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Balance between activating NKG2D, DNAM-1, NKp44 and NKp46 and inhibitory CD94/NKG2A receptors determine natural killer degranulation towards rheumatoid arthritis synovial fibroblasts

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Summary

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and synovial hyperplasia leading to progressive joint destruction. Fibroblast-like synoviocytes (FLS) are central components of the aggressive, tumour-like synovial structure termed pannus, which invades the joint space and cartilage. A distinct natural killer (NK) cell subset expressing the inhibitory CD94/NKG2A receptor is present in RA synovial fluid. Little is known about possible cellular interactions between RA-FLS and NK cells. We used cultured RA-FLS and the human NK cell line Nishi, of which the latter expresses an NK receptor repertoire similar to that of NK cells in RA synovial fluid, as an *in vitro* model system of RA-FLS/NK cell cross-talk. We show that RA-FLS express numerous ligands for both activating and inhibitory NK cell receptors, and stimulate degranulation of Nishi cells. We found that NKG2D, DNAM-1, NKp46 and NKp44 are the key activating receptors involved in Nishi cell degranulation towards RA-FLS. Moreover, blockade of the interaction between CD94/NKG2A and its ligand HLA-E expressed on RA-FLS further enhanced Nishi cell degranulation in co-culture with RA-FLS. Using cultured RA-FLS and the human NK cell line Nishi as an *in vitro* model system of RA-FLS/NK cell cross-talk, our results suggest that cell-mediated cytotoxicity of RA-FLS may be one mechanism by which NK cells influence local joint inflammation in RA.

Keywords: CD94/NKG2A; fibroblast-like synoviocytes; natural killer cytotoxicity; NKG2D.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and progressive destruction of cartilage and bone.[1] The healthy synovial lining that encapsulates the joint cavity is only a few cell layers thick and is composed of two main cellular subsets termed fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes. In RA the synovial membrane becomes hyperplastic, including a tumour-like proliferation of activated FLS (RA-FLS) together with infiltrating leucocytes such as macrophages, T cells, natural killer (NK) cells and B cells. Together, these cells form a synovial pannus, which aggressively spreads into the joint cavity, invading the cartilage and bone, which subsequently results in structural tissue damage.[2]

The RA-FLS play an essential role in driving cartilage erosion.[3] As recently reviewed by Niedermeier *et al.*,[4] these cells can directly infiltrate cartilage and release mediators responsible for proteolytic matrix degradation, including matrix metalloproteinases. Moreover, RA-FLS contribute to RA pathogenesis by production of pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-15 and granulocyte–macrophage colony-stimulating

factor, as well as secretion of chemokines, including stromal cell-derived factor 1 (CXCL12), IP10 (CXCL10), monocyte chemotactic protein 1 (MCP1), IL-8 (CXCL8), and RANTES (CCL5).[5–9]

Natural killer cells are bone marrow-derived lymphocytes that are important in immune defence. Upon activation, they rapidly produce a range of cytokines and mediate cytotoxic responses against infected, stressed or malignantly transformed cells.[10–13] It is becoming increasingly appreciated that NK cells may play a role in the modulation of a multitude of immune responses through their capacity to promote differentiation of monocytes into osteoclasts and dendritic cells, and to promote polarization of T-cell and B-cell responses.[14–17] Furthermore, recent studies in mice have shown that NK cells eliminate subsets of activated T cells via cell-mediated cytotoxic responses.[18,19] Natural killer cell-mediated killing of activated T cells is dampened by the interaction with its antigen (in mouse this is Qa-1, the mouse equivalent of human HLA-E) expressed on activated T cells, and inhibitory CD94/NKG2A receptors expressed on NK cells and subsets of $CD8^+$ T cells.[20] Consistent with *in vitro* data, disrupting the interaction between CD94/NKG2A and Qa-1 in mouse models of RA and multiple sclerosis (e.g. in collagen-induced arthritis and experimental-allergic encephalomyelitis models), using antibodies against CD94/NKG2A or Qa-1 ameliorated disease,[18,19,21] and in one study in experimental-allergic encephalomyelitis it led to a reduction in the number of follicular helper T cells and T helper 17 cells.[19] Therapeutic enhancement of NK cell-mediated cytotoxic responses therefore appears to be beneficial in certain experimental models of human chronic inflammatory diseases.

Human NK cells may be divided into two main subsets based on surface expression of CD56 and CD16 (FcγRIII). $CD56^{dim}CD16^+$ NK cells make up approximately 90% of peripheral blood (PB) NK cells, whereas the remaining 10% are $CD56^{bright}$ $CD16^{dim/-}$ NK cells.[22–24] The $CD56^{bright}$ subset is, however, the dominant subset in secondary lymphoid organs such as tonsils and lymph nodes.[25,26] Moreover, NK cells with a $CD56^{bright}$ phenotype are over-represented at inflammatory sites in numerous diseases,[27,28] including synovial fluid (SF) of RA patients.[29,30]

Previous studies suggest that RA-FLS promote the migration, activation and survival of PB NK cells *in vitro*.[31] Based on the finding that the vast majority of RA SF NK cells display an activated phenotype *in vivo*, together with the observation that RA-FLS express MHC class I-chain related protein A (MIC-A) and MIC-B, ligands for the activating NK cell receptor NKG2D,[32,33] we investigated the cross-talk that takes place between NK cells and RA-FLS *in vitro*. Using the human NK cell line Nishi, we investigated whether RA-FLS can stimulate NK cell degranulation that reflects NK cell-mediated cytotoxicity *in vitro*, and if so, which receptor–ligand interactions were involved.

Materials and methods

Ethics statement

Collection of human tissue, SF and blood samples was approved by the local ethics committee at each site (listed below for each sample) and informed consent was obtained from all participating patients.

Clinical RA synovial samples

Synovial tissue biopsies for the isolation of RA-FLS were provided by Dr Henning Bliddal and Dr Martin Andersen from the Rheumatology Department, Frederiksberg Hospital, Copenhagen, Denmark, and obtained from the small joints of RA patients undergoing arthroscopic synovectomy (eight patients, approved by The Ethics Committee for Copenhagen and Frederiksberg Municipalities, Denmark). Samples of SF for *in vitro*-derived RA-FLS and enrichment of autologous NK cells were collected from patients at the Rheumatology Clinic, Rigshospitalet Hospital (Copenhagen, Denmark) by Dr Bo Baslund and Dr Lars Juul (two patients, approved by The Ethics Committee for Copenhagen, Denmark). Paired SF and PB samples for cell surface staining of NK cells were from the Rheumatology unit of Karolinska University Hospital (eight patients, approved by the Ethics Review Board in Stockholm). Synovial fibroblasts from non-inflamed tissue of healthy donors without RA were purchased from Asterand (two donors, under full compliance with all UK Human Tissue Authority Codes of Practice and directives).

