CD56+ NK cells (representative dot plots of 1 subject from each group are shown) and (C) the frequency of IFN- γ + cells within subsets of CD56^{dim} and CD56^{bright} NK cells (representative dot plots from 1 HC are shown) was assessed. The horizontal line in the scatter dot plots indicates the median; error bars indicate interquartile ranges. Statistical significance: * p < 0.05. SN: seronegative; HC: healthy control; PBMC: peripheral blood mononuclear cells; SP: seropositive; SAP: seropositive arthralgia patients; RA: rheumatoid arthritis; IFN: interferon; NK: natural killer; PMA: phorbol myristate acetate.

NK cells can be divided into 2 phenotypically and functionally distinct subsets based on CD56 expression (Figure 1A). The absolute numbers of CD56^{dim} NK cells were decreased in both SP arthralgia (median 0.19×10^6 cells/ml) and SP RA (median 0.17×10^6 cells/ml) compared to HC (median 0.27×10^6 cells/ml; Figure 1C). The

frequencies of CD56^{dim} NK cells (within total CD3- cells), however, were not altered (Supplementary Figure 1B, available online at jrheum.org). In contrast, the absolute numbers of CD56^{bright} NK cells were not different in SP arthralgia (median 0.014×10^6 cells/ml) or SP RA (median 0.018×10^6 cells/ml) when compared to HC (median 0.022

$\times 10^6$ cells/ml). SN RA patients showed a specific increase of CD56^{bright} NK cells in both their absolute numbers (median 0.040×10^6 cells/ml) and frequencies (median 4.44% of CD56^{bright} cells within CD3⁻) when compared to HC (Figure 1D; Supplementary Figure 1C).

The mean age of patients with SN RA was higher than the mean age of the HC (Table 1). To exclude the possibility that the observed outcome is confounded by the age difference, a multiple linear regression analysis was performed. After adjusting for age, the previously observed differences in the absolute numbers and the frequencies of CD56^{bright} NK cells between patients with SN RA and HC ($p < 0.005$ and $p = 0.028$, respectively) remained statistically significant (analysis not shown).

Next, we assessed whether the decline of NK cell numbers was associated with markers of general inflammation (C-reactive protein, erythrocyte sedimentation rate) or disease-specific characteristics (28-joint Disease Activity Score, anti-CCP, or RF level). We found a weak negative correlation between RF level and the absolute numbers of total NK cells ($p = 0.034$ and $r = -0.23$; data not shown).

NK cells from patients with SP RA showed decreased IFN- γ expression. Because we found the NK cell pool altered in SAP and patients with early SP RA, we next investigated their functionality by analyzing intracellular expression of CD107a and IFN- γ following PMA/Ca ionophore stimulation *in vitro*. Spontaneous expression of these markers did not differ among the groups (data not shown). NK cells from recently diagnosed patients with SP RA showed a decreased potency to produce IFN- γ compared to HC (median 51.4% vs 58.9% IFN- γ + cells within CD3-CD56+ NK cells in SP RA and HC, respectively; Figure 2A). This was not observed for NK cells from SAP. When analyzing the contribution of the different NK cell subsets to the impaired capacity for IFN- γ production in SP RA, we noted that both the CD56^{dim} and CD56^{bright} subsets showed a reduced capacity for IFN- γ production in SP RA but not SP arthralgia (Figure 2C). Thus, the lesser capacity for IFN- γ production by NK cells from SP RA does not seem to be caused by the decline of CD56^{dim} NK cells. No statistically significant differences in CD107a expression were observed between the studied groups (Figure 2B).

NK cell function was also analyzed indirectly by assessing the surface expression of receptors with an activating (NKG2D, CD57), inhibitory (CD94/NKG2A), or activating/inhibitory role (KIR2DL4). In SP RA, significantly higher frequencies of NKG2D+ NK cells were found when compared to HC (median 74.1% vs 63.1% NKG2D+ cells within CD3-CD56+ NK cells in SP RA and HC, respectively). This was observed within the CD56^{dim} NK cell subset only. No other differences in the frequencies of NKG2D+, CD57+, CD94/NKG2A+, or KIR2DL4+ NK cells between the studied groups were observed (Supplementary Figure 3, available online at jrheum.org).

