Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a CD56\textsuperscript{bright},CD94\textsuperscript{bright},CD158\textsuperscript{negative} phenotype

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Background. Natural killer (NK) cells play an important role in several animal models of autoimmunity by modulating T-cell responses, but it is unclear whether human NK cells have similar functions.

Methods. We characterized the phenotype of NK cells in synovial fluid (SF) and peripheral blood (PB) of patients with rheumatoid arthritis (RA) and in healthy control subjects using flow cytometry and quantitative PCR.

Results. The proportions of NK cells in PB and SF of RA patients were not significantly different from those in healthy PB. However, the SF NK cell phenotype was strikingly different, with increased CD94 and CD56 densities and greatly reduced proportions of cells expressing CD158a\textsubscript{b}. These cells also had reduced mRNAs coding for CD158a\textsubscript{b} and low perforin levels compared with RA PB and healthy PB NK cells.

Conclusions. We identified a novel phenotype of SF NK cells that is of potential significance in RA. Experiments are now under way to determine the function of these SF NK cells and their potential role in RA.

KEY WORDS: NK cells, Rheumatoid arthritis, Synovial fluid, Killer immunoglobulin-like receptors, CD158, CD94.

Rheumatoid arthritis (RA) is an autoimmune condition in which a chronic inflammatory response develops, particularly in synovial joints, in the absence of an identifiable infectious agent. There is compelling evidence that activation of T cells is central to the development of the inflammation [1–3], but the mechanisms leading to the long-standing T-cell activation are poorly understood. In several animal models of autoimmunity, natural killer (NK) cells have been shown to control T-cell activation. In both the EAE (experimental autoimmune encephalomyelitis) [4] and the Staphylococcus aureus-induced arthritis [5] disease models, depletion of NK cells before disease induction resulted in an increase in disease activity. NK cell-mediated suppression of antigen-specific T-cell responses has also been demonstrated in vitro, both in mice [4] and in rats [6]. In RA, several studies have investigated the potential involvement of NK cells. All of these detected NK cell-like lymphocytes in the joints but conclusions about their cytotoxicity were conflicting [7–9]. Furthermore, these studies were all carried out before receptors regulating NK cell activity were defined. Better understanding of the biology of NK receptors has led to a greater knowledge of their physiological role, and consequently their role in disease can now be investigated.

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The receptors involved in the regulation of NK cell activity fall into three categories. Natural cytotoxicity receptors (NCR) [10], including Nkp30 [11], Nkp44 [12] and Nkp46 [13], bind mostly undefined ligands on potential target cells. The resulting activating signal is essential for NK cell-mediated cytotoxicity [13], and at least one NCR-NCR ligand interaction is necessary to initiate NK cell-mediated cytotoxicity against a given target cell [11].

In addition, cytotoxicity is also regulated by a combination of C-type lectin-like and immunoglobulin (Ig) superfamily member receptors. These receptor families contain both activating and inhibitory forms, and recognize HLA class I molecules and structurally related proteins as their ligands. The inhibitory signal elicited by the interaction of MHC class I molecules and their inhibitory receptors protect the cell from NK cell-mediated damage [14]. As proposed by the ‘missing self’ hypothesis [15], NK cells eliminate target cells which, due to malignant transformation or viral infection, have lost the expression of MHC class I molecules [14]. The inhibitory killer Ig-like receptors (KIRs), mainly present on NK cells [16], include KIR2DL1-3 receptors (recently designated CD158a/b1/b2 [17]), which interact with HLA-C locus products [18]; KIR2DL4 (CD158d), recognizing HLA-G [19]; the three-domain KIR3DL (CD158e1/k) receptors, binding to various HLA-A and B proteins; and KIR2DL5 (CD158f), with as yet undefined ligand(s) [20–23]. The C-type lectin-like CD94/NKG2A/B heterodimers indirectly recognize certain HLA-class I molecules through their leader peptides, which are presented by the non-classical MHC class I molecule HLA-E [24]. The inhibitory signal elicited via the above receptors in NK cells is dominant over activating signals and results in the survival of the target cell [25].