Isolation and culture of cells

The RA-FLS were derived from human synovial tissue as previously described.[34] Isolated RA-FLS and healthy FLS were cultured in RPMI-1640 GlutaMAX™ medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2% heat-inactivated human serum (Sigma-Aldrich, St Louis, MO), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 nM basic fibroblast growth factor (Invitrogen, Carlsbad, CA).[35] For two experiments (Fig. 5e), RA-FLS were isolated from RA SF by adding SF directly to the above cell culture medium, after which RA-FLS adhered to the tissue culture flask. The RA-FLS were passaged at 90% confluency (approximately every 4–7 days) and passaged no more than five times for use in experiments. Nishi is a human NK cell line derived from the peripheral blood mononuclear cells of a boy with chronic active Epstein–Barr virus infection complicated with NK cell leukaemia.[36] Nishi NK cells

were grown in 24-well plates in IMDM GlutaMAX™ medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2% heat-inactivated human serum (Sigma-Aldrich), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml recombinant human IL-15 (PeproTech, Rocky Hill, NJ). NK cells were enriched from RA SF by centrifuging SF, resuspending cells in the above-described medium and enriching for NK cells by negative selection magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched SF NK cells were cell surface stained and analysed by flow cytometry; or expanded in the same culture medium for up to 3 weeks before co-culture with autologous SF-derived RA-FLS. Mononuclear cells were prepared from paired RA PB and RA SF samples by Ficoll separation (Pharmacia, Stockholm, Sweden), frozen in 10% DMSO (Sigma-Aldrich) and 90% fetal calf serum (Life Technologies, Carlsbad, CA), and stored in liquid nitrogen for further analysis by flow cytometry.

Flow cytometry

The following antibodies were used for surface staining of either RA-FLS (detached using 2 mM EDTA) or NK cells at 10 μg/ml or 1 : 100 dilution, together with Near-IR LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen): FITC anti-CD3 (HIT3A, BD Biosciences, Franklin Lakes, NJ), FITC-conjugated anti-CD158a/b (EB6, BD Biosciences), FITC-conjugated anti-CD158b1/b2 (DX27, BD Biosciences), FITC-conjugated anti-CD158e1 (DX9, BD Biosciences), FITC-conjugated anti-DNAX accessory molecule-1 (DNAM-1; DX11, BD Biosciences), phycoerythrin (PE) -conjugated anti-NKG2D (1D11, BD Biosciences), PE-conjugated anti-CD85j (HP-F1, BD Biosciences), PE-conjugated anti-CD244 (2B4, BD Biosciences), PE-Cy7-conjugated anti-CD56 (B159, BD Biosciences), anti-HLA-E (3D12, eBioscience, San Diego, CA), anti-MHC class I (DX17, BD Biosciences), PE-conjugated anti-CD48 (BJ40, BioLegend, San Diego, CA), anti-MIC-A (159227, R&D Systems, Minneapolis, MN), anti-MIC-B (236511, R&D Systems), anti-UL-16 binding protein 1 (ULBP-1; 170818, R&D Systems), anti-ULBP-2/5/6 (165903, R&D Systems), anti-ULBP-3 (166510, R&D Systems), anti-CD112 (R2.525, BD Biosciences), PE-conjugated anti-CD155 (SKII.4, BioLegend), allophycocyanin (APC) -conjugated anti-CD54 (HA58, BD Biosciences), anti-KIR2D (NKVFS1, Miltenyi Biotec), PE-conjugated anti-CD55 (A10, BD Biosciences), APC-conjugated anti-CD68 (Y1/82A, BD Biosciences), anti-HLA-G (87G, BioLegend) and PE-conjugated anti-NKG2C (134591, R&D Systems). Uncojugated antibodies were detected by subsequent incubation with APC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Mouse IgG1, mouse IgG2a and mouse IgG2b were used as isotype controls (R&D Systems). The specificities of the anti-ULBP-1, -2 and -3 monoclonal antibodies (mAbs; R&D Systems) were tested on BA/F3 or K562 cells transfected with ULBP-1, -2, -3, -4 or -6. We found that the anti-ULBP-1 and anti-ULBP-3 mAbs specifically recognize only ULBP-1 and ULBP-3, respectively, whereas anti-ULBP-2 binds ULBP-2, ULBP-5 and ULBP-6 (data not shown). Flow cytometry was performed on a BD LSR II or BD FACSCanto II (BD Biosciences). Data were analysed using FlowJo software (TreeStar Inc., Ashland, OR).

NK degranulation assays

Adherent RA-FLS (passaged up to five times) were trypsinized (Gibco) and seeded at 3×10^4 cells/well in flat-bottom 96-well plates. The next day, NK cells were added at 9×10^4 cells/well. Cells were co-cultured for 5 hr at 37° with FITC-conjugated anti-CD107a mAb (H4A3, BD Biosciences), FITC-conjugated anti-CD107b mAb (H4B4, BD Biosciences) and BD GolgiStop™ containing monensin (BD Biosciences). The following mouse anti-human mAbs were added at 10 μg/ml where indicated: anti-NKG2D (149810, R&D Systems), anti-NKG2A (humanized mAb Z270 (NNC0141-0100), isotype human IgG4, Novo Nordisk A/S, Måløv, Denmark), anti-LIR-1 (GHI/75, BioLegend), anti-2B4 (C1.7, BioLegend), anti-DNAM-1 (DX11, BioLegend), anti-NKp30 (P30-15, BioLegend), anti-NKp44 (P44-8, BioLegend), anti-NKp46 (9E2, BioLegend) and anti-lymphocyte function-associated antigen 1 (LFA-1/CD11a; HI111, BioLegend). Mouse IgG1 (R&D Systems), mouse IgG2b (R&D Systems) and human IgG4 (Sigma-Aldrich) were used as isotype controls. It was verified that none of the antibodies resulted in CD107a/b expression when added to NK cells alone (data not shown). Following the 5-hr CD107 assay, non-adherent cells were stained with Peridinin chlorophyll protein-conjugated anti-CD3 (SK7, BD Biosciences), Pacific Blue-conjugated anti-CD16 (3G8, BD Biosciences), PE-conjugated anti-NKG2A (Z199, Beckman Coulter, Pasadena, CA), Alexa Fluor 700 anti-CD56 (B159, BD Biosciences) and Near-IR LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen). NK cells were gated as viable, single, $CD3^-$ $CD56^+$ $CD16^+$ cells. Results are expressed as % $CD107a/b^+$ NK cells.

RA-FLS 'clearance' assays

The RA-FLS were trypsinized (Gibco), counted and seeded at 1×10^4 cells/well in 48-well plates, grown to confluency (approximately $4{\cdot}8 \times 10^4$ cells/well) for 72 hr, and Nishi were added at the indicated effector : target ratios overnight. In another experiment, RA-FLS were seeded at 2×10^4 cells/well in 96-well plates, the following day, 6×10^3 cells/well Nishi were added, RA-FLS and Nishi were co-cultured overnight. Following overnight

co-cultures of RA-FLS and Nishi, non-adherent cells were removed by washing with PBS, and the remaining adherent cells were fixed with 4% paraformaldehyde and stained with Eosin. The image analysis was performed using a Series CTL-ImmunoSpot S5 Micro Analyzer (Cellular Technology, Bonn, Germany). Images were analysed and quantified using ImmunoSpot software (CTL-Europe, Bonn, Germany).

