

BAFF from Bone Marrow-Derived Mesenchymal Stromal Cells of Rheumatoid Arthritis Patients Improves Their B-Cell Viability-Supporting Properties

(rheumatoid arthritis / bone marrow / mesenchymal stromal cells / B cells / BAFF)

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Abstract. Mesenchymal stromal cells (MSCs) represent a unique cell type with anti-proliferative effects on activated T and B cells. Based on our observation of differences between rheumatoid arthritis and osteoarthritis bone marrow B cells we hypothesized that rheumatoid arthritis bone marrow MSCs may enhance B-cell survival. We aimed to compare the effect of rheumatoid arthritis and osteoarthritis bone marrow-derived MSCs (rheumatoid arthritis MSCs, osteoarthritis MSCs) on the survival of healthy donor purified B cells. Rheumatoid arthritis and osteoarthritis MSCs were isolated from patients undergoing hip replacement surgery, and cultured *in vitro* for 2–5 passages. Washed cells were co-cultured with CD20⁺ B cells for 30–90 hours. Cell survival was analysed using 7-amino-actinomycin D labelling by flow cytometry. Expression of mRNA and protein was determined by RT-PCR and flow cytometry. Co-culture with both rheumatoid arthritis MSCs and osteoar-

thritis MSCs significantly enhanced B-cell survival, the effect being more prominent in rheumatoid arthritis MSCs. Both types of MSCs displayed expression of B cell-activating factor mRNA and protein. Blocking B cell-activating factor signalling from MSCs by specific anti-B cell-activating factor and anti-B cell-activating factor receptor antibodies weakly reversed the effect of MSCs on B-cell survival mainly in rheumatoid arthritis MSCs. MSC interaction with B cells provides stimuli for B-cell survival and therefore may contribute to the pathogenesis of rheumatoid arthritis. MSC-derived factors other than B cell-activating factor are likely to contribute to this effect. This feature is more prominent in rheumatoid arthritis MSCs, possibly due to the B cell-activating factor.

Introduction

Rheumatoid arthritis (RA) is a chronic, progressive, inflammatory, systemic disorder primarily affecting the synovium, cartilage and bone of diarthrodial joints, resulting in their destruction, severe disfigurement and loss of function (Pavelka et al., 2003). The autoimmune character of RA is well documented and widely accepted; however, the exact pathophysiological mechanisms are still incompletely understood.

There are several lines of evidence showing that bone marrow (BM) is actively involved in RA and that its role goes far beyond just being a source of immune cells, possibly acting as a secondary lymphoid organ with important implications for the pathogenesis of RA. Although not specific to RA, signal intensity variations on magnetic resonance imaging (MRI) scans (increased signal intensity in T2 and STIR-weighted fat-suppressed images) in the vicinity of affected joints even at early stages of RA have been described as “bone marrow oedema” (Ostergaard et al., 2003; McQueen et al., 2003,

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Abbreviations: Ab – antibody, APC – antigen-presenting cell, ARA – American Rheumatism Association, BAFF – B cell-activating factor, BCMA – B-cell maturation antigen, BM – bone marrow, CIA – collagen-induced arthritis, FLS – fibroblast-like synoviocyte, FSC – forward-scatter, GC – germinal centre, Ig – immunoglobulin, mAb – monoclonal antibody, MRI – magnetic resonance imaging, MSC – mesenchymal stromal cell, PBMC – peripheral blood mononuclear cell, OA – osteoarthritis, RA – rheumatoid arthritis, RF – rheumatoid factor, SLE – systemic lupus erythematosus, SSC – side-scatter, TACI – transmembrane activator calcium modulator cyclophilin ligand interactor.

2006). This suggests that BM reacts to or possibly participates in the inflammatory process in its neighbourhood. The presence of widening vascularized canals connecting the synovium (Marinova-Mutafchieva et al., 2002) and BM and the presence of BM-derived mesenchymal stromal cells (MSCs) in the pannus (Jimenez-Boj et al., 2005) confirm that intense communication between these two seemingly separated compartments must be taking place even at early stages of RA. Also, at later stages of joint inflammation, the pannus can invade the BM space (Jimenez-Boj et al., 2005). There are only limited data on the consequences of the physical contact of inflamed synovium with BM and the interactions between them have remained unexplored so far.