Both KIR and C-type lectin-like receptors exist in activating as well as inhibitory forms. These activating receptors also recognize MHC class I molecules or structurally related proteins as their ligands [26]. The role of these receptors in the immunosurveillance of emerging tumours or malignantly transformed cells remains poorly understood [27]. The activating receptors encoded by KIR2DS1 and 2 (CD158h and j) are recognized by the same monoclonal antibodies as the related inhibitory molecules KIR2DL1-3 (CD158a and CD158b1/2).

Apart from their role in regulating NK cell-mediated cytotoxicity, CD158 receptors have also been shown to modulate T-cell responses, including antigen-dependent cell proliferation, cytokine production and CTL-mediated cytotoxicity [28, 29]. It has been hypothesized that inhibitory forms of CD158 are found on potentially autoreactive T cells, and these receptors are involved in preventing autoreactive responses [30]. This hypothesis, and the observation that NK cells can modulate T-cell activation, suggest that these cells have the potential to play a significant role in the pathological process of RA and prompted us to study NK cells and T cells expressing CD158 (KIR2DL1-3/S1-2) receptors in RA.

**Materials and methods**

**Cell populations**

The study was approved by the South Sefton research ethics committee. Thirty-six matched synovial fluid (SF) and peripheral blood (PB) samples, one unpaired PB sample and three unpaired SF samples were collected from patients with RA who fulfilled the revised American College of Rheumatology (ACR) criteria for the disease. The ratio of female to male patients was 2.3:1 and the age range 32–87 yr, with a mean age of 56.4 yr. Control PB samples were obtained from healthy laboratory staff and ovarian follicular fluid samples from patients undergoing in vitro fertilization at the Liverpool Women’s Hospital. Mononuclear cells were separated from the samples using density gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden), and NK cells were separated from other mononuclear cells using negative magnetic separation (StemSep®, StemCell Technologies, Meylan, France). The purified samples contained 90–95% CD56+ cells according to flow cytometric analysis.

**Flow cytometry**

The antibodies used for cell surface antigen staining by twocolour flow cytometry were: anti-CD3–FITC (UCHT1; Dako, Cambridge, UK) anti-CD158a (KIR2DL1/S1, EB6 unconjugated; Serotec, Oxford, UK), anti-CD158b (KIR2DL2/L3/82, GL183 unconjugated; Serotec) anti-CD94 (HP-3D9 unconjugated; Pharmingen, San Diego, CA, USA), anti-CD56–PE (N901; Immunotech, Marseilles, France), isotype-matched control (IgG1–PE; catalogue no. 0670, Immunotech), goat anti-mouse IgG–PE conjugate (P-9670 Sigma, St Louis, MO, USA), sheep anti-mouse IgG–FITC (fluorescein isothiocyanate) conjugate (F-2883 Sigma) (both secondary antibodies), anti-CD56–FITC (Becton Dickinson, Franklin Lanes, NJ, USA), anti-CD158a–PE (Immunotech, Marseilles) and anti-CD158b–PE (Immunotech). Aliquots (2 × 105 cells) were incubated on ice with the appropriate antibodies for 30 min. Where necessary, this was followed by two washing steps with phosphate-buffered saline (PBS) and further incubation on ice with a secondary antibody. Further washing steps were carried out before analysis on a Epics XL-MCL flow cytometer (Coulter, High Wycombe, UK). Lymphocytes were gated based on their forward and side scatter characteristics.