Statistical analysis

Statistical significances were determined by an analysis of variance test, and a Tukey post-test (determined using GraphPad Prism software; GraphPad Prism, San Diego, CA). All values are given as average ± SEM. Statistical significance is defined as the following: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Results

Synovial fibroblasts express multiple ligands for NK cell receptors

We first evaluated whether synovial fibroblasts derived during surgery of non-inflamed joints from healthy donors without RA are potential targets for NK cells. The synovial lining layer contains both FLS and macrophage-like synoviocytes.[37] The complement-regulatory molecule CD55 has previously been shown to specifically mark FLS located in the synovial lining,[38,39] whereas CD68 is a well-known macrophage marker that is present in macrophage-like synoviocytes. Flow cytometry analyses showed that cultured synoviocytes derived from non-inflamed healthy synovial tissue express CD55, but not CD68, consistent with the FLS phenotype (Fig. 1a). We found that

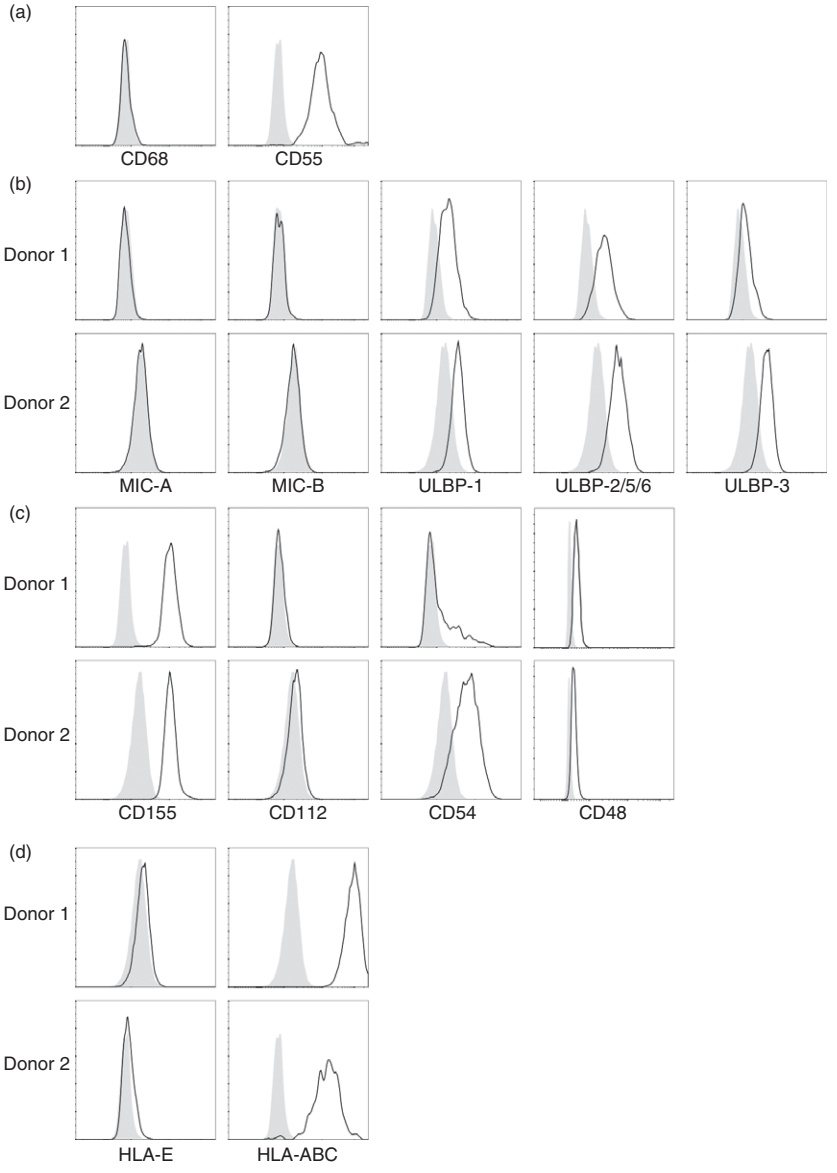

Figure 1. Synovial fibroblasts from non-inflamed synovium express numerous ligands for natural killer (NK) cell receptors. Synovial fibroblasts derived from tissue of non-inflamed joints were surface stained for expression of (a) fibroblast-marker CD55 and macrophage-marker CD68, ligands for activating NK cell receptors (b) NKG2D [MHC class I-chain related protein A,B (MIC-A,B) and UL-16 binding protein 1 (ULBP-1; ULBP-2/5/6,3)] and (c) DNAX accessory molecule 1 (DNAM-1; CD155 and CD112), lymphocyte function-associated antigen 1 (LFA-1; CD54) and 2B4/CD244 (CD48); and (d) ligands for inhibitory NK cell receptors. Filled histograms represent isotype-matched control immunoglobulin. Figure shows stainings from two donors.

FLS from non-inflamed synovium from two donors express numerous ligands for several activating NK cell receptors, including ULBP-1, ULBP-2/5/6 and ULBP-3, but not MIC-A or MIC-B, all known ligands for the activating NK cell receptor NKG2D (Fig. 1b). Among the ligands for the activating receptor DNAM-1, FLS from donors without RA expressed CD155 (poliovirus receptor) but not CD112 (poliovirus-related receptor 2). Efficient cytolytic function and cytokine production by NK cells are dependent on the adhesion molecule integrin β_2 LFA-1 binding to CD54 (intercellular adhesion molecule 1) on target cells,[40] and we detected high levels of CD54 on non-inflamed FLS; as well as CD48, a ligand for the activating 2B4 (CD244) receptor (Fig. 1c). Next we analysed whether non-inflamed FLS express MHC class I ligands, which can be recognized by inhibitory NK cell receptors, and we found that FLS expressed high levels of classical MHC class I molecules (i.e. HLA-A, -B and -C), but not HLA-E, the latter of which is the ligand for the inhibitory NK cell receptor CD94/NKG2A (Fig. 1d).

To assess whether synovial fibroblasts from an inflammatory setting express similar ligands we raised several short-term adherent cell lines from the small joints of RA synovial tissue biopsies. We confirmed that tissue-derived RA-FLS cultured *in vitro* are $CD68^-$ $CD55^+$ (shown in Fig. 2a for a representative donor) and similar to non-inflamed FLS, the RA-FLS also express the NKG2D ligands ULBP-1, ULBP-2/5/6 and ULBP-3 (Fig. 2b), as well as the DNAM-1 ligand CD155, the LFA-1-ligand CD54 and the 2B4 (CD244) ligand CD48 (Fig. 2c). We detected expression of the NKG2D ligand MIC-A in two of nine RA donors. Furthermore, we found that RA-FLS express both high levels of classical MHC class I molecules (HLA-A, -B, -C), and HLA-E, the ligand for CD94/NKG2A (Fig. 2d).

