Chapter 4

Altered NK-cell subsets in seropositive arthralgia and early rheumatoid arthritis patients are associated with autoantibody status

Paulina Chalan¹,³, Johan Bijzet¹, Bart-Jan Kroesen²,³, Annemieke M.H. Boots¹,³#, Elisabeth Brouwer¹,³#

Departments of ¹Rheumatology & Clinical Immunology, ²Laboratory Medicine ³Groningen Research initiative on healthy Ageing and Immune Longevity (GRAIL) University of Groningen, University Medical Center Groningen, Groningen, the Netherlands. # contributed equally.

Submitted
Chapter 4

Abstract

Objective

The role of NK-cells in the immunopathogenesis of RA is unclear. Therefore, numerical and functional alterations of CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK-cells in the early stages of RA development were studied.

Methods

Whole blood samples from newly diagnosed, treatment-naïve, seropositive and seronegative RA patients (SP RA, n=45 and SN RA, n=12), seropositive arthralgia patients (SAP, n=30) and healthy controls (n=41) were assessed for numbers and frequencies of T-cells, B-cells and NK-cells. Seropositive status was defined as anti-cyclic citrullinated peptide antibodies [ACPA] and/or rheumatoid factor [RF] positive. 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 SAP but not in SN RA. Also, NK-cells from SP RA showed a decreased potency for IFN-\(\gamma\) production. A selective decrease of CD56\textsuperscript{dim}, but not CD56\textsuperscript{bright} NK-cells in SP RA and SAP was observed. This prompted investigation of CD16 (Fc\(\gamma\)RIIIa) triggering in NK-cell apoptosis and cytokine expression. In vitro, CD16 triggering induced apoptosis of CD56\textsuperscript{dim}, but not CD56\textsuperscript{bright} NK-cells from healthy controls which was augmented by adding IL-2. Also, CD16 triggering in the presence of IL-2 stimulated IFN-\(\gamma\) expression by CD56\textsuperscript{dim} NK-cells.

Conclusions

The decline of CD56\textsuperscript{dim} NK-cells in SAP and SP RA and the in vitro apoptosis of CD56\textsuperscript{dim} NK-cells upon CD16 triggering, suggest a functional role of IgG-containing autoantibody (ACPA and/or RF)-immune complexes in this process. Moreover, CD16-triggered cytokine production by CD56\textsuperscript{dim} NK-cells may contribute to the pro-inflammatory state as seen in SAP and SP RA.
Introduction

Rheumatoid arthritis (RA) is a chronic autoinflammatory 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 (1-4). This poses a challenge for understanding the primary events in immune dysregulation involved in RA development. Current data support a role of IL-17 in the early phases of RA (3, 5). Despite the originally postulated pathogenicity of IFN-γ, several reports demonstrated its protective role in the development of collagen-induced arthritis (CIA), a mouse model of RA (6-8). The exact protective mechanism of IFN-γ in RA is currently not fully known (9). NK-cells are primary IFN-γ producers (9) by which they connect to the adaptive immune response and favor Th1 cell polarization in the course of an inflammatory response (10-12). NK-cell depletion was found to accelerate CIA onset which was associated with an impaired IFN-γ-dependent regulation of the Th17 response (7). Also, NK-cells contribute to immune tolerance through 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, NCAM) (15). CD56dim 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 expression (16). This suggests a primary role for CD56dim NK-cells in killing of autoreactive cells. In addition, CD56dim cells are more effective in antibody-dependent cellular cytotoxicity (ADCC) when compared to the CD56bright subset, as a result of higher surface expression of FcγRIIIa (CD16). CD56bright 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 fluid (19), psoriatic plaques (20)) CD56bright NK-cells have been shown to outnumber CD56dim cells. CD56bright NK-cells may also have an immunoregulatory role due to an increased ability (compared to CD56dim subset) to produce pro- and anti-inflammatory cytokines (15, 16, 21-25).