These data suggest an altered functionality of the peripheral NK cell pool in SP RA, but not in SP arthralgia.

CD56^{dim} and CD56^{bright} NK cell subsets: different susceptibility to Fc γ RIIIa-induced apoptosis. We next assessed whether the reduced NK cell numbers in SP arthralgia and SP RA might be explained by immune complex-mediated induction of NK cell apoptosis by Fc γ RIIIa (CD16) triggering. In line with prior studies, CD56^{dim} NK cells demonstrated higher proportions of CD16+ cells than did CD56^{bright} NK cells (Supplementary Figure 4). CD56^{dim} and CD56^{bright} NK cells were sorted from the blood of healthy volunteers (Figure 3A) and incubated with RAGG or agonistic anti-CD16 antibody in the presence or absence of recombinant IL-2. RAGG has been demonstrated to bind Fc γ R on NK cells and to mirror RF immune complexes²⁷. Apoptosis was assessed by DiOC₆(3) uptake analysis. Anti-CD16 alone and RAGG in the presence of IL-2 enhanced apoptosis of cultured CD56^{dim} NK cells [median 14.14% and 21.89% DiOC₆(3)^{low} cells, respectively, Figure 3B] compared to the control [medium alone, 8.53% DiOC₆(3)^{low} cells]. IL-2 alone had no effect on the number of apoptotic CD56^{dim} cells *in vitro*. Apoptosis of CD56^{dim} NK cells by anti-CD16 antibody was further increased in the presence of IL-2 [35.06% DiOC₆(3)^{low} cells]. In contrast to CD56^{dim} NK cells, anti-CD16, IL-2+anti-CD16, or IL-2+RAGG did not enhance apoptosis of CD56^{bright} NK cells (Figure 3C).

Our data show that CD56^{dim} but not CD56^{bright} NK cells undergo apoptosis upon Fc γ RIII-triggering *in vitro*, with the number of apoptotic cells increasing further upon the addition of IL-2.

CD56^{dim} and CD56^{bright} NK cell subsets: different propensity to produce IFN- γ and TNF- α following Fc γ RIIIa triggering. We next assessed the effect of CD16 triggering on cytokine production by NK cells from healthy donors. Sorted CD56^{dim} and CD56^{bright} NK cells (Figure 3A) were cultured with medium alone, IL-2, RAGG, anti-CD16, IL-2+RAGG, or IL-2+anti-CD16, and the supernatant was assessed for the production of IFN- γ , TNF- α , and other proinflammatory cytokines such as IL-12, IL-4, IL-5, and IL-6. In the presence of IL-2+RAGG or IL-2+anti-CD16, CD56^{dim} NK cells produced IFN- γ and TNF- α (Figure 4A and Figure 4B). Increases in the production of IL-12 and IL-4 by CD56^{dim} NK cells were also observed upon IL-2+RAGG culture (Figure 4C and Figure 4D). No statistically significant increase in cytokine expression was observed for CD56^{bright} NK cell cultures (Figure 4).

DISCUSSION

We show a decline of NK cells in recently diagnosed RA and in patients with SP arthralgia, representing subjects at risk of progressing toward RA²⁵. By stratifying our RA cohort according to autoantibody status, we found the NK cell decrease associated with SP but not SN RA. The decline in