Overall, FLS from the non-inflamed synovium of healthy donors as well as RA-FLS express numerous ligands for both activating and inhibitory NK cell receptors, and are hence capable of forming a cytolytic synapse. Interestingly, it has recently been shown that both mouse and human NKG2D ligands are subject to regulation by proliferative signals, as serum starvation or inhibition of proliferation (via inhibition of cyclin-dependent kinases by Roscovitine) reduced expression of the NKG2D ligand RAE-1ε on primary mouse fibroblasts and tumour cell lines, as well as MICA/B and ULBP-2 on a human tumour cell line.[41] In our hands, serum starvation affected the expression of the detected ligands by RA-FLS in a variable pattern (see Supporting information; Fig. S1a). However, we found that inhibition of proliferation by Roscovitine did reduce, but not abrogate, the expression of ULBP-1 and ULBP-2/5/6 by RA-FLS (Fig. S1b). This suggests that the expression of CD155, CD54, HLA-E and HLA-A, -B, -C on RA-FLS is not regulated by proliferative signals, whereas the expression of ULBP-1 and ULBP-2/5/6 is partially regulated in connection with proliferation.

Characterization of SF NK cell receptor repertoire

It has been previously reported that RA SF NK cells primarily exhibit a $CD56^{bright}$ phenotype, and are phenotypically similar to the $CD56^{bright}$ NK cell subset in PB.[29,30] We characterized the receptor repertoire of NK cells from paired peripheral blood mononuclear cells and SF mononuclear cells (SFMC) samples from RA patients, and confirmed that RA SF NK cells exhibit a $CD56^{bright}$ phenotype and express LIR-1 and KIR to a lower frequency and CD94/NKG2A to a higher frequency, compared with RA PB NK cells (Fig. 3). Furthermore, similarly to RA PB NK cells, RA SF NK cells are primarily $DNAM\text{-}1^+$ $NKG2D^+$, whereas the expression of 2B4 or NKG2C were more heterogeneous in the RA SF NK cell population compared with NK cells derived from PB (Fig. 3).

We next wished to analyse the receptor–ligand interactions between NK cells and RA-FLS. Due to difficulties in isolating sufficient numbers of RA SF NK cells to perform such experiments, we evaluated whether the human NK cell line Nishi could serve as an appropriate effector cell model. We analysed the expression of NK cell receptors on Nishi cells and found that they are phenotypically nearly identical to RA SF NK cells, in that Nishi cells express the activating receptors NKG2D, DNAM-1, 2B4 and LFA-1 (Fig. 4a). Previous studies have shown that RA SF NK cells also express the natural cytotoxicity receptors (NCRs) NKp30 and NKp44, the latter of which is an activation marker.[42] Both NKp30 and NKp44, as well as NKp46, were also expressed by Nishi cells (Fig. 4a). Similarly to RA SF NK cells, Nishi cells are also $KIR2D^-CD94/NKG2A^+$ (Fig. 4b). In contrast, although we could only detect a small subset of RA SF NK cells positive for LIR-1, all Nishi cells express this inhibitory receptor (Figs 3 and 4). Based on these phenotypic similarities, we reasoned that Nishi cells could mimic RA SF NK cells in an *in vitro* set-up aimed at investigating the receptor–ligand interactions between NK cells and RA-FLS.

Nishi cells induce clearance of RA-FLS in an *in vitro* culture

To assess NK cell interactions with RA-FLS, the latter were grown to confluence. Subsequently Nishi were added at different effector : target (E : T) ratios for a 24-hr co-culture. As shown in Fig. 5(a), an almost complete disappearance of adherent RA-FLS was observed at an E : T of 1 : 1, whereas decreasing the E : T ratio led to a gradual increase in the number of adherent RA-FLS (Fig. 5a). These results suggest that Nishi cells are mediating 'clearance' of adherent RA-FLS *in vitro*, and although previous