For intracellular staining of perforin, the mononuclear cells were counted, and 5 × 105 cells were dispensed into flow cytometry tubes. The cells were washed with PBS + 1% fetal calf serum (FCS) and then fixed using 100 μl of 1% paraformaldehyde (BDH, Poole, UK) incubated for 20 min at room temperature with vortex mixing every few minutes. Saponin buffer (PBS + 1% FCS + 0.1% saponin) was then used to wash the cells. The cell pellet was resuspended in a total volume of 200 μl of saponin buffer before adding 10 μl of mouse anti-human perforin R-PE-conjugated antibody (clone S9; catalogue no. 65995X, Pharmingen) or the mouse R-phycocerythrin (R-PE)-conjugated isotype control (catalogue no. 66375X, Pharmingen). The antibody was incubated with the cells for 30–40 min at room temperature in the dark.

Following this incubation, the cells were washed with saponin buffer. The membrane was then restored by incubating the cells for 10 min with PBS + 1% FCS. The cells were spun down and the cell pellet was resuspended in the residual volume left in the tube. Four microlitres of the mouse anti-human CD56–FITC-conjugated antibody (clone NCAM16.2; catalogue no. 340410, Becton Dickinson) or mouse anti-human IgG1–FITC-conjugated negative control antibody (catalogue
no. PN IM0639, Immunotech) was added and incubated with the cells for 30 min on ice in the dark. The cells were washed twice with PBS + 1% FCS and then resuspended in PBS before analysis on the Coulter XL-MCL flow cytometer.

Stimulation of NK cells with IL-2 or serum/synovial fluid supernatant

Paired purified SF mononuclear cells and peripheral blood mononuclear cells (PBMCs) were resuspended in standard tissue culture medium (RPMI 1640; Life Technologies, Paisley, UK) + 10% FCS (Harlan Sera-Lab, Loughborough, UK) + l-glutamine (2 mM) + penicillin (50 U/ml)/streptomycin (50 µg/ml) (both from Life Technologies) and incubated with or without interleukin (IL) 2 (100 U/ml; I-2644, Sigma) in tissue culture flasks. The expression of CD94 and CD158a or without interleukin (IL) 2 (100 U/ml; I-2644, Sigma) in tissue culture flasks. The expression of CD94 and CD158a receptors was determined on CD3+ cells (10.7%) and CD158b (KIR2DL1-3 and KIR2DS1-2) was assessed after 24 h by flow cytometry.

In similar experiments, purified SF mononuclear cells from RA patients were resuspended in either standard tissue culture medium (RPMI + 10% FCS + l-glutamine + penicillin/streptomycin) or in a 1:1 mixture of RPMI medium and autologous plasma. In parallel, PBMCs collected from the same patients were resuspended either in the standard medium or a 1:1 mix of RPMI and the cell-free supernatant of the SF from the same patient. Following overnight incubation, cell surface expression of CD158 (KIR2DL1-3/SI-2) and CD94 receptors was analysed as previously.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from $5 \times 10^5$ mononuclear cells or purified NK cells using the RNeasy Blood kit (Qiagen, Crawley, UK). cDNA was then generated using the Reverse Transcription System (Promega, Madison, WI, USA) with oligo(dT) primers. The KIR2DL3 quantitative polymerase chain reaction (PCR) was carried out in a volume of 25 µl, containing 1 µl of cDNA, 5 pmol of each primer and 0.5 U Red Hot Taq DNA polymerase, reaction buffer 289 (AbGene, Epsom, UK). Triplicate reactions were run for 50 cycles at 95°C for 30 s, 60°C for 1 min and 72°C for 30 s, using a Rotor-gene four-colour instrument (Corbett Instruments, Cambridge, UK) with DNA detected using SYBR Gold dye (Biogene, Cambridge, UK) at a concentration of 1:100 000. Ten-fold dilutions of plasmid DNA, were used to generate the standard curve.