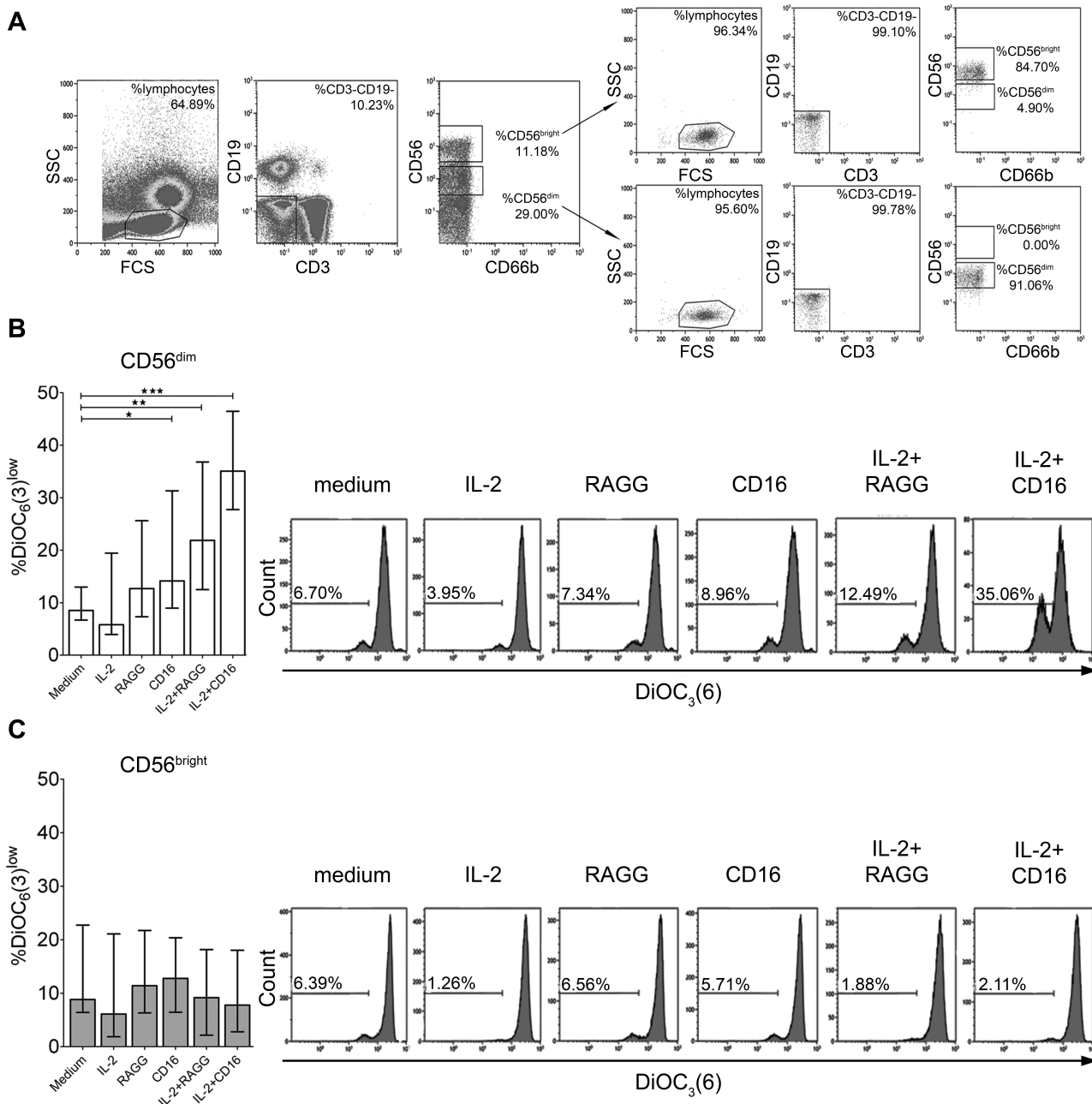


Figure 3. CD56^{dim} but not CD56^{bright} NK cells undergo apoptosis upon FcγRIIIa triggering. **A.** The gating strategy used in sorting for CD56^{dim} and CD56^{bright} NK cells and the reanalysis of the purity of sorted NK cell subsets are shown (median 85% and 96%, respectively). CD56^{dim} and CD56^{bright} NK cells from HC (n = 6) were cultured with IL-2, RAGG, anti-CD16, IL-2+RAGG, IL-2+anti-CD16, or medium alone. Frequencies of DiOC₆(3)^{low} cells within **(B)** CD56^{dim} and **(C)** CD56^{bright} events and representative histograms of 1 HC are shown. The horizontal line in the bar plots indicates the median; error bars indicate interquartile ranges. Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001. HC: healthy control; NK: natural killer; IL: interleukin; RAGG: heat-aggregated rabbit immunoglobulin G; SSC: side scatter; FCS: forward scatter.