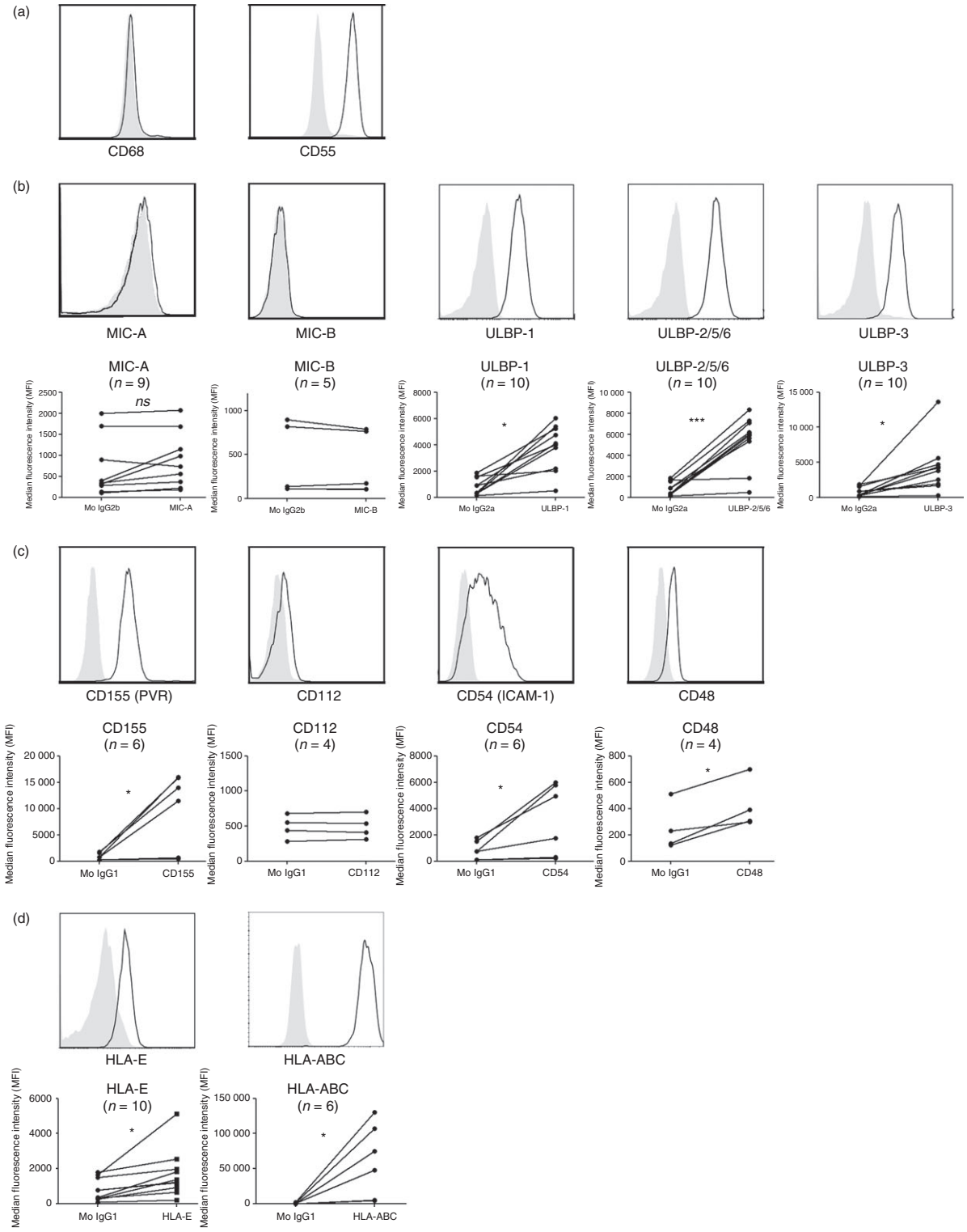

Figure 2. Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) express numerous ligands for natural killer (NK) cell receptors. RA-FLS derived from overgrowth of RA synovial tissue were surface stained for expression of (a) fibroblast-marker CD55 and macrophage-marker CD68 (histograms are representative of $n = 3$ to $n = 5$ donors), ligands for activating NK cell receptors (b) NKG2D [MHC class I-chain related protein A,B (MIC-A,B) and UL-16 binding protein 1 (ULBP-1; ULBP-2/5/6,3) and (c) DNAX accessory molecule 1 (DNAM-1; CD155 and CD112), lymphocyte function-associated antigen 1 (LFA-1; CD54) and 2B4/CD244 (CD48); and (d) ligands for inhibitory NK cell receptors. Filled histograms represent isotype-matched control immunoglobulin. Histograms in (b) to (d) are representative of the number of donors indicated in plots of median fluorescence intensity (MFI) beneath.

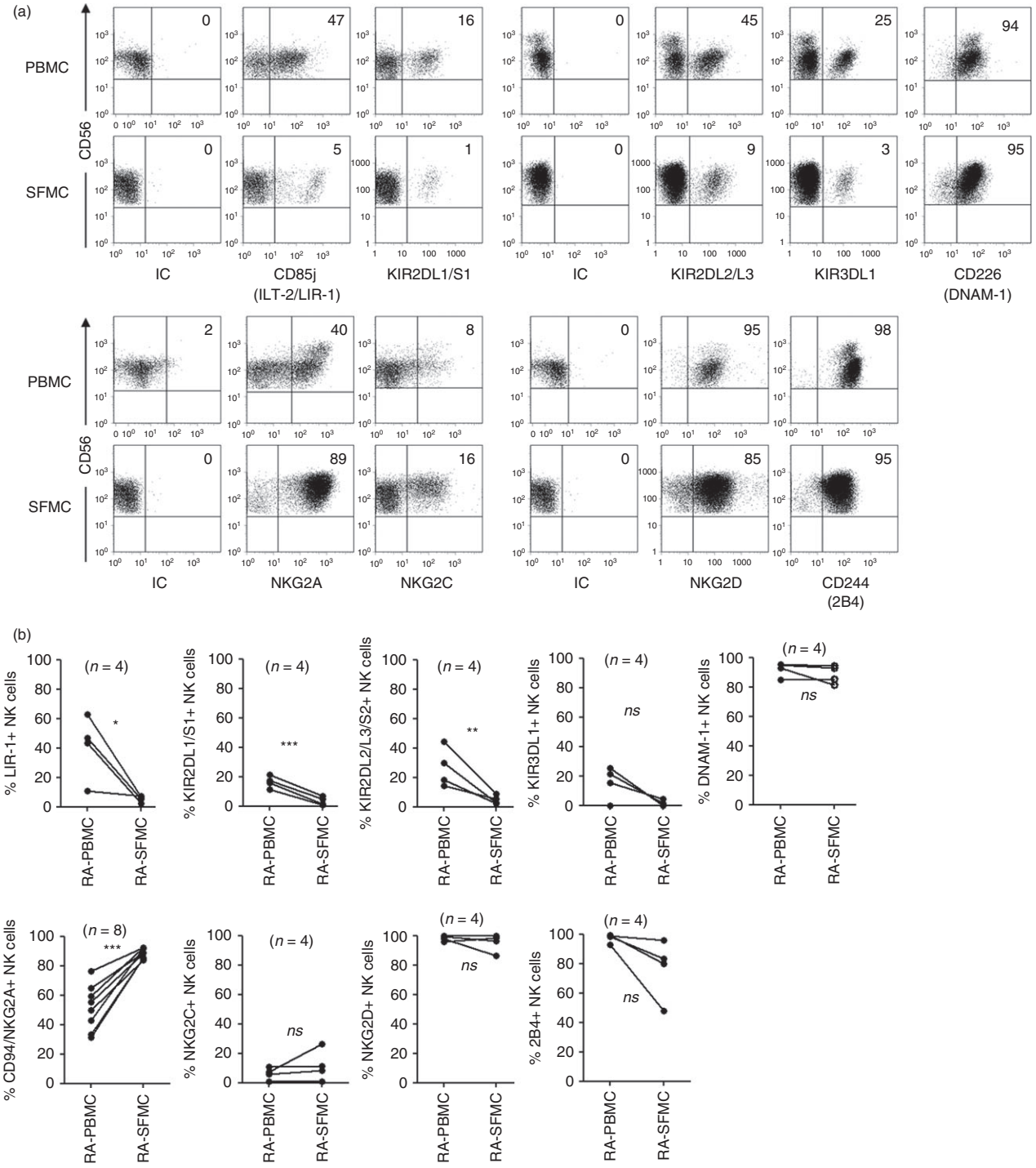

Figure 3. Expression of natural killer (NK) cell receptors on paired rheumatoid arthritis synovial fluid mononuclear cell (RA SFMC) and peripheral blood mononuclear cell (PBMC) samples. Frozen samples of paired SFMC and PBMC from $n = 4$ to $n = 8$ RA donors were stained for expression of various NK cell receptors as indicated. Dot plots in (a) are representative examples of NK receptor expression and (b) shows % NK cells positive for a given receptor, relative to isotype-matched control immunoglobulin. NK cells were gated as viable, single, $CD3^-$ $CD14^-$ $CD56^+$ cells.

studies suggest that adherent cells detach following apoptosis induction *in vitro*,[43] further experiments are required to confirm that this clearance of RA-FLS is indeed a result of Nishi NK cell-mediated cytotoxicity.

Assuming that this 'clearance' of RA-FLS is dependent on receptor–ligand interactions between RA-FLS and Nishi cells, we used an anti-human NKG2A mAb to block the interaction between the inhibitory receptor CD94/

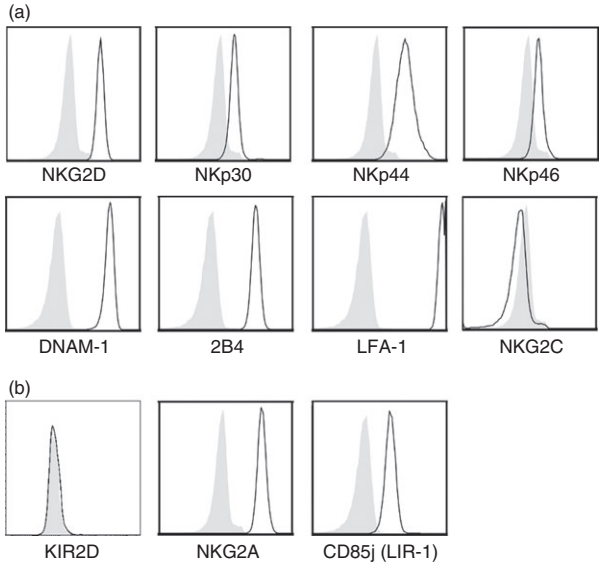

Figure 4. Expression of natural killer (NK) cell receptors by the Nishi NK cell line. Nishi NK cells were stained for surface expression of (a) activating and (b) inhibitory NK cell receptors (black line) or isotype-matched control immunoglobulin (filled histogram). Nishi NK cells were gated as viable, single $CD56^+$ cells.