Housekeeping genes were measured using the Lightcycler h-HPRT (hypoxanthine phosphoribosyl transferase) and h-G6PDH (glucose-6-phosphate dehydrogenase) housekeeping gene sets (Roche Diagnostics, Mannheim, Germany). The supplied RNA standards were reverse-transcribed using random hexamers. Triplicate reactions of each standard (from $5 \times 10^5$ to 50 molecules/reaction) were used to generate a standard curve in a 12.5 µl volume containing 0.25 U Red Hot Taq DNA polymerase, reaction buffer 289, 2 mM MgCl2; and the Lightcycler detection mix (containing the PCR primers, the dNTPs and the two labelled FRET probes for detection of the PCR product). The cycling conditions for both housekeeping genes were 50 cycles at 95°C for 30 s, 55°C for 45 s and 72°C for 30 s. PCR product was detected by the binding of the two probes after 15 s of the annealing phase (excitation wavelength 470 nm, detection wavelength 660 nm).

DNA cloning and sequencing

PCR products were cloned into the pGEM-T Easy vector (Promega), purified using the Wizard SVplus Miniprep plasmid extraction kit (Promega) and the size of the inserted gene was confirmed by PCR. The DNA was sequenced on an ABI (Foster City, CA, USA) automated DNA sequencer at the School of Tropical Medicine, University of Liverpool, in order to confirm PCR amplification of the primer specific mRNA. Bulk cultures of the KIR2DL3 plasmid were purified using the QiaFilter Maxi kit (Qiagen) and quantified by spectrophotometry for use as a standard in the real-time PCRs, as above.

Statistical analysis

Differences between experimental groups were tested non-parametrically using the Mann–Whitney U-test for unpaired samples and the Wilcoxon signed rank test for paired samples.

Results

Phenotypic characterization of peripheral blood and synovial fluid NK cells in RA

The phenotype of lymphocytes present in SF and PB was analysed by two-colour flow cytometric analysis (Fig. 1). CD56+CD94+ NK cells (Fig. 1a) represented 7.7 ± 4.1% of all gated lymphocytes in the PB of healthy volunteers, 8.0 ± 5.3% in the PB of RA patients and 8.2 ± 5.3% in the SF of the patients; all these values were within the normal range, with no statistical difference [31]. However, while the percentages of cells expressing CD94+ and CD56+ in the PB and SF samples was not significantly different, SF NK cells expressed these molecules at much higher densities on their cell surface. The mean fluorescence intensity (MFI) for CD56 was 22.2 ± 11.3 (arbitrary units) for SF samples, 8.8 ± 2.1 for normal PB lymphocytes and 10.6 ± 3.5 for PB lymphocytes from RA patients (both P < 0.0005). Similarly, the MFI for CD94 was significantly elevated in RA SF (15.4 ± 5.7) compared with RA PB lymphocyte samples (8.7 ± 2.1; P < 0.0001) and normal PB lymphocytes (10.2 ± 2.7; P < 0.013) (Fig. 1a). The increase in MFI for CD56 and CD94 was not a generalized increase in all cell surface markers on SF lymphocytes compared with matched PB lymphocytes, as the MFI for CD3 expression on the PB lymphocytes was 49.7 ± 11.5, while the MFI for CD3 expression on SF lymphocytes was 36.1 ± 8.9.

The expression of CD158a/b (KIR2DL1-3/SI-2) receptors was determined on CD3+ lymphocytes from healthy controls and matched PB lymphocytes and SF samples from RA patients (Figs 1b, c and 2A). In the healthy control group, 4.5 ± 2.3% of lymphocytes were CD3+CD158a+ and 6.2 ± 1.3% were CD3+CD158b+. The total of CD158a+ and CD158b+ cells (10.7%) exceeded the number of CD94+ cells (7.7 ± 4.1%) as expected, given that a single NK cell can express both CD158a and CD158b receptors simultaneously. These percentages fall within the range reported in the literature [31, 32]. In the PB lymphocytes of RA patients, the percentages of both CD158a+ and CD158b+ cells appeared reduced (Fig. 2A). While this reduction was not statistically significant for CD158a, the difference in CD158b expression between the PB of the healthy controls and patients reached statistical significance (P = 0.003).
NK cells from the SF of the patients showed dramatically reduced expression of CD158a and CD158b receptors (Fig. 2A). Only $0.4 \pm 0.5$ and $0.7 \pm 0.6\%$ of these cells expressed CD158a and CD158b respectively, compared with $3.0 \pm 2.5$ and $4.0 \pm 3.6\%$ of cells in matched PB lymphocytes ($P<0.0001$ in both cases) (Fig. 2A).