NK cells may be explained by a selective decrease of CD56^{dim} NK cells as a result of apoptosis induction through FcγR triggering by IgG-containing immune complexes. In line with published data^{27,28,29,30,31,32}, we observed the occurrence of FcγR-dependent (anti-CD16-induced) apoptosis of NK cells, augmented by IL-2, *in vitro*. We demonstrated

differential susceptibility of CD56^{dim} and CD56^{bright} NK cell subsets to FcγR-induced cell death.

The decline of NK cells in SP arthralgia and in recently diagnosed SP RA suggests that this alteration may contribute to disease development rather than represent the consequence of longterm inflammation. Most of the published

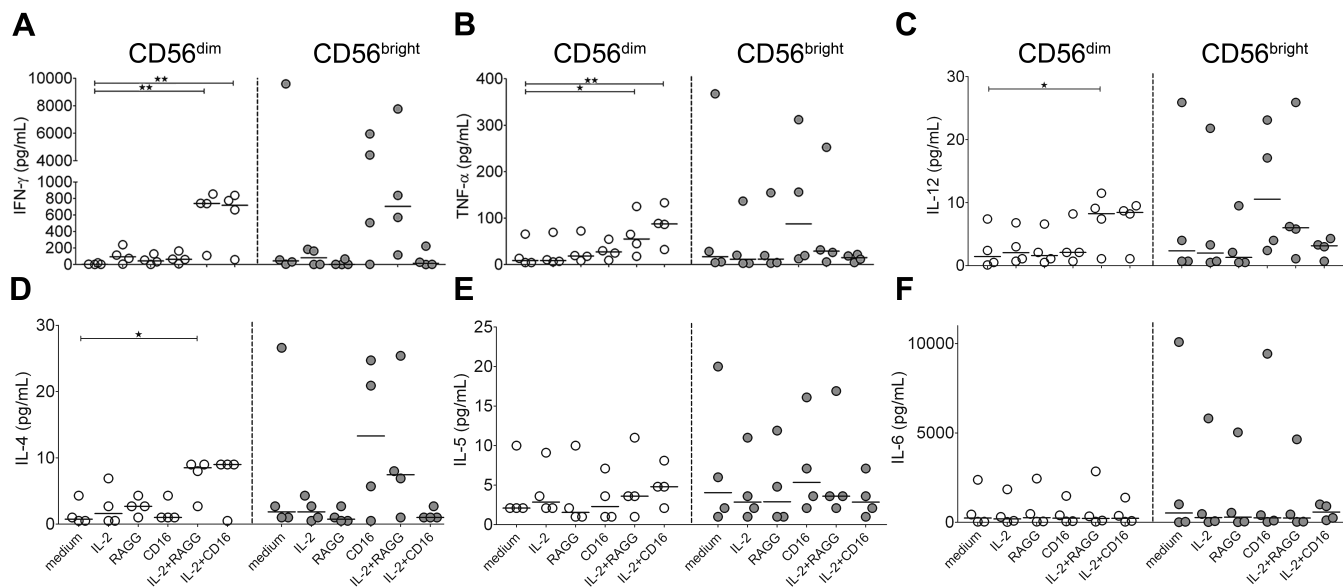


Figure 4. CD56^{dim} but not CD56^{bright} NK cells produce IFN- γ , TNF- α , IL-12, and IL-4 upon Fc γ RIIIa triggering. CD56^{dim} and CD56^{bright} NK cells were sorted and cultured as described in Figure 3. After 16 h, supernatants from various culture conditions of CD56^{dim} and CD56^{bright} NK cells (from 4 HC used for Figure 3) were collected. Levels of (A) IFN- γ , (B) TNF- α , (C) IL-12, (D) IL-4, (E) IL-5, and (F) IL-6 were assessed using 6-plex cytokine assay. The horizontal line in the scatter dot plots indicates the median. Statistical significance: * $p < 0.05$, ** $p < 0.01$. HC: healthy control; NK: natural killer; IL: interleukin; RAGG: heat-aggregated rabbit immunoglobulin G; IFN: interferon; TNF: tumor necrosis factor.

data describe similarly decreased NK cell numbers in later stages of RA^{33,34,35}. The use of NSAID was found to have no effect on peripheral NK cell numbers³⁶. Thus, NSAID are unlikely causal to the NK cell decline in SP arthralgia and SP RA.