Given their potentially initiating capacity in skewing and regulating the immune response, we aimed to investigate the role of NK-cells in early stages of RA development. We studied newly diagnosed, treatment-naïve RA patients and seropositive arthralgia patients (anti-cyclic citrullinated peptide antibodies [ACPA] and/or rheumatoid factor [RF]) positive, without synovitis [SAP]). Previous studies show that 35% of SAP develop RA after approximately 1 year of follow-up (26, 27). In the present study we focused on CD56dim and CD56bright NK-cell subsets, specifically in relation to their differential function in the immune response.

Material and methods

Patients

Thirty SAP were defined based on seropositivity for RF (serum levels ≥ 15 IU/mL) and/or ACPA (serum levels ≥ 10 IU/mL), arthralgia in at least one joint and lack of arthritis. Also, 45 early RA patients
seropositive for ACPA and/or RF, 12 early seronegative RA patients (ACPA- and RF-) and 41 healthy controls (HC) were included in the study (Table 1). All RA patients, fulfilling 1987 or 2010 American College of Rheumatology (ACR) classification criteria for RA, were included in the study at the time of diagnosis, before start of treatment with DMARDs. SAP and RA patients received non-steroidal anti-inflammatory drugs (NSAIDs) 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).

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

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>SAP</th>
<th>SP RA</th>
<th>SN RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>41</td>
<td>30</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>Age [yrs]; mean (SD)</td>
<td>50.3 (11.7)</td>
<td>50.7 (14.4)</td>
<td>57.4 (14.0)</td>
<td>64.3 (8.4)</td>
</tr>
<tr>
<td>Gender; % female (n)</td>
<td>68.3 (28)</td>
<td>70.0 (21)</td>
<td>80.0 (36)</td>
<td>75.0 (9)</td>
</tr>
<tr>
<td>CRP [mg/l]; median (range)</td>
<td>nd</td>
<td>5.0 (5.0-29.0)</td>
<td>12.0 (5.0-108.0)</td>
<td>16.5 (5.0-57.0)</td>
</tr>
<tr>
<td>ESR [mm/h]; median (range)</td>
<td>nd</td>
<td>12.0 (2.0-69.0)</td>
<td>24.0 (2.0-96.0)</td>
<td>38.5 (11.0-88.0)</td>
</tr>
<tr>
<td>DAS28; mean (SD)</td>
<td>na</td>
<td>na</td>
<td>4.8 (1.4)</td>
<td>5.0 (1.4)</td>
</tr>
<tr>
<td>ACPA positive; % (n)</td>
<td>nd</td>
<td>93.3 (28)</td>
<td>91.1 (41)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>RF positive; % (n)</td>
<td>nd</td>
<td>83.3 (25)</td>
<td>91.1 (41)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>Erosions; % (n)</td>
<td>na</td>
<td>na</td>
<td>22.2 (10)</td>
<td>16.7 (2)</td>
</tr>
</tbody>
</table>

HC = healthy controls; SAP = seropositive arthralgia patients; SP RA = seropositive rheumatoid arthritis; SN RA = seronegative rheumatoid arthritis; ACPA = anti-cyclic citrullinated proteins antibodies; RF = rheumatoid factor; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; DAS28 = disease activity score 28; na = not applicable; nd = not defined