NKG2A and its ligand HLA-E, expressed on RA-FLS. We reasoned that blocking this inhibitory signal in an overnight Nishi/RA-FLS co-culture would lead to increased activation of Nishi cells, and therefore also to an increased clearance of RA-FLS, as CD94/NKG2A is the primary inhibitory NK cell receptor expressed by both RA SF NK cells and Nishi cells. As shown in Fig. 5(b), blocking the interaction between CD94/NKG2A and its ligand HLA-E with an anti-NKG2A mAb did indeed lead to increased clearance of RA-FLS.

Nishi cells degranulate in response to RA-FLS via multiple activating receptors

To confirm that Nishi/RA-FLS interactions result in activation of NK cell functions, i.e. cytotoxic responses against RA-FLS, we analysed cell surface expression of CD107a [lysosomal-associated membrane protein (LAMP-1)], and CD107b (LAMP-2), molecules, which become exposed on the NK cell surface following exocytosis of lytic granules,[44] and which are known to correlate with specific target cell lysis. As shown in Fig. 5(c), Nishi cells cultured alone do not degranulate (1·4 ± 0·2% are $CD107a/b^+$), but an average of 12·0 ± 1·4% Nishi cells degranulate in response to RA-FLS, supporting the theory that RA-FLS trigger Nishi NK cell activation. In an identical set-up using FLS derived from the non-inflamed synovium of two healthy donors, we found that non-RA FLS can also stimulate Nishi NK cell degranulation, as 15·1% and 6·2% of Nishi NK cells are $CD107a/b^+$ following co-culture with healthy donors 1 and 2, respectively (see Supporting information, Fig. S2).

Successful triggering of NK cell functions results from an integration of both activating and inhibitory signals.[45] To evaluate which receptor–ligand interactions are involved in the NK/RA-FLS cross-talk, a set of neutralizing mAbs targeting various activating and inhibitory receptors was added to the co-cultures described above. The antibodies were chosen to cover a panel of NK cell receptors shown to be expressed on RA SF NK cells, as well as on Nishi cells (Fig. 5d). The addition of these antibodies, or immunoglobulin-matched isotype controls, to Nishi cells in the absence of RA-FLS did not result in Nishi cell degranulation (data not shown). As shown in Fig. 5(d), the addition of anti-NKG2D reduced Nishi cell degranulation by an average of 46·4% ± 6·5%, relative to isotype control. Moreover, masking the NCRs NKp44 and NKp46 reduced degranulation by 21·8% ± 2·5% and 49·2% ± 9·0%, respectively. The activating receptor DNAM-1 also plays an important role in the interaction between Nishi cells and RA-FLS, as adding an anti-DNAM-1 mAb to the co-culture reduced degranulation by 57·3% ± 7·9%. Furthermore, we found that blocking the interaction between the inhibitory CD94/NKG2A receptor and its ligand HLA-E enhanced Nishi cell degranulation towards RA-FLS by 82·9% ± 20·0% relative to isotype control (Fig. 5d). Masking the NCR NKp30, the 2B4 receptor or the inhibitory LIR-1 receptor did not affect Nishi degranulation towards RA-FLS (data not shown). Overall, this suggests that NKG2D, DNAM-1, NKp44 and NKp46 are involved in Nishi degranulation towards RA-FLS, and that RA-FLS expression of the ligand for CD94/NKG2A, HLA-E, inhibits Nishi cell degranulation.

IL-15-activated RA SF NK cells degranulate in response to autologous RA-FLS in *in vitro* co-cultures

Another approach taken to assess the interaction between RA-FLS and RA SF NK cell cross-talk was to derive *in vitro* RA-FLS and RA SF NK cells from the same SF sample to operate in an autologous set-up. We were able, from SF samples from two RA patients, to derive RA-FLS and enrich the SFMC for RA SF NK cells using IL-15-stimulated SF cultures for up to 3 weeks. We reasoned that IL-15 stimulation would mimic the inflammatory conditions in the RA joint as IL-15 has been shown to be in abundance in an RA joint.[46,47] These IL-15-stimulated RA-SFMC were co-cultured with autologous RA-FLS in a CD107 assay, and NK cells were subsequently identified as $CD3^-$ $CD56^+$ IL-15-activated SFMC. These IL-15-stimulated RA SF NK cells degranulated in response to autologous RA-FLS, and masking CD94/NKG2A with an anti-NKG2A mAb led to a significant increase in NK cell degranulation in both samples (Fig. 5e). This suggests