Previously, NK cell depletion was found to accelerate the onset and augment the severity of CIA. Following the decline of NK cells, the decrease in systemic IFN- γ levels led to an expansion of Th17 cells directly involved in CIA induction. Further, the NK cell decrease was associated with plasma cell development and increased systemic levels of IgG autoantibodies⁷. This, together with the decline of NK cells in SP RA but not in SN RA, suggests a protective role for NK cells in the development of seropositive RA.

Despite similarly reduced NK cell numbers in SP arthralgia and early untreated SP RA, the decline of IFN- γ expression was observed in the latter group only. Thus, as shown for CIA⁷, the progression of pre-RA to overt disease may be associated with a reduction of NK cells as well as their functional impairment in production of IFN- γ . This would confirm the beneficial role of IFN- γ in arthritis pathology as shown in CIA^{6,7,8} and RA³⁷.

The decline of peripheral NK cell numbers in SP but not in SN patients as well as the previously reported induction of NK cell apoptosis by Fc γ R triggering^{27,28,31} suggested a role of RA-related IgG-containing autoantibodies in this process. The majority of anti-CCP are IgG³⁸ and can be bound by IgM RF³⁹. A study by Boros, *et al* showed that IgM from RA sera was reactive with Fc γ RIII⁴⁰. Further, about half of patients

with RA have RF in a form of small IgG complexes⁴¹, which are efficiently bound by Fc γ RIIIa⁴².

We confirmed induction of NK cell apoptosis by agonistic anti-CD16 antibody and RAGG *in vitro*, a process that was enhanced by addition of IL-2^{27,28,31}. We observed a higher sensitivity of sorted CD56^{dim} NK cells to Fc γ R crosslinking-induced apoptosis, which is likely the result of the higher expression of CD16 compared to CD56^{bright} cells¹⁵ (Supplementary Figure 4, available online at jrheum.org). Differential susceptibility of CD56^{dim} and CD56^{bright} NK cells to Fc γ R-dependent apoptosis corresponds to the decline of circulating CD56^{dim} but not CD56^{bright} NK cells in the SP patients *in vivo*. It is unlikely that the decline of CD56^{dim} NK cells in the periphery is a result of a preferential recruitment of this population to the joints because SF-derived NK cells were mainly of the CD56^{bright} phenotype (data not shown¹⁹).

We observed an increased number of CD56^{bright} NK cells in SN RA. Prior data suggest that the expansion of CD56^{bright} NK cells is more specific for autoimmune diseases such as systemic lupus erythematosus⁴³ or multiple sclerosis⁴⁴ than RA and was associated with increased levels of type I interferons.

As shown^{29,30}, CD16 triggering of CD56^{dim} NK cells induced production of IFN- γ and TNF- α , cytokines implicated in RA pathogenesis. This process was augmented by the addition of IL-2. Similarly, we observed increased expression of Th1-specific (IL-12) and Th2-specific (IL-4) cytokines by CD56^{dim} NK cells cultured with RAGG and IL-2. This suggests that CD56^{dim} NK cells may be primarily

responsible for the enhanced expression of a spectrum of various cytokines, although this feature has previously been attributed to CD56^{bright} NK cells^{15,16,21,22,23,24}. Increased IFN- γ and TNF- α expression by CD56^{bright} compared to CD56^{dim} was previously seen following stimulation with combinations of monocyte-derived cytokines^{21,22}, PMA/ionomycin^{16,21}, or whole bacterial pathogen²⁴. Involvement of CD16 in the modulation of CD56^{dim} NK cell cytokine expression has also been demonstrated^{45,46,47}. Thus, proinflammatory cytokine production cannot be exclusively attributed to CD56^{bright} NK cells. Depending on the available stimulus, both NK cell subsets can produce cytokines.