Analysis of circulating leukocyte populations

Whole blood was analyzed using BD MultiTest™ TruCount method with reagents detecting CD45, CD3, CD4, CD8, CD19, CD16/CD56, according to the manufacturer's instructions (BD Biosciences, Breda, The Netherlands). 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 mononuclear cells (PBMC) by Lymphoprep™ (Axis-Shield, Oslo, Norway) 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, Vienna, Austria), CD56 FITC, CD16 Alexa Fluor700, CD94 APC, NKG2D PE-Cy7 (BioLegend, San Diego, CA, USA), NKG2A PerCP (R&D Systems, Abingdon, UK), KIR2DL4 (Exbio Praha, Vestec, Czech Republic).
To assess NK-cell IFN-γ expression, thawed PBMC were resuspended in RPMI-1640 containing 10% FBS and 0.6% gentamicin (Life Technologies, Bleiswijk, The Netherlands) at a concentration of 10^6 cells/100 μL. 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 μg/mL (both from Sigma-Aldrich, Zwijndrecht, The Netherlands) and BD GolgiPlug™ (BD Biosciences) diluted 1:1000. After 4h at 37°C, PBMC were stained with the following antibodies: CD3 eFluor605NC, CD56 PE (eBioscience), CD69 PE-Cy (BioLegend), FasL (CD178) Alexa Fluor 647 (AbD Serotec, Puchheim, Germany). 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% gentamycin at a concentration of 10^6 cells/100 μL. Cells were stimulated with PMA at the same concentrations as mentioned above in the presence of 0.5 µg of anti-CD107a Brilliant Violet 421 (BioLegend) antibody. After 1h, 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 hours. After washing, PBMC were stained with antibodies: CD3 eFluor605NC and CD56 PE (eBioscience). PBMC were analyzed using 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% BSA and incubated with antibodies: CD3 eF450, CD56 PE and CD19 PE-Cy7 (eBioscience). NK-cell subsets: CD3-CD19-CD56dim and CD3-CD19-CD56bright were isolated by fluorescence-activated cell sorting using MoFlo Astrios sorter (Beckman Coulter, Woerden, The Netherlands, Fig.3A). Sorted CD56dim and CD56bright NK-cells were resuspended in RPMI with 0.6% gentamicin and 5% FBS (Lonza) to a concentration of 5x10^5 cells/mL and incubated for 16h at 37°C in 96-well flat-bottom polystyrene plates (Thermo Fisher Scientific). Culture conditions included 1000 U/mL human recombinant IL-2 (PeproTech, London, UK), heat-aggregated rabbit IgG (RAGG; Sigma-Aldrich) at a final concentration of 100 µg/mL, anti-CD16 antibody (clone 3G8; BioLegend) at a final concentration of 1 µg/mL, both IL-2 and RAGG, both IL-2 and anti-CD16 or medium alone. RAGG was prepared as described (28).

**Analysis of CD56dim and CD56bright NK-cell apoptosis in vitro**

After 16h incubation, cell suspensions of sorted CD56dim and CD56bright NK-cells were centrifuged, supernatant was collected and stored at -20°C until analysis. Cell pellets were washed with PBS and resuspended with 3,3’-dihexyloxacarbocyanine 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 LSR II flow cytometer (BD Biosciences). Data analysis was performed with Kaluza® analysis software (Beckman Coulter).

Detection of cytokines in supernatants from cultured CD56$^{\text{dim}}$ and CD56$^{\text{bright}}$ NK-cells

Levels of IFN-γ, 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, Birmingham, UK).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics 20 (SPSS, Chicago, IL, USA). Normally distributed data were analyzed using an unpaired t test. Skewed data were analyzed using the Mann-Whitney 2-tailed test. Paired samples were analyzed with the Wilcoxon signed-rank test. P<0.05 was considered statistically significant.

Results

Seropositive patients (SAP and RA), but not seronegative RA patients, are characterized by a decline of 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 (SAP, early SP RA and early SN RA) and HC (Fig.1, Suppl. Fig.1). The number of NK-cells was significantly decreased in SAP and SP RA compared to HC (median 0.21 in SAP and 0.19 in SP RA vs. 0.30 x10$^6$ NK-cells/mL in HC). A similar decrease was observed for the NK-cell percentage within the total CD45$^+$ pool (median 10.15% in SAP and 10.90% in SP RA vs. 13.64% in HC). In contrast, the absolute number and the proportion of NK-cells were not altered in early SN RA patients (median 0.33 x10$^6$ NK-cells/mL and 19.31%, respectively) (Fig.1A). No significant alterations in the number of other circulating lymphocyte subsets were observed between patients and HC (Suppl.Fig.1).