The bone marrow stroma has long been considered to be merely a framework providing a supportive milieu for the maturation and differentiation of haematopoietic cell lines. Mesenchymal stromal cells (multipotent MSCs) are part of the BM stroma and constitute a small fraction (0.001–0.01 %) of the total BM cell population (Friedenstein et al., 1974). MSCs can be isolated and expanded from various tissues (postnatal BM, fat tissue, placenta and others), thus representing a network of MSCs in the entire organism that in times of need can be replenished with functionally active MSCs migrating from BM to affected tissues. Also, MSCs are precursors of synovial fibroblasts that have key functions in chronic synovial inflammation of RA. The ability of MSCs to escape recognition by alloreactive cells and thus to surpass the MHC barrier between individuals is probably not just a passive process, but involves active cellular and molecular mechanisms. Thus, MSCs are considered to be potential suppressors of the immune system (Maccario et al., 2005) with possible implications for the treatment of autoimmune diseases.

Although auto-antibody (rheumatoid factor, anti-citrullinated protein Ab) production has long been recognized to precede first clinical signs and to correlate with disease severity of RA, only the recent success of B cell-depleting biologic therapies (De Vita et al., 2002; Edwards et al., 2004) has moved B cells into the focus of interest of basic research. B cell-deficient mice do not develop collagen-induced arthritis (CIA) (Svensson et al., 1998) – a widely accepted experimental model of RA. B cells serve as critical antigen-presenting cells (APCs) that can process and present auto-antigens in association with high-risk MHC II antigens (HLA-DRB1*0401) to autoreactive T cells (Hill et al., 2003) and the functional activity of T cells is fundamentally dependent on the presence of B cells in the synovium (Takemura et al., 2001). Although CD20⁺ B cells represent a minority in the mononuclear infiltrate of rheumatoid synovial tissue, they are the main cellular component of T-cell/B-cell aggregates and GC-like structures. GCs occur in synovial tissue of 10–25 % of RA patients and their presence correlates with increased Ig production and a more severe course of disease. B cells play an active role in the formation and maintenance of GCs by producing lymphotoxins (LT- α , LT- β) and also in the induc-

tion of proinflammatory factor production (IL-1 β , matrix metalloproteinases, chemokines CCL2, CCL5, CCL8, and adhesion molecules) by fibroblast-like synoviocytes (Braun et al., 2004). Under appropriate stimulation conditions, B cells themselves produce proinflammatory cytokines that promote leukocyte infiltration, neoangiogenesis and synovial hyperplasia (IL-4, IFN- γ), amplify the ongoing immune response (TNF- α , IL-6) (Schneider et al., 1999) or even stimulate B cells via a feedback loop (IL-10), thus perpetuating chronic inflammation (Takemura et al., 2001). Hence the contribution of B cells to the pathogenesis of RA goes far beyond the production of auto-antibodies.

B-cell activation factor (BAFF) from the TNF family (BAFF, BLyS, TALL-1, THANK, zTNF-4) is a type 2 trans-membrane protein, expressed by myeloid lineage cells (macrophages, monocytes, dendritic cells, neutrophils). BAFF can be proteolytically processed by subtilisin-like furin family-like proteases and secreted as a soluble ligand that can act as a systemic cytokine (Schneider et al., 1999). BAFF belongs to the superfamily of TNF ligands that regulate immune responses and immune-mediated tissue injury and is known to be a vital survival factor that supports B-cell generation, differentiation, maturation as well as maintenance and function of the mature B-cell pool (Loder et al., 1999; Do et al., 2000; Ng et al., 2005). All B cells from the late transitional stage through to the GC B cell require BAFF-induced signalling through the BAFF receptor (BAFF-R) for survival. Counts of late transitional, mantle-zone and follicular B cells as well as serum Ig levels are profoundly reduced in BAFF^{-/-} mice (Gross et al., 2001; Schiemann et al., 2001; Sasaki et al., 2004). Conversely, BAFF-transgenic mice have increased numbers of peripheral B cells with an activated phenotype and produce high levels of Ig (IgG, IgM, IgA and IgE) including anti-DNA Ab and RF leading to systemic lupus erythematosus (SLE) and Sjögren syndrome-like symptoms (Mackay et al., 1999; Khare et al., 2000). Hence, BAFF is an effective modulator of peripheral B-cell homeostasis and a potential driver of B-cell hyperplasia and autoantibody production.