We also report that CD56^{dim} NK cells in early SP RA are enriched with NKG2D+ cells compared to HC. Activating receptor NKG2D has been implicated in the development of autoimmunity⁴⁸, and NKG2D blocking had beneficial effects on clinical, histological, and immunological variables (including reduction of IL-17 expression) in CIA⁴⁹. This indicates that, next to the loss of protective function, CD56^{dim} NK cells in SP RA may also display disease-promoting functions.

Our study focused on the systemic NK cell alterations, as it has been suggested that RA does not begin at the level of the joint but is preceded by systemic inflammation⁵⁰. Our data suggest a role of circulating CD56^{dim} NK cells in the pathogenesis of SP RA. On the other hand, enrichment of CD56^{bright} NK cells in synovial fluid and tissue of longstanding RA¹⁹ indicates a role for these cells in the local inflammatory process in late-stage RA. However, it remains to be established whether CD56^{bright} NK cells in the affected joints display inflammation-promoting or immunoregulatory properties.

We propose that the interaction between NK cells and RA-specific IgG-containing immune complexes is an early event in disease development. This is in line with the notion that the emergence of anti-CCP and RF autoantibodies occurs years before RA onset³. Fc γ RIIIa triggering of CD56^{dim} NK cells by autoantibody-immune complexes could result in cytokine expression and a higher sensitivity to apoptosis of the CD56^{dim} NK cell subset. The latter process is accelerated by IL-2, which, similar to ACPA and RF, was found increased in the periphery at the pre-RA stage³. Activation of CD56^{dim} NK cells may thus contribute to early systemic inflammation as seen in SP arthralgia and SP RA. Moreover, the decline of CD56^{dim} NK cells may allow for uncontrolled expansion of autoimmune cells contributing to RA development.

Also, our results demonstrate differences in the systemic immune profiles between SP and SN RA, adding to the notion that SP RA and SN RA may represent different disease entities.

ONLINE SUPPLEMENT

Supplementary data for this article are available online at jrheum.org.

REFERENCES

- Nielen MM, van Schaardenburg D, Reesink HW, Twisk JW, van de Stadt RJ, van der Horst-Bruinsma IE, et al. Simultaneous development of acute phase response and autoantibodies in preclinical rheumatoid arthritis. *Ann Rheum Dis* 2006;65:535-7.
- Karlson EW, Chibnik LB, Tworoger SS, Lee IM, Buring JE, Shadick NA, et al. Biomarkers of inflammation and development of rheumatoid arthritis in women from two prospective cohort studies. *Arthritis Rheum* 2009;60:641-52.
- Kokkonen H, Soderstrom I, Rocklov J, Hallmans G, Lejon K, Rantapaa Dahlqvist S. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. *Arthritis Rheum* 2010;62:383-91.
- Masi AT, Rehman AA, Elmore KB, Aldag JC. Serum acute phase protein and inflammatory cytokine network correlations: comparison of a pre-rheumatoid arthritis and non-rheumatoid arthritis community cohort. *J Innate Immun* 2013;5:100-13.
- Cascao R, Moura RA, Perpetuo I, Canhao H, Vieira-Sousa E, Mourao AF, et al. Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis. *Arthritis Res Ther* 2010;12:R196.
- Kelchtermans H, Struyf S, De Klerck B, Mitera T, Alen M, Geboes L, et al. Protective role of IFN-gamma in collagen-induced arthritis conferred by inhibition of mycobacteria-induced granulocyte chemotactic protein-2 production. *J Leukoc Biol* 2007;81:1044-53.
- Lo CK, Lam QL, Sun L, Wang S, Ko KH, Xu H, et al. Natural killer cell degeneration exacerbates experimental arthritis in mice via enhanced interleukin-17 production. *Arthritis Rheum* 2008;58:2700-11.
- Lee J, Lee J, Park MK, Lim MA, Park EM, Kim EK, et al. Interferon gamma suppresses collagen-induced arthritis by regulation of Th17 through the induction of indoleamine-2,3-deoxygenase. *PLoS One* 2013;8:e60900.
- Pollard KM, Cauvi DM, Toomey CB, Morris KV, Kono DH. Interferon-gamma and systemic autoimmunity. *Discov Med* 2013;16:123-31.
- Scharton-Kersten T, Afonso LC, Wysocka M, Trinchieri G, Scott P. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *J Immunol* 1995;154:5320-30.
- Morandi B, Bougras G, Muller WA, Ferlazzo G, Munz C. NK cells of human secondary lymphoid tissues enhance T cell polarization via IFN-gamma secretion. *Eur J Immunol* 2006;36:2394-400.
- Caligiuri MA. Human natural killer cells. *Blood* 2008;112:461-9.
- French AR, Yokoyama WM. Natural killer cells and autoimmunity. *Arthritis Res Ther* 2004;6:8-14.
- Shi FD, Van Kaer L. Reciprocal regulation between natural killer cells and autoreactive T cells. *Nat Rev Immunol* 2006;6:751-60.
- Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001;22:633-40.
- Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G, et al. CD56^{bright} cells differ in their KIR repertoire and cytotoxic features from CD56^{dim} NK cells. *Eur J Immunol* 2001;31:3121-7.
- Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, et al. CD56^{bright} natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* 2003;101:3052-7.
- Ferlazzo G, Thomas D, Lin SL, Goodman K, Morandi B, Muller WA, et al. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J Immunol* 2004;172:1455-62.
- Pridgeon C, Lennon GP, Pazmany L, Thompson RN, Christmas SE, Moots RJ. Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a CD56^{bright},CD94^{bright},CD158^{negative}