NK-cells can be divided into 2 phenotypically and functionally distinct subsets based on CD56 expression (Fig.1B). The absolute number of CD56$^{\text{dim}}$ NK-cells was decreased in both SAP (median 0.19 x10$^6$ cells/mL) and SP RA (median 0.17 x10$^6$ cells/mL) compared to both HC (median 0.27 x10$^6$ cells/mL) and SN RA (median 0.29 x10$^6$ cells/mL). The frequencies of CD56$^{\text{dim}}$ NK-cells (within total CD3$^-$ cells), however, were not altered (Fig.1B). In contrast, the absolute numbers of CD56$^{\text{bright}}$ NK-cells were not different in SAP (median 0.014 x10$^6$ cells/mL) or SP RA (median 0.018 x10$^6$ cells/mL) when compared to
HC (median 0.022 x10^6 cells/mL). SN RA patients showed a specific increase of CD56^bright NK-cells in both their absolute number (median 0.040 x10^6 cells/mL) and frequency (median 4.44% of CD56^bright cells within CD3-) when compared to HC (2.01%), SAP (1.99%) and SP RA patients (2.55%) (Fig.1 D).

The mean age of SN RA patients 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 number and the frequency of CD56^bright NK-cells between HC and SN RA patients (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 (CRP, ESR) or disease-specific characteristics (DAS28, ACPA or RF level). We found a weak negative correlation between RF level and the absolute number of total NK-cells (p=0.034 and r=-0.23; data not shown).

**NK-cells from seropositive RA patients showed decreased IFN-γ expression**

As we found the NK-cell pool altered in SAP and early SP RA patients, 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 between the groups (data not shown). NK-cells from recently diagnosed SP RA patients 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; Fig.2A). This was not observed for NK-cells from SAP. 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 (Fig.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). CD56^+ NK-cells from SP RA showed significantly higher expression of NKG2D when compared to HC (median 74.1% vs. 63.1% NKG2D^+ cells within CD3-CD56^+ NK-cells in SP RA and HC, respectively). This alteration was observed within both CD56^dim and CD56^bright NK-cell subsets. No other differences in the expression of NKG2D or CD57, CD94/NKG2A and KIR2DL4 between the studied groups were observed (Suppl.Fig.2). These data suggest an altered functionality of the peripheral NK pool in SP RA, but not in SAP.
Chapter 4

Figure 1. Decrease of NK-cells in SAP and newly diagnosed, seropositive RA.

(A) Absolute number and frequency of CD56+CD16+ NK-cells in the blood of HC (n=33), SAP (n=30), early SP RA (n=44) and SN RA (n=11). (B) Representative dot plots from HC, SAP, SP RA and SN RA patient showing the gating strategy to analyze CD56\text{dim} and CD56\text{bright} cells within the CD3-CD56+ NK-cell population. The absolute number and the frequency of (C) CD56\text{dim} and (D) CD56\text{bright} NK-cells was assessed using PBMC from HC (n=32), SAP (n=28), early SP RA (n=43) and early SN RA patients (n=10). Statistical significance: * p <0.05; ** p <0.001.
Altered NK-cell subsets in SAP and RA

**Figure 2.** NK-cells from early SP RA show decreased intracellular expression of IFN-γ.

PBMC from HC (n=8), SAP (n=7), early SP RA (n=9) and SN RA (n=8) were stimulated for 4h with PMA (50 ng/mL) and calcium ionophore (1.6 µg/mL). After gating for CD3-CD56+ cells, the frequency of (A) IFN-γ+ and (B) CD107a+ cells was assessed. Representative dot plots of 1 subject from each group are shown. Statistical significance: * p <0.05.