Since B cells play an active role in the pathogenesis of RA, MSCs are being considered for therapeutic purposes in RA and direct interactions of MSCs and B cells in the inflammatory processes leading to RA seem to be inevitable, we explored the nature of interactions between B lymphocytes and MSCs. For this purpose, mixed cultures of BM-derived MSCs and B cells were set up and the activation status and viability of B cells were investigated by flow-cytometric analysis. We observed that MSCs significantly enhanced the viability of co-cultured B cells without affecting their activation status – an effect that was more prominent in MSCs derived from RA patients. In an attempt to identify the carrier of the survival signal transmitted from MSC to B cells, the production of BAFF in BM and MSCs was analysed. We could demonstrate that BAFF expression is a constant feature of MSCs, which however may only

in part be responsible for the improved survival observed in our *in vitro* system.

Material and Methods

Patients

Altogether MSCs from 31 patients were included in our study of which MSCs from 12 patients were used for co-culture experiments. Among RA patients (N = 7) of which 5 were male and 2 female, there was 1 patient diagnosed with juvenile chronic arthritis and the patients were aged 23–61 years (mean 40.1 years, median 39 years). RA patients met the American Rheumatism Association (ARA) criteria (Arnett et al., 1988). MSCs from osteoarthritis (OA) patients, which are generally accepted as controls, were used in parallel experiments to RA MSCs. Among OA patients (N = 5) there was 1 male and 4 females, their ages were 32–75 years (mean 57.4 years, median 61 years). For flow-cytometric analysis of BAFF expression, MSCs from RA (N = 6; age 24–60, mean 41.8, median 42 years; 4 females, 2 males) and OA (N = 6; age 32–75, mean 53.8, median 50 years; 4 females, 2 males) patients were used, of which 3 were identical with MSCs used in co-culture experiments. For culture experiments, MSCs originated from passages 1–5 (median 2) and their viability at plating ranged between 60–90 % (median 72.5 %; mean \pm SD 70.2 ± 10.5 ; quartiles 60–75 %). MSCs from 2 RA and 3 OA patients were used for two separate co-culture experiments on different occasions resulting in a total of 9 culture experiments with RA MSCs and 8 culture experiments with OA MSC, each carried out in triplicate. For semi-quantitative analysis of BAFF mRNA expression MSCs from 10 patients (RA N = 5, OA N = 5) were used.

For ELISA analysis of BAFF, paired serum and bone marrow supernatant samples (N = 32) from 16 OA and 16 RA patients as well as serum samples from healthy controls (N = 7) and patients with active RA (N = 15) were analysed. Also, synovial fluid aspirates from RA patients (N = 26) were subjected to detection of BAFF by ELISA.

The study was approved by the Institute of Rheumatology, Warsaw Ethics Committee and all patients gave informed consent. The study was carried out in compliance with the Helsinki Declaration.

MSC isolation and culture

MSCs were isolated from femoral bone marrow harvested by aspiration from the site of surgery in patients with RA or OA undergoing hip replacement surgery. Sodium citrate was used as anticoagulant. The aspirate was washed with phosphate-buffered saline (PBS, Lublin, Poland) and cultured in plastic 75 cm² flasks (Nunc, Roskilde, Denmark) with standard Dulbecco's modified Eagle's medium (DMEM, Invitrogen-Gibco, Carlsbad, CA) supplemented with 15% foetal calf serum (FCS, Biochrom, Berlin, Germany), 25 μ g/ml PlasmocinTM

(Cayla, Toulouse, France) and amphotericin 2.5 mg/ml (Sigma, Munich, Germany) for 5 days in 5% CO₂ at 37 °C. Next, the non-adherent haematopoietic cell fraction was discarded. MSCs were cultured to confluence and passaged at 1:3/1:2 ratio after trypsinization (10x trypsin-EDTA solution, Sigma) and cultured under conditions as stated above. Some MSCs for co-culture experiments were derived from samples frozen in liquid nitrogen for up to 26 months. MSCs for experiments were derived from passages 1–5, at which time they were a homogenous population of fibroblast-like cells negative for the expression of monocyte/macrophage-specific molecules CD14 (Pharmingen, San Diego, CA) and leukocyte-specific CD45 (Pharmingen) (less than 10 %) and positive for the expression of MSC-characteristic surface molecules CD105 (Serotec, Kidlington, UK) and CD166 (Pharmingen) (more than 90 %), as determined by flow-cytometric analysis (FACScalibur, Becton Dickinson, Sunnyvale, Canada).