- phenotype. *Rheumatology* 2003;42:870-8.
20. Ottaviani C, Nadorri F, Bedini C, de Pita O, Girolomoni G, Cavani A. CD56brightCD16(-) NK cells accumulate in psoriatic skin in response to CXCL10 and CCL5 and exacerbate skin inflammation. *Eur J Immunol* 2006;36:118-28.
 21. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 2001;97:3146-51.
 22. Li Z, Lim WK, Mahesh SP, Liu B, Nussenblatt RB. Cutting edge: in vivo blockade of human IL-2 receptor induces expansion of CD56(bright) regulatory NK cells in patients with active uveitis. *J Immunol* 2005;174:5187-91.
 23. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology* 2009;126:458-65.
 24. Batoni G, Esin S, Favilli F, Pardini M, Bottai D, Maisetta G, et al. Human CD56bright and CD56dim natural killer cell subsets respond differentially to direct stimulation with *Mycobacterium bovis* bacillus Calmette-Guerin. *Scand J Immunol* 2005;62:498-506.
 25. van de Stadt LA, Witte BI, Bos WH, van Schaardenburg D. A prediction rule for the development of arthritis in seropositive arthralgia patients. *Ann Rheum Dis* 2013;72:1920-6.
 26. Pilling D, Tucker NM, Gomer RH. Aggregated IgG inhibits the differentiation of human fibrocytes. *J Leukoc Biol* 2006;79:1242-51.
 27. Ortaldo JR, Mason AT, O'Shea JJ. Receptor-induced death in human natural killer cells: involvement of CD16. *J Exp Med* 1995;181:339-44.
 28. Eischen CM, Schilling JD, Lynch DH, Krammer PH, Leibson PJ. Fc receptor-induced expression of Fas ligand on activated NK cells facilitates cell-mediated cytotoxicity and subsequent autocrine NK cell apoptosis. *J Immunol* 1996;156:2693-9.
 29. Jewett A, Cavalcanti M, Bonavida B. Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells. *J Immunol* 1997;159:4815-22.
 30. Anegón I, Cuturi MC, Trinchieri G, Perussia B. Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J Exp Med* 1988;167:452-72.
 31. Azzoni L, Anegón I, Calabretta B, Perussia B. Ligand binding to Fc gamma R induces c-myc-dependent apoptosis in IL-2-stimulated NK cells. *J Immunol* 1995;154:491-9.
 32. Warren HS, Kinnear BF. Quantitative analysis of the effect of CD16 ligation on human NK cell proliferation. *J Immunol* 1999;162:735-42.
 33. Shibatomi K, Ida H, Yamasaki S, Nakashima T, Origuchi T, Kawakami A, et al. A novel role for interleukin-18 in human natural killer cell death: high serum levels and low natural killer cell numbers in patients with systemic autoimmune diseases. *Arthritis Rheum* 2001;44:884-92.
 34. Aggarwal A, Sharma A, Bhatnagar A. Role of cytolytic impairment of natural killer and natural killer T-cell populations in rheumatoid arthritis. *Clin Rheumatol* 2014;33:1067-78.