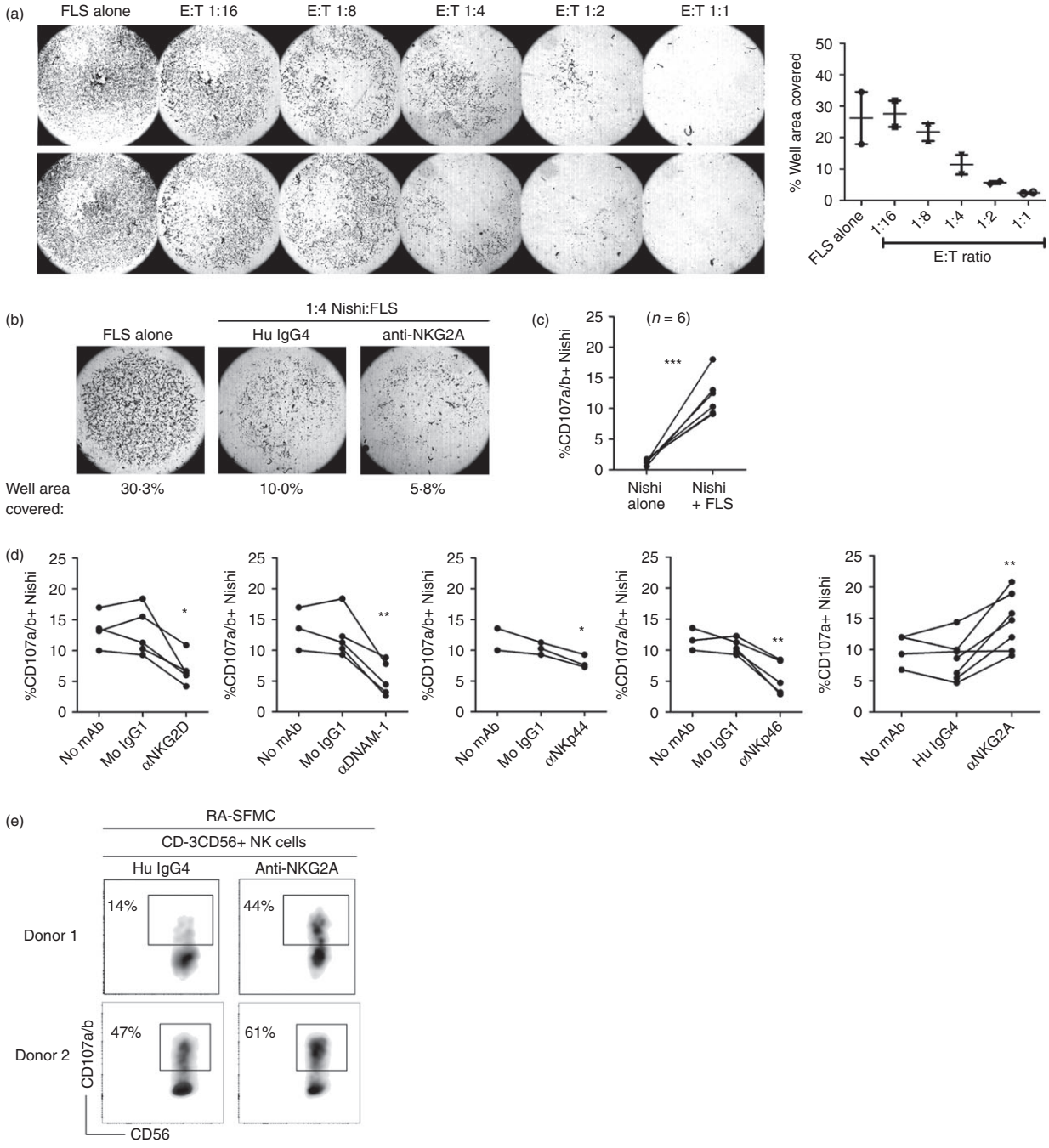

Figure 5. NKG2D, DNAX accessory molecule 1 (DNAM-1), NKp44 and NKp46 are involved in Nishi natural killer (NK) cell degranulation towards rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). (a) RA-FLS were seeded at 1×10^4 cells/well in 48-well plates, grown to confluency (approximately $4{\cdot}8 \times 10^4$ cells/well) for 72 hr, and Nishi were added at the indicated ratios overnight. Adherent cells were fixed with 4% paraformaldehyde and stained with eosin. An ImmunoSpot Image Analyzer was used to take images and quantify % of well area covered. Figure and graph are duplicates of one donor, and representative of $n = 2$ donors. (b) RA-FLS were seeded at 2×10^4 cells/well in 96-well plates, the following day 6×10^3 cells/well Nishi were added to the approximately $4{\cdot}8 \times 10^4$ RA-FLS/well giving an effector : target ratio of 1 : 4. RA-FLS and Nishi were co-cultured overnight. Wells were analysed as described. Representative of $n = 3$. (c, d) RA-FLS were seeded at 3×10^4 cells/well in 96-well plates, the following day 9×10^4 Nishi/well were added. Nishi and RA-FLS were co-cultured as described and blocked with monoclonal antibodies (mAbs) or isotype-matched control immunoglobulin as indicated. Nishi were identified as viable, single $CD3^-$ $CD16^-$ $CD56^+$ cells. Data points are a combination of several experiments and a total of $n = 8$ donors. (e) RA synovial fluid mononuclear cells (SFMC) were enriched for NK cells by antibody-coated bead separation and cultured in interleukin-15 (IL-15) for up to 3 weeks. RA-FLS were seeded at $1{\cdot}5 \times 10^4$ cells/well in 96-well plates, the following day $4{\cdot}5 \times 10^4$ autologous enriched $CD3^-$ $CD56^+$ RA SF NK cells were added per well. NK cells and FLS were co-cultured as described. Results from two donors are shown. NK cells were identified as viable, single $CD3^-$ $CD56^+$ cells.

that RA SF NK cells, which have been IL-15-activated *in vitro*, can also degranulate in response to RA-FLS, a process that is further enhanced upon blocking CD94/NKG2A.

Discussion

It is becoming increasingly appreciated that NK cells play an important role in regulating adaptive immune responses. In this study, we have set-up a model system to investigate NK cell/RA-FLS interactions *in vitro*. We have shown that the human NK cell line Nishi degranulates upon co-culture with RA-FLS *in vitro*, and found that DNAM-1 and NKG2D are key activating NK cell receptors involved in mediating this response, because blocking these receptors with mAbs drastically reduced Nishi NK cell degranulation. NKG2D and DNAM-1 are well-characterized receptors, which have previously been shown to play a role in NK cell-mediated lysis of macrophages,[48] tumour cells,[49] microglia,[50] dendritic cells,[51] activated $CD4^+$ T cells[52–56] and regulatory T cells.[57] The NCRs NKp44 and NKp46 are also involved in NK cell recognition of RA-FLS, because blocking these receptors also reduced Nishi cell degranulation.

We detected ligands for NKG2D and DNAM-1 on RA-FLS from all donors. Of the NKG2D ligands, we detected surface expression of ULBP-1 and ULBP-2/5/6, but not MIC-A/B, despite previous studies showing expression of MIC-A/B in RA synoviocytes by immunohistochemistry.[32,33] This discrepancy could be due to a low level of MIC-A/B expression, which can be detected by immunohistochemistry but not by flow cytometry. Our findings suggest that further studies using ULBP- and MIC-specific mAbs should be performed to conclude which members of the NKG2D-ligand family are present at inflammatory sites in RA. Endogenous ligands for the NCRs NKp44 and NKp46 are still obscure, although viral haemagglutinins have been proposed as possible ligands,[58,59] but attempts to identify the ligand(s) on RA-FLS have so far been unsuccessful. Ligands for activating NK cell receptors are typically induced upon cellular stress, viral infection or tumorigenesis.[60] Our data suggest that the expression of the NKG2D ligands ULBP-1 and ULBP-2/5/6 on RA-FLS can be regulated by proliferative signals, which supports the theory that RA-FLS may indeed express these ligands *in vivo*, as RA-FLS are highly proliferative in RA synovium.[61] A recent report using mouse fibroblasts[41] showed that the expression of these ligands is also affected by serum starvation. In our hands there was only a variable effect of serum starvation on ligand expression. Interestingly, we could detect expression of the same ligands for activating NK receptors on FLS derived from non-inflamed synovium of two healthy donors, suggesting that the expression of these ligands on RA-FLS may not be disease-specific, as they may also be up-regulated as a cellular stress response following isolation, culture and passaging of FLS *in vitro*. Indeed, our finding that Nishi NK cells also degranulate in response to FLS derived from non-inflamed synovium, despite a limitation of only having FLS from two healthy donors in this study, emphasizes that further experiments are required to analyse freshly isolated FLS from both RA and healthy donors, which have not been cultured *in vitro*, for expression of these ligands, as well as their ability to trigger NK cell cytotoxicity. It remains to be seen whether the pathogenic characteristics of RA-FLS are a result of the chronic inflammatory environment, or whether these cells are intrinsically abnormal. A previous study, using a SCID mouse model of RA, showed that RA-FLS are able to degrade normal articular cartilage in the absence of immune cells and a pro-inflammatory environment,[3] suggesting that RA-FLS are of an autonomous, activated phenotype.