PBMC and B-cell isolation

PBMCs were isolated from citrated leukocyte concentrate of healthy adult blood donors by density gradient centrifugation using Ficoll/Hypaque (Pharmacia Biotech AB, Uppsala, Sweden). After repeated washing with PBS to deplete thrombocytes, PBMCs were suspended in cooled (4 °C) MACS buffer. MACS buffer was prepared from 20% BSA, PBS and sodium citrate. The cells were incubated with sub-saturating concentrations of mouse anti-human CD20-MACS beads (MACS, Miltenyi, Auburn, CA) to isolate B cells by positive selection according to the manufacturer's instructions. Positive CD20⁺ B cells were purified by passing the PBMC suspension through MACS LS columns. The resultant population of B cells contained > 95 % CD19-positive cells, as estimated by flow-cytometric analysis using anti-CD3, anti-CD19 antibodies. B cells were suspended in RPMI 1640 medium (Invitrogen-Gibco or Biomed, Lublin) enriched with 10% heat-inactivated FCS, penicillin G (200 U/ml), streptomycin (20 ng/ml, Polfa Tarchomin® S.A., Warsaw, Poland), kanamycin (30 μ g/ml, Sigma) and HEPES buffer (1 mM, Invitrogen) at 37 °C.

Co-culture conditions

For phenotypic analysis MSCs from passages 1–5 were seeded at 3×10^4 cells per well onto 24-well plastic culture dishes (Nunc) in DMEM medium (Invitrogen-Gibco) enriched with 15% FCS, penicillin G (200 IU/ml), streptomycin (20 ng/ml), amphotericin (2 mg/ml), PlasmocinTM (25 μ g/ml) and cultured for 1–2 days in humidified atmosphere and 5% CO₂ at 37 °C until adherence. After that time, DMEM medium was aspirated carefully without washing and isolated B cells suspended in RPMI 1640 medium (Invitrogen-Gibco or Biomed) supplemented with 10% heat-inactivated FCS and penicillin G (200 IU/ml), streptomycin (20 ng/ml), amphotericin (2.5 mg/ml), PlasmocinTM (25 μ g/ml) and HEPES buffer at 37 °C were seeded onto the adherent MSC

monolayer or into empty wells at a concentration of $3\text{--}5 \times 10^5$ B cells per 1 ml and cultured in humidified atmosphere and 5% CO₂ at 37 °C for 30, 60 and 90 h. All experiments were performed in triplicate. After the designated time, B cells and MSCs were harvested by extensive washing with cooled (4 °C) FACS buffer and aspiration to secure harvesting of all B cells, including those underneath the MSC monolayer. Complete removal of all cells from wells was checked microscopically. Trypsinization was not used due to concerns with a possible adverse effect on B-cell viability.

To interfere with BAFF signalling, mouse anti-human BAFF antibody (anti-BAFF, R&D, Minneapolis, MN) and goat anti-human BAFF receptor antibody (anti-BAFF-R, R&D) was used in blocking experiments. The respective doses of antibodies were derived from the neutralization dose (N_{50}) as provided by the manufacturer. Mouse IgG_{2B} and goat IgG class antibodies (R&D) were used as controls for anti-BAFF and anti-BAFF-R, respectively. After careful aspiration of DMEM medium from MSC cultures a minimal volume (200 µl) of enriched RPMI 1640 medium and anti-BAFF (20 µg/ml) or control antibody (20 µg/ml) were added and incubated in humidified atmosphere and 5% CO₂ at 37 °C for 30 min to neutralize surface BAFF on MSCs. Only then, isolated B cells in enriched RPMI 1640 were added to a final volume of 1 ml at a concentration of $3\text{--}5 \times 10^5$ B cells per 1 ml and cultured in humidified atmosphere and 5% CO₂ at 37 °C for 60 h. For BAFF receptor neutralization, DMEM medium from MSC cultures was aspirated carefully and 500 µl of enriched RPMI 1640 were added to MSCs. To block BAFF receptor on B-cell surfaces, isolated B cells were incubated with anti-BAFF-R antibodies and control antibodies at a concentration 15 µg/ml and incubated in humidified atmosphere and 5% CO₂ at 37 °C for 30 min. After that time, B cells were added to MSCs at a concentration of $3\text{--}5 \times 10^5$ B cells per 1 ml and cultured in humidified atmosphere and 5% CO₂ at 37 °C for 60 h.