 35. Conigliaro P, Triggianese P, Perricone C, Chimenti MS, Di Muzio G, Ballanti E, et al. Restoration of peripheral blood natural killer and B cell levels in patients affected by rheumatoid and psoriatic arthritis during etanercept treatment. *Clin Exp Immunol* 2014;177:234-43.
 36. Forre O, Thoen J, Helgetveit K, Haile Y. Non-steroidal anti-inflammatory drugs in rheumatoid arthritis. Effects on clinical parameters and cellular immunity. *Inflammation* 1984;Suppl 8:S109-13.
 37. Page CE, Smale S, Carty SM, Amos N, Lauder SN, Goodfellow RM, et al. Interferon-gamma inhibits interleukin-1beta-induced matrix metalloproteinase production by synovial fibroblasts and protects articular cartilage in early arthritis. *Arthritis Res Ther* 2010;12:R49.
 38. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP antibodies: the past, the present and the future. *Nat Rev Rheumatol* 2011;7:391-8.
 39. Reparón-Schuijt CC, van Esch WJ, van Kooten C, Schellekens GA, de Jong BA, van Venrooij WJ, et al. Secretion of anti-citrulline-containing peptide antibody by B lymphocytes in rheumatoid arthritis. *Arthritis Rheum* 2001;44:41-7.
 40. Boros P, Odin JA, Chen J, Unkeless JC. Specificity and class distribution of Fc gamma R-specific autoantibodies in patients with autoimmune disease. *J Immunol* 1994;152:302-6.
 41. Mageed RA, Kirwan JR, Thompson PW, McCarthy DA, Holborow EJ. Characterisation of the size and composition of circulating immune complexes in patients with rheumatoid arthritis. *Ann Rheum Dis* 1991;50:231-6.
 42. Edwards JC, Cambridge G. Rheumatoid arthritis: the predictable effect of small immune complexes in which antibody is also antigen. *Br J Rheumatol* 1998;37:126-30.
 43. Schepis D, Gunnarsson I, Eloranta ML, Lampa J, Jacobson SH, Karre K, et al. Increased proportion of CD56bright natural killer cells in active and inactive systemic lupus erythematosus. *Immunology* 2009;126:140-6.
 44. Saraste M, Irjala H, Airas L. Expansion of CD56Bright natural killer cells in the peripheral blood of multiple sclerosis patients treated with interferon-beta. *Neurol Sci* 2007;28:121-6.
 45. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010;115:2167-76.
 46. Juelke K, Killig M, Luetke-Eversloh M, Parente E, Gruen J, Morandi B, et al. CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells. *Blood* 2010;116:1299-307.
 47. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* 2010;116:3865-74.
 48. Shegarfi H, Naddafi F, Mirshafiey A. Natural killer cells and their role in rheumatoid arthritis: friend or foe? *ScientificWorldJournal* 2012;2012:491974.
 49. Andersson AK, Sumariwalla PF, McCann FE, Amjadi P, Chang C, McNamee K, et al. Blockade of NKG2D ameliorates disease in mice with collagen-induced arthritis: a potential pathogenic role in chronic inflammatory arthritis. *Arthritis Rheum* 2011;63:2617-29.
 50. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. *PLoS One* 2012;7:e35296.