To determine if the lack of CD158a/b receptors on the SF NK cells merely reflected changes associated with the extravasation of NK cells, we also analysed six ovarian follicular fluid samples. NK cells from this site expressed CD158a and b in proportions comparable to PB lymphocytes (Fig. 2B). Furthermore, while the MFI of CD94 expression on ovarian follicular fluid NK cells (mean $\pm$ s.d. $13.2 \pm 1.5$) was elevated to levels similar to those on SF NK cells, the CD56 MFI was relatively low ($14.9 \pm 1.3$), as on the NK cells from the PB lymphocytes from RA patients and healthy controls. Thus, the CD56$^{bright}$,CD94$^{bright}$,CD158a$^{-}$ and b-deficient phenotype was characteristic of NK cells found in the RA SF and not a universal phenomenon associated with extravasation.

To illustrate that the reduction in the percentage of CD3 lymphocytes expressing CD158 corresponded to a reduction on NK cells, five further paired samples of PB and SF from RA patients were dual-stained with anti-CD56-FITC and anti-CD158a/b-PE. On average, the gated PB lymphocytes in these samples contained $16.7 \pm 6.1\%$ CD56$^{+}$ lymphocytes and the SF lymphocyte population included $13.6 \pm 7.7\%$ CD56$^{+}$ cells. As found previously, there was no statistical difference in the proportions of NK cells in the PB and SF lymphocyte populations. The percentage of lymphocytes that were both CD56$^{+}$ and CD158a$^{+}$ or CD158b$^{+}$ was analysed. The SF showed a lower percentage of NK cells expressing CD158a or CD158b ($0.4 \pm 0.2$ and $0.8 \pm 0.4\%$ respectively) compared with matched PB samples ($1.7 \pm 2.1$ and $5.5 \pm 1.9\%$ respectively). This reduction reached statistical significance for CD158b ($P=0.03$), but did not quite reach statistical significance for CD158a ($P=0.06$) using the Wilcoxon statistical test.

**Decreased CD158 expression on synovial fluid NK cells is not due to proteolytic activity**

To test whether the observed decrease of CD158 expression on SF NK cells was due to proteolytic cleavage of these receptors from the cell surface, we isolated PBMC from patients (expressing CD158 receptors) and incubated them overnight in medium containing 50% autologous cell-free SF supernatant. This incubation did not produce any detectable decrease in
surface expression of CD158a, CD158b or CD94 (data not shown), which suggests that proteolysis by enzymes present in the SF did not affect receptor expression. Furthermore, overnight incubation either in tissue culture medium containing 100 U/ml IL-2 or in RPMI medium containing 50% autologous plasma failed to detectably increase cell surface expression of CD158a/b receptors on SF lymphocytes (data not shown). This again argues against receptor loss due to proteolytic mechanisms, and also suggests that these receptors are not simply masked by autoantibodies or soluble class I molecules.