We could not find a correlation between expression level of HLA-E on RA-FLS, and the effect of blocking with an anti-NKG2A mAb. This is not surprising, however, as HLA-E is only expressed at the cell surface in conjunction with bound peptides, typically leader peptides derived from signal sequences of classical MHC class I molecules.[62] The nature of the bound peptide affects the stability and cell surface expression of the HLA-E/peptide complex,[63] which subsequently determines the extent to which it is recognized by NKG2A, or an anti-HLA-E antibody. The expression level of HLA-E detected by flow cytometry is therefore not necessarily indicative of the strength of its engagement with NKG2A.

Rheumatoid arthritis SF NK cells are known to exhibit a $CD56^{bright}$ $NKG2A^+KIR^-$ phenotype[30] and produce high levels of cytokines following IL-2 stimulation.[29] Although PB $CD56^{bright}$ NK cells are typically cited as being less cytotoxic compared with $CD56^{dim}$ NK cells due to lower levels of perforin, granzymes and cytolytic granules,[64] numerous studies have shown that, following cytokine activation, $CD56^{bright}$ NK cells are equally, if not more, cytotoxic compared with $CD56^{dim}$ NK cells.[23,56,65] Indeed, we found not only that RA SF NK cells have a $CD56^{bright}$ $NKG2A^+KIR^-$ phenotype, but also that they are $LIR\text{-}1^-$ $DNAM\text{-}1^+$ $NKG2D^+$. Natural killer cells with a $CD56^{bright}$ phenotype are found at inflammatory sites in a number of diseases,[27,30,66–69] and an expansion in $CD56^{bright}$ NK cells has been associated with clinical remission in autoimmune diseases such as multiple sclerosis,[70–72] implicating an immune-regulatory role for $CD56^{bright}$ NK cells in chronic inflammation. In addition to their $CD56^{bright}$ $NKG2A^+$ KIR^- phenotype,[30] RA SF NK cells have also been shown to be $CD69^+$ $NKp44^+$,[29] suggesting an activated phenotype. Under steady-state conditions, NK cells in healthy donors are resting, and hence $CD69^-$ $NKp44^-$, highlighting an important difference between RA SF NK cells and healthy resting NK

cells. This suggests that despite our finding that Nishi NK cells also degranulated in response to healthy FLS *in vitro*, this is unlikely to happen *in vivo*, for two reasons: first, normal non-inflamed synovium is relatively acellular and contains very few lymphocytes[73] and it is therefore unlikely that there would be any NK cells present; and second, NK cells under resting conditions will not elicit cytotoxicity towards healthy FLS, even if the latter express ligands for activating NK cell receptors, as NK cells require cytokines for efficient activation. Further studies are required to assess whether primary RA SF NK cells, as well as primary resting NK cells from healthy donors, can kill freshly isolated FLS from both healthy donors and RA patients *ex vivo*. The accumulation of $CD56^{bright}$ NK cells in RA SF may be a result of preferential recruitment from blood, local differentiation, or selective survival. $CD56^{bright}$ NK cells have been shown to have a higher resistance to apoptosis and to survive better in oxidant-rich surroundings,[74,75] such as in an inflamed RA joint.

The net outcome of the cellular interactions between NK cells and RA-FLS in an inflamed synovium remains unclear. A previous study has shown that cell–cell contact between RA-FLS and NK cells stimulates NK cell survival, migration, and interferon-γ production, as well as the production of pro-inflammatory chemokines and matrix metalloproteinases.[31] RA-FLS constitutively express IL-15,[76,77] a cytokine that enhances NK cell cytotoxicity and proliferation.[78] These studies imply that RA-FLS/NK contact-dependent and -independent interactions may influence joint inflammation at numerous levels. Despite a low number of donors available in this study, our results suggest that RA-FLS can stimulate degranulation of Nishi NK cells as well as autologous IL-15-stimulated RA SF NK cells *in vitro*. Further studies are required to confirm that NK cell degranulation leads to RA-FLS apoptosis, and not simply cell detachment *in vitro*. One can speculate that NK cells may also be able to kill RA-FLS in an inflamed RA joint *in vivo*, and that exploitation of the cytotoxic potential of NK cells by blocking CD94/NKG2A with an anti-NKG2A mAb may potentially yield an opportunity for therapeutic treatment of chronic inflammation. Indeed, interrupting the interaction between Qa-1 (the equivalent of HLA-E in rodents) and CD94/NKG2A, either with a genetic mutation or with a blocking anti-NKG2A $F(ab')_2$ fragment, in mouse models of multiple sclerosis (experimental-allergic encephalomyelitis) and RA (collagen-induced arthritis), resulted in reduced activated $CD4^+$ T-cell numbers and disease amelioration in a perforin-dependent fashion,[18,19,21] supporting the hypothesis that promoting NK cell-mediated elimination of pro-inflammatory immune cells may also be a potential treatment strategy in humans. A prerequisite, however, for NK cell-mediated killing is expression of relevant ligands for activating receptors, and further studies by immunohistochemistry are required to confirm that RA-FLS express the relevant ligands for activating and inhibitory NK cell receptors *in situ*. Furthermore, as this study was based on a model system of Nishi NK cell degranulation towards cultured RA-FLS *in vitro*, further functional studies are required to confirm that primary RA SF NK cells can kill freshly isolated RA-FLS *ex vivo*.

The cellular interactions between NK cells, RA-FLS, and other immune cells in the synovium of a chronically inflamed joint, and how these interactions subsequently influence disease progression, remain unclear. This study is the first to suggest that NK cells may play a role in the elimination of RA-FLS, a process that is enhanced upon blocking the ability of HLA-E to engage the inhibitory CD94/NKG2A NK cell receptor. One can speculate that NK cell-mediated cytotoxicity of RA-FLS may therefore be one mechanism through which NK cells are involved in the regulation of local inflammation in RA.

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Pieter Spee at the time of the study was employed by Novo Nordisk A/S where the studies were performed and owns shares in Novo Nordisk A/S.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of serum-free culture conditions and inhibition of proliferation on expression of ligands on rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). (a) RA-FLS were cultured under normal conditions (RPMI + 10% fetal calf serum + 2% human serum + 2 nM basic fibroblast growth factor) or in FibroGRO serum-free media for 5 days, and were surface stained for expression of ligands as indicated. Plots indicate median fluorescence intensity (MFI) for $n = 4$ donors. (b) RA-FLS were cultured under normal conditions or with the addition of 25 μM Roscovitine to inhibit proliferation for 5 days, and subsequently surface stained for expression of ligands as indicated. Plots indicate MFI for six donors.

Figure S2. Nishi natural killer (NK) cells degranulate in response to fibroblast-like synoviocytes (FLS) derived from non-inflamed synovium of two healthy donors. FLS were seeded at 3×10^4 cells/well in 96-well plates, the following day 9×10^4 Nishi/well were added. Nishi and FLS were co-cultured as described. CD107a/b expression was detected on Nishi cells by flow cytometry. Nishi were gated as viable, single CD3$^-$ CD16$^-$ CD56$^+$ cells. Nishi cultured alone (without FLS) did not express CD107a/b (data not shown).