Stimulation of MSCs

MSCs from passages 1–5 were seeded at 1×10^5 cells per well onto 6-well plastic culture dishes (Nunc) in DMEM medium (Invitrogen-Gibco) enriched with 15% heat-inactivated FCS and cultured in humidified atmosphere and 5% CO₂ at 37 °C. Stimulating cytokines from following sources and at following final concentrations were added at the onset of culture; TNF α (R&D, 10 ng/ml), IL-15 (R&D, 10 ng/ml), IFN- γ (R&D, 10 ng/ml). After a 60 h culture MSCs were harvested using 10 \times trypsin-EDTA solution (Sigma) 40 \times diluted with 0.9% NaCl solution.

BAFF ELISA

The Quantikine Human BAFF/BLyS/TNFSF13B ELISA (R&D) was used to detect BAFF in synovial fluid, serum and BM supernatants according to manufacturer's instructions.

RT-PCR

Total RNA was isolated from cultured MSCs (with and without stimulation by inflammatory cytokines) using the TRISOL-reagent (Invitrogen, Carlsbad, CA) or the NucleoSpin RNA II (Promega, Madison, WI) RNA isolation system according to the manufacturer's instructions. MSCs were harvested using 10 \times trypsin-EDTA solution (Sigma) 40 \times diluted with 0.9% NaCl solution. First-strand cDNA was obtained by RT-PCR from MSC mRNA after adjustment of spectrophotometrically measured concentration of isolated mRNA. The amplification of the housekeeping gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) in a PCR using 5'-ACGGATTTGGTTCGTATTGGGC-3' and 5'-TTGACGGTGCCATGGAATTTG-3' as forward and reverse primers, respectively, was used to check for the presence of cDNA in our samples after RT-PCR. For BAFF-PCR a mix containing 10 \times PCR buffer, dNTP, sterile deionized water, Taq polymerase, 0.75 mM MgCl₂ and 0.25 pmol/µl forward and reverse primers for a final volume of 50 µl for each reaction was used. Amplification primers for BAFF were 5'-ACCGCGGACTGAAAATCT-3' and 5'-TCCCATGGCGTAGTCTTATC-3'. Each sample was run in duplicate at following thermal conditions; 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s followed by final extension for 10 min at 72 °C. The resultant 303-bp product was analysed on a 1.5% agarose gel and its amount was assessed semi-quantitatively by detection of net intensity using Kodak 1D Image analysis (Eastman Kodak, Rochester, NY). The relative amount of BAFF mRNA level in each sample was determined as a ratio of BAFF mRNA/GAPDH mRNA net fluorescence intensity. The sequence of the 303-bp product was confirmed by sequencing carried out in the Institute of Biochemistry and Biophysics, Warsaw, Poland.

Flow-cytometric analysis

After harvesting, cells (MSCs and B cells) were washed with cooled (4 °C) FACS buffer (PBS without Mg²⁺/Ca²⁺ containing 1% BSA and 0.1% sodium azide). Single- and double-colour cell-surface fluorescence was performed according to standard direct immunofluorescence techniques using isotype-matched unrelated murine mAbs as negative controls (BD Biosciences, San Jose, CA). Cells were reacted with saturating concentrations of various mAbs at 4 °C for 30 min. FITC-, PE- and APC-conjugated Abs from following sources were used for single- and dual-colour analyses of cell-surface antigens; FITC-conjugated anti-CD3 (BD Biosciences), FITC-conjugated anti-CD105 (AbD Serotec), PE-conjugated anti-CD19 (BD Biosciences), PE-conjugated anti-CD45 and anti-CD166 (BD Biosciences), and APC-conjugated anti-CD14 (BD Biosciences). Fluorescence was analysed in a FACSCalibur® flow cytometer (Becton Dickinson). For B-cell analysis, gating was set according to forward and wide-angle light scatter pa-

rameters to exclude MSCs and subcellular particles from acquisition data. Controls were incubated with individual isotype-control Abs, and these cells were used to set the lymphocyte gates. B-cell viability was analysed by 7-amino-actinomycin D (7-AAD) incorporation during a 10 s period at 4 °C shortly before data acquisition. Non-viable cells were brightly positive for 7-AAD. To identify the BAFF protein in MSCs, MSCs were incubated with saturating concentrations of human BAFF, Ig class:mouse IgG1 (R&D) antibody and stained intracellularly and extracellularly and subsequently conjugated with goat anti-mouse IgG conjugated with APC (BD Biosciences) after permeabilization with 0.1% saponine. Identification of MSCs was based on FSC/SSC parameters and CD105 positivity. Acquired data were processed by Cell Quest® software (Becton Dickinson) and analysed as percentages of positive cells.