**CD56^dim** and **CD56^bright** NK-cell subsets: different susceptibility to FcγRIIIa-induced apoptosis

We next assessed if the reduced NK-cell numbers in SAP and SP RA might be explained by immune complex-mediated induction of NK-cell apoptosis via FcγRIIIa (CD16)-triggering. CD56^dim and CD56^bright NK-cells were isolated from the blood of healthy volunteers and incubated with rabbit aggregated IgG (RAGG) or agonistic anti-CD16 antibody in the presence or absence of recombinant IL-2. RAGG has been demonstrated to bind FcγR on NK-cells and to mirror RF immune complexes (29). Apoptosis was assessed by DiOC₆(3) uptake analysis. RAGG and anti-CD16 alone enhanced apoptosis of cultured CD56^dim NK-cells (median 12.68% and 14.14% DiOC₆(3)^low cells, respectively, Fig.3A,B) compared to the control (medium alone, 8.53% DiOC₆(3)^low cells). Apoptosis of CD56^dim NK-cells was further increased in the presence of IL-2 (35.06% DiOC₆(3)^low cells). IL-2 alone had no effect on the number of apoptotic cells in vitro. In contrast to CD56^dim NK-cells, RAGG or anti-CD16 did not enhance apoptosis of CD56^bright NK-cells (Fig.3A,C).

Previously, culture of NK-cells with anti-CD16 antibody was found to induce apoptosis, cytotoxicity, proliferation, TNF-α expression and phenotypic changes, such as upregulation of CD69 and FasL (30, 31). Thus, we assessed whether NK-cells isolated from seropositive patients (SAP and SP RA) showed
increased expression of CD69 and FasL, indicating functional FcγRIII-triggering in vivo. Both CD69 and FasL were expressed at a higher level by CD56\textsuperscript{dim} (median 3.9% and 2.0%, respectively) than CD56\textsuperscript{bright} (median 2.1% and 0.3%, respectively) cells in healthy controls. In SP RA, the percentages of CD69+ and FasL+ cells within CD56\textsuperscript{dim} NK-cells were significantly increased (6.7%, p=0.027 and 2.3%, p=0.036; respectively) when compared with HC (Fig.3D,E). A similar pattern was observed for CD56\textsuperscript{dim} NK-cells in SAP but this did not reach statistical significance.

In conclusion, our in vitro data show that CD56\textsuperscript{dim} and not CD56\textsuperscript{bright} NK-cells undergo apoptosis upon FcγRIII-triggering in vitro, with the number of apoptotic cells increasing further upon addition of IL-2.

**Figure 3.** CD56\textsuperscript{dim} but not CD56\textsuperscript{bright} NK-cells undergo apoptosis upon FcγRIIIa-triggering.

(A) Gating strategy used in sorting for CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK-cells and re-analysis of the purity of sorted NK-cell subsets are shown (median 85% and 96%, respectively). CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK-cells were cultured with...
IL-2, RAGG, anti-CD16, IL-2 and RAGG, IL-2 and anti-CD16 or medium alone. Frequency of DiOC₆(3)low cells within (B) CD56dim and (C) CD56bright events. Representative histograms from 1 HC are shown. (D) CD69 and (E) FasL expression within non-stimulated CD56dim and CD56bright NK-cells from HC (n=8), SAP (n=7), SP RA (n=9) and SN RA (n=8). Representative dot plots from 1 HC are shown. Statistical significance: * p <0.05.

**CD56dim and CD56bright NK-cell subsets: different propensity to produce IFN-γ following FcγRIIIa triggering**

We next assessed the effect of CD16 triggering on cytokine production by NK-cells from healthy donors. Sorted CD56dim and CD56bright NK-cells were cultured with medium alone, IL-2, RAGG, anti-CD16, IL-2+RAGG or IL-2+anti-CD16 and supernatant was used to assess the production of IFN-γ, TNF-α, IL-12, IL-4, IL-5 and IL-6. IL-2+RAGG or IL-2+anti-CD16 stimulation induced significant upregulation of IFN-γ production, specifically in CD56dim NK-cells. The median fold increases in IFN-γ levels when compared to cultures with medium alone were 161 and 126 times, respectively. A trend (p=0.057) toward increased TNF-α production by CD56dim NK-cells cultured in the presence of IL-2 and anti-CD16 was also observed. No statistically significant increase in cytokine expression was observed for CD56bright NK-cell cultures (Fig. 4).