**NK cells in RA synovial fluid contain reduced levels of CD158a and CD158b mRNAs**

We next investigated the levels of mRNAs coding for KIR2DL molecules by quantitative PCR using purified NK cell samples. Due to the difficulty of obtaining sufficient NK cells from the SF and the small volume of the patient blood samples, only six RA PB and seven RA SF samples were measured, four of which were paired. The KIR2DL3 mRNA was significantly reduced in SF NK cells compared with healthy PB NK cells, irrespective of whether the samples were normalized against G6PDH (glucose-6-phosphate dehydrogenase) ($P = 0.02$) or HPRT (hypoxanthine phosphoribosyl transferase) ($P < 0.001$) (Fig. 3a and b). In both cases the medians of the healthy PB NK cells and RA PB NK cells were virtually identical. All four paired samples showed reduced levels in the SF NK cells compared with the paired RA PB NK cells, regardless of which housekeeping gene was used (Fig. 3c and d). The quantitative PCR reaction amplified only KIR2DL3 molecules, as confirmed by cloning and sequencing of the PCR product. Other primer sets amplifying KIR2DL2 or all of KIR2DL1-3 showed a similar degree of reduction (data not shown).

**NK cells in RA synovial fluid contain less perforin than matched peripheral blood NK cells**

The percentage of gated lymphocytes that showed positive fluorescence for both CD56 and perforin was
measured in nine paired RA PB and SF samples and in nine healthy PB samples. A reduction in the percentage of NK cells containing perforin was found in the SF (5.7 ± 3.9%) compared with the matched PB (13.3 ± 6.1%) \((P=0.02, \text{Wilcoxon test})\). Healthy PB showed percentages similar to those of RA PB (14.9 ± 6.3%) (no significant difference, Wilcoxon test) (Fig. 4).

Expression of NK-cell receptors on T cells of the peripheral blood and synovial fluid of RA patients

The monoclonal antibody staining experiments were designed to give information not only on NK cell phenotypes but also on the expression of NK cell markers on T cells. As shown in Table 1, the percentage of CD56+ cells in the lymphocyte population was 62.2 ± 19.1% in the PB of RA patients, significantly lower than in healthy PB (73.1 ± 6.5%) \((P=0.003)\). The RA SF also had fewer T cells in the gated lymphocyte population (61.5 ± 25.5%) than normal PB samples. However, this difference was not statistically significant and the range of values was much wider, both in the RA PB and SF samples (Table 1). The percentages of T cells expressing NK cell markers CD94, CD158a and CD158b showed wide variations between individuals. T cells expressing CD94 were somewhat fewer in RA PB lymphocytes (1.6%) than in PB of healthy controls (2.8%) and in matched RA SF (2.3%). The proportions of CD158a-expressing T cells in healthy PB, PB of RA patients and SF were similar (0.2, 0.5 and 0.4% respectively), while there was an increase in the mean percentage of CD158b+ T cells in the PB and SF of patients with RA compared with the PB of healthy volunteers. However, the CD158b result was biased by one patient who had unusually high levels of CD3+,CD158b+ cells in their PB and SF lymphocytes (13.2 and 11.0% respectively).

**Discussion**

Apart from their cytotoxic activity, NK cells secrete a variety of cytokines and modulate T-cell responses, both in vitro and in animal models of autoimmunity [4–6]. However, a similar regulatory role for NK cells in human autoimmune conditions remains to be proven. In the present study, we report the consistent presence of substantial numbers of NK cells in the SF of affected joints in RA patients that exhibit an unusual phenotype. More than 90% of SF NK cells were devoid of the receptors CD158a and CD158b, while both CD56 and CD94 were expressed at high densities. NK cells with this phenotype are rarely observed in PBL [25]. A recent study by Dalbeth and Callan [36] also showed a decrease in expression of KIR/KAR receptors in the SF of a
group of 21 RA patients. This group of workers stained with a panel of monoclonal antibodies, including antibodies against KIR3DL1 and KIR3DL2, which probably accounts for the modest decrease seen in their data compared with ours. The predominance of the cells we observed in the SF is the result either of their development in situ, selective accumulation, or of proliferation.

The loss of KIR receptors as a result of down-regulation due to extended contact with their ligands has been documented [33]. However, under experimental conditions stimulation of these receptors led to a decrease in cell surface density rather than the complete loss seen in the RA patients. Furthermore, the observation that NK cells in the follicular fluid express normal levels of CD158 receptors shows that this loss was not the result of extravasation.