Statistical analysis

Each figure shows data from at least four independent experiments where viable cell numbers are presented as the median and lower (25 percentile) and upper (75 percentile) quartile values of all culture triplicates. For the calculation of median values of net intensity of RT-PCR products, data from at least four independent stimulation experiments were used. Statistical analysis of differences in viable cell number and proliferative responses was performed using the data analysis software system STATISTICA 6.0 (StatSoft Inc., Tulsa, OK). Mann-Whitney U test was used to analyse all data. Values of $P < 0.05$ were considered to be statistically significant.

Results

Mixed cultures of MSCs and isolated CD20⁺ B cells without any stimulation by antigen, proliferation-inducing agents or inflammatory cytokines were used to demonstrate the effects of MSC interaction with B cells. No significant differences in the activation status of unstimulated B cells as assessed by the expression of CD86 on CD19⁺ B cells were observed upon co-culture of B cells with MSCs as compared to B cells cultured in medium alone (data not shown).

Isolated B cells survived poorly in medium alone, which was readily seen by the early appearance of a population of lymphocytes of altered size and granularity in forward-scatter (FSC) and side-scatter (SSC) plots in flow-cytometric analysis. Interestingly, in spite of the increased nutritional demands in mixed MSC/B-cell cultures, we repeatedly observed a marked reduction of this population and hence supposed an improvement of B-cell viability upon co-culture with MSCs. To assess this observation more specifically, incorporation of 7-AAD into non-viable cells was used to assess the proportion of viable CD19⁺ B cells in a lymphocyte gate adjusted according to FSC/SSC parameters (Fig. 1). In fact, the sole presence of MSCs without any additional survival or stimulatory factors was sufficient to reverse the ratio of viable and dead B cells at 60 h of co-culture; the median viability of B cells cultured for 60 h in medium alone ($N = 8$) was 34.2 % (mean 35 ± 14 %, quartiles 30.1–43.3 %), while for B cells co-cultured with MSCs ($N = 17$) was 63.9 % (mean 62.8 ± 12.3 %, quartiles 52.8–74.4 %) ($P < 0.001$). Division of data from all experiments of B cells co-cultured with MSCs according to their origin (OA MSC $N = 8$, RA MSC $N = 9$) revealed significant differences in the survival of B cells cultured with RA MSCs (median 68.3, mean 69.0 ± 8.0 %, quartiles 63.9–76.2 %) and OA MSCs (median 52.8, mean 56 ± 15 %, quartiles 47.4–62.0 %) ($P < 0.05$) (Fig. 2). Even after separation of MSC groups the ability of both OA MSCs and RA MSCs to support B-cell survival remained significant as compared to B cells cultured alone ($P < 0.05$ and $P < 0.0001$, respectively). Hence, we concluded that MSCs, irrespective of their origin, are able to support B-cell survival; however, in MSCs derived from BM of RA patients this feature is significantly more prominent. In line with this observation, we observed the ability of MSCs to retard B-cell death in two time-course experiments with the assessment of B-cell viability at 30, 60 and 90 h of co-culture with MSCs (data not shown). Further, we explored whether BM-derived MSCs are able to express functional BAFF similarly to fibroblast-like synoviocytes of mesenchymal origin (Ohata et al., 2005) and thus could be responsible for the improved survival of B cells observed at co-culture with MSCs.

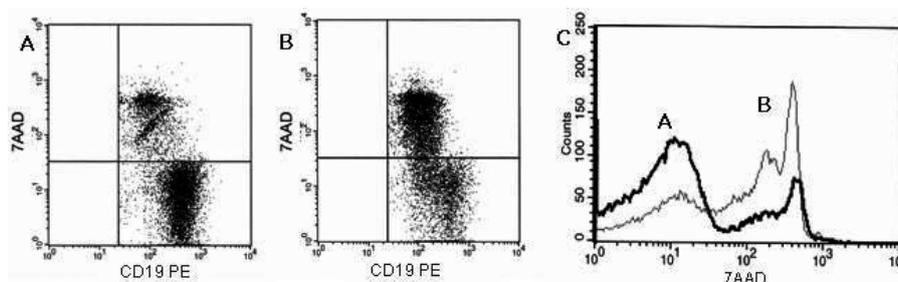


Fig. 1. MSCs support B-cell survival *in vitro*. Representative experiment showing the increase in viability of CD19⁺ B cells cultured for 60 h in co-culture with MSCs (A) as compared to B cells in medium alone (B) detected by 7-AAD incorporation. Corresponding histogram plot of A and B (C).