Taken together, the observed decline of CD56dim NK-cells in SP RA and SAP may be mechanistically explained by RF/ACPA immune complex-mediated induction of CD16-dependent apoptosis. Moreover, CD16 triggered cytokine production by CD56dim NK-cells may contribute to the early pro-inflammatory state as seen in SAP and SP RA.

**Figure 4. CD56dim but not CD56bright NK-cells produce IFN-γ upon FcγRIIIa-triggering.**

CD56dim and CD56bright NK-cells were sorted and cultured as described in Fig.3. After 16h, supernatants from various culture conditions of CD56dim and CD56bright NK-cells (from 4 HC used for Fig.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. Statistical significance: * p <0.05.
Discussion

We show a profound decline of NK-cells in recently diagnosed RA and in seropositive arthralgia patients, representing subjects at risk of progressing towards RA (26, 27). By stratifying our RA cohort according to autoantibody status, we found the NK-cell decrease associated with seropositive, but not seronegative RA. Moreover, the decline in NK-cells may be explained by a selective decrease of CD56<sup>dim</sup> NK-cells as a result of apoptosis induction via FcγR triggering by IgG-containing immune complexes. In line with published data (29-34), 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<sup>dim</sup> and CD56<sup>bright</sup> NK-cell subsets to FcγR-induced cell death.

The robust decline of NK-cells in SAP and in recently diagnosed SP RA suggests that this alteration may contribute to disease development rather than represent the consequence of long-term inflammation. Most of the published data describe similarly decreased NK-cell numbers in later stages of RA (35-37). The use of NSAIDs was found to have no effect on peripheral NK-cell numbers (38). Thus, NSAIDs are unlikely causal to the NK-cell decline in SAP 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. Furthermore, the NK-cell decrease was associated with plasma cell development and increased systemic levels of IgG autoantibodies (7). This, together with the here described decline of NK-cells in SP RA, but not in SN RA, suggests a protective role for NK-cells in RA development.

Despite similarly reduced NK-cell numbers in SAP and early SP RA, the decline of IFN-γ expression was observed in the latter group only. Thus, as shown for CIA (7), the progression of pre-RA to overt disease may be associated with a reduction of NK-cells as well as their functional impairment. This would confirm the beneficial role of IFN-γ in arthritis pathology as shown in CIA (6-8) and RA (39).

The decline of peripheral NK-cell numbers in seropositive, but not in seronegative patients, as well as the previously reported induction of NK-cell apoptosis by FcγR-triggering (29, 30, 33) suggested a role of RA-related IgG-containing autoantibodies in this process. The majority of ACPAs are IgG (40) and can be bound by IgM RF (41). A study by Boros et al showed that IgM from RA sera was reactive with FcγRIII (42). Furthermore, about half of RA patients have RF in a form of small IgG complexes (43), which are efficiently bound by FcγRIIIa (44).

We confirmed induction of NK-cell apoptosis by agonistic anti-CD16 antibody and rabbit aggregated IgG in vitro, a process that was enhanced by addition of IL-2 (29, 30, 33). We observed a higher sensitivity of sorted CD56<sup>dim</sup> NK-cells to FcγR crosslinking-induced apoptosis, which is likely the result of the higher expression of CD16 compared to CD56<sup>bright</sup> cells (15). Differential susceptibility of CD56<sup>dim</sup> and CD56<sup>bright</sup> NK-cells to FcγR-dependent apoptosis corresponds to the decline of circulating CD56<sup>dim</sup> but not CD56<sup>bright</sup> NK-cells in the seropositive patients in vivo. It is unlikely that the decline of CD56<sup>dim</sup> NK-
cells in the periphery is a result of a preferential recruitment of this population to the joints as SF-derived NK-cells were mainly of the CD56\textsuperscript{bright} phenotype (data not shown, (19)).