The presence of CD158NK cells in the SF could be the result of the selective loss of this receptor. As the SF is rich in proteolytic enzymes, it is possible that the loss of CD158 receptors is the result of selective proteolysis. Alternatively, soluble HLA class I molecules or other factors could bind to and mask the receptors, rendering them undetectable by monoclonal antibodies. However, coinoculation studies of RA PB lymphocytes with matched SF supernatant and incubation of the SF lymphocytes with tissue culture medium or matched PB serum did not alter the proportions or intensity of receptor expression. Furthermore, in all these instances the levels of mRNAs coding for the receptors would be expected to be normal or even elevated, whereas we observed that the level of expression of mRNA for KIR2DL3 was drastically reduced in SF NK cells. Thus, the reduction of CD158a/b expression on SF NK cells was due to low mRNA levels, resulting in low protein production.

In vitro experiments have shown that, in the presence of IL-15, early thymocytes developed into NK cells, which were CD94+ but did not express CD158a/b receptors [34]. The phenotype of NK cells found in SF was compatible with this developmental pathway, and high IL-15 levels are present in joints affected by RA [35]. However, the early thymocytes used in these in vitro experiments are unlikely to be present in the peripheral circulation. Indeed, in preliminary experiments the incubation of PBMCs from RA patients in the presence of IL-15 for 16 h did not result in an increase in the proportion of CD94+CD158NK cells (C. Pridgeon, unpublished data). However, an increase in the expression of CD56 following incubation for 10 days with a combination of IL-12 and IL-15 has been reported recently [36].

While practically all NK cells in PB are positive for CD56, CD56NK cells represent a relatively small proportion of NK cells. The lack of CD158 receptors and elevated expression of CD94 are characteristic features of the CD56NK SF NK cells. It has been reported that CD56NK PB NK cells show reduced cytotoxic activity [37] because of low perforin and granzyme levels as well as the failure of CD56NK cells to bind to potential target cells [38]. Our observation of decreased percentages of perforin-positive NK cells in the SF compared with the PB supports this. Additionally, CD56NK NK cells have been reported to produce significantly higher amounts of cytokines upon stimulation than their CD56NK counterparts [39]. The composition of the resulting cytokine mix is highly dependent on the stimulation used in vitro. Our preliminary experiments indicated that non-stimulated SF NK cells produce IL-10 and transforming growth factor β (TGF-β), suggesting that SF NK cells have the potential to contribute significantly to the development and maintenance of the cytokine milieu in the arthritic joints.

T cells infiltrating involved joints in patients with RA predominantly express a memory phenotype [2, 3] and attempts to identify expanded antigen-specific T-cell receptor (TCR) populations have been unsuccessful. Such inflammation may therefore result from erroneous regulatory pathways rather than direct antigen-driven accumulation. It has been proposed that T cells expressing inhibitory NK-cell receptors represent a potentially autoreactive cell population that is controlled by the presence of the inhibitory NK-cell receptors [30]. However, our results show that double-positive CD3+CD158NK cells are rare, representing less than 3% of all lymphocytes.

Some studies have recently focused on cell populations with proven or assumed T-cell regulatory capacity in various human autoimmune disorders, such as CD161+ TCR Vα24NK cells and CD4+CD25+ regulatory T cells [40–42]. However, these cell subsets represent a very small portion of the infiltrating lymphocytes, whereas the NK cells described here represent a much larger lymphocyte subset. Thus, their role in T-cell regulation, either by cytokine production or direct cell–cell interaction, is likely to be more significant, especially given that NK cells with the CD56NK phenotype have a known cytokine-producing role [39].

This study has yielded a consistent and unusual NK cell phenotype from affected joints of patients with RA that has the potential to be of profound significance in modulating inflammation in this disease. Definition of the exact cytokine production profiles and functional capacity of these cells is currently under way. This may provide clues to understanding the crucial immunoregulatory pathways in RA.

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