Our hypothesis of autoantibody-mediated stimulation of NK-cells in vivo is supported by the increased expression of CD69 and FasL by non-stimulated CD56\textsuperscript{dim} NK-cells in SP RA patients. Upregulation of both these markers has been demonstrated upon in vitro culture of isolated NK-cells or NK-cell clones in the presence of anti-CD16 antibody (30, 31). FasL, expressed upon FcγRIII-triggering, has been shown to facilitate NK-cell apoptosis in an autocrine manner (30), thereby contributing to enhanced cell death.

We observed an increased number of CD56\textsuperscript{bright} NK-cells in seronegative RA. Prior data suggests that the expansion of CD56\textsuperscript{bright} NK-cells is more specific for autoimmune diseases such as systemic lupus erythematosus (SLE) (45) or multiple sclerosis (46) than RA and was associated with increased levels of type I interferons. As suggested by Meyer, in contrast to “classical” ACPA- and RF- positive RA with a central role of TNF-\textgreek{a}, seronegative RA shares more similarities with SLE (47).

As shown previously (31, 32), CD16 triggering of CD56\textsuperscript{dim} NK-cells induced production of IFN-\textgreek{g} and TNF-\textgreek{a}, cytokines implicated in RA pathogenesis. This process was augmented by the addition of IL-2. We observed that the subset of CD56\textsuperscript{dim} NK-cells may be primarily responsible for the enhanced cytokine expression, although this feature has previously been attributed to CD56\textsuperscript{bright} NK-cells (15, 16, 21-25). Increased IFN-\textgreek{g} and TNF-\textgreek{a} expression by CD56\textsuperscript{bright} compared to CD56\textsuperscript{dim} was previously seen following stimulation with combinations of monocyte-derived cytokines (21-23), PMA/ionomycin (16, 22) or whole bacterial pathogen (25). Involvement of CD16 in the modulation of CD56\textsuperscript{dim} NK-cell cytokine expression has also been demonstrated (48-50). Thus, pro-inflammatory cytokine production cannot be exclusively attributed to CD56\textsuperscript{bright} NK-cells. Depending on the available stimulus, both NK-cell subsets can produce cytokines.

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 ACPA and RF autoantibodies occurs years before RA onset (3). FcγRIIIa- triggering of CD56\textsuperscript{dim} NK-cells by autoantibody-immune complexes could result in activation and cytokine expression. Persistent FcγRIIIa-triggering, however, in a pro-inflammatory environment may lead to loss of function and a higher sensitivity to apoptosis of the CD56\textsuperscript{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 stage (3). Activation of CD56\textsuperscript{dim} NK-cells may thus contribute to the pro-inflammatory state as seen in SAP and SP RA. Moreover, the decline of CD56\textsuperscript{dim} NK-cells may allow for uncontrolled expansion of autoimmune cells contributing to RA development.

Also, our results demonstrate differences in the systemic immune profile between seropositive and seronegative RA adding to the notion that SP RA and SN RA may represent different disease entities.
Chapter 4

Supplementary Figure 1. Numbers of circulating T-cells and B-cells in patients and controls.

Absolute numbers of (A) CD3+CD4+ T-cells, (B) CD3+CD8+ T-cells, (C) CD19+ B-cells and the relative frequencies within CD45+ lymphocytes of (D) CD3+CD4+ T-cells, (E) CD3+CD8+ T-cells and (F) CD19+ B-cells was assessed in the peripheral blood of HC (n=33), SAP (n=30), early RA SP (n=44) and SN RA (n=11). Statistical significance: * p <0.05.

Supplementary Figure 2. CD56^{dim} and CD56^{bright} NK-cell subsets from SP RA are characterized by an increased NKG2D expression.

Frequencies of (A) CD94+NKG2A+, (B) NKG2D+, (C) CD57+ and (D) KIR2DL4+ cells within total CD56+, CD56^{dim} and CD56^{bright} NK-cell subsets was assessed using PBMC from HC (n=15), SAP (n=16), early SP RA (n=20) and SN RA (n=11). Statistical significance: * p <0.05.

References