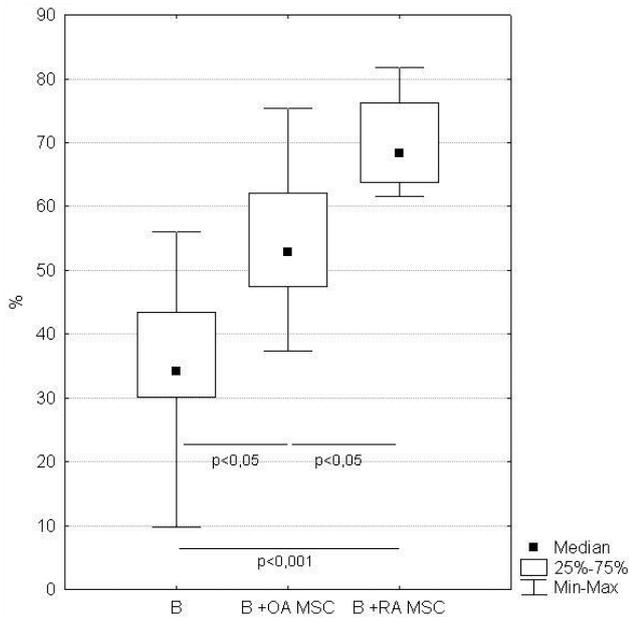


Fig. 2. MSCs support B-cell survival *in vitro* – summary of data. Plot of viability data from all B-cell culture experiments (N = 25) without (N = 8) and with co-cultured MSCs from 17 different experiments (RA MSC N = 9 and OA MSC N = 8) showing significant differences in the proportion of viable cells between all groups (B cells cultured alone (B) (median 34.2; quartiles 30.1–43.3 %), B cells cultured with OA MSCs (B+OA MSC) (median 52.8; quartiles 47.4–62.0 %) and RA MSCs (B+RA MSC) (median 68.3, quartiles 63.9–76.2 %).

First, ELISA assay was used to detect BAFF. Serum levels of BAFF protein in controls, and patients with OA, chronic and active RA (median; mean \pm SD; quartiles: controls: 527.3; 507.5 \pm 88.2; 462.1–568.0 pg/ml; OA: 685.0; 741.3 \pm 233.3; 574.8–809.3 pg/ml; chronic RA: 578.6; 624.5 \pm 224.1; 507.7–726.4 pg/ml, active RA: 741.1; 893.6 \pm 413.9; 537.4–1220.0 pg/ml) were significantly different between OA patients and controls ($P < 0.01$) and active RA patients and controls ($P < 0.05$) (Fig. 3a). Although high individual values of serum BAFF were noted in the active RA group, due to great variance, the difference as compared to chronic RA patients did not reach statistical significance. BAFF levels were significantly higher in serum as compared to bone marrow in both RA (median; mean \pm SD; quartiles: 606.1; 624.5 \pm 224.1; 528.9–980.4 pg/ml vs. 149.5; 166.3 \pm 149.5; 87.2–242.7 pg/ml; $P < 0.0001$) and OA patients (685.0; 741.3 \pm 233.3; 574.8–809.3 vs. 194.2; 242.9 \pm 194.2; 143.4–295.0 pg/ml; $P < 0.0001$). Of note, levels in samples of synovial fluid from the vicinity of affected joints of RA patients were significantly higher than in blood and BM supernatants of RA patients (3416.5; 3095.5 \pm 1069.7; 2453.0–3958.0 pg/ml vs. 606.1; 754.7 \pm 351.8; 528.9–980.4 pg/ml and 149.5; 166.3 \pm 85.0; 87.2–242.7 pg/ml; $P < 0.0001$) (Fig. 3b), suggesting that the inflamed synovia may be the primary source of elevated BAFF levels observed in RA patients. Samples of synovial fluid of OA patients were not available for analysis.

In order to explore the possibility that also BM itself may contribute to BAFF levels measured in BM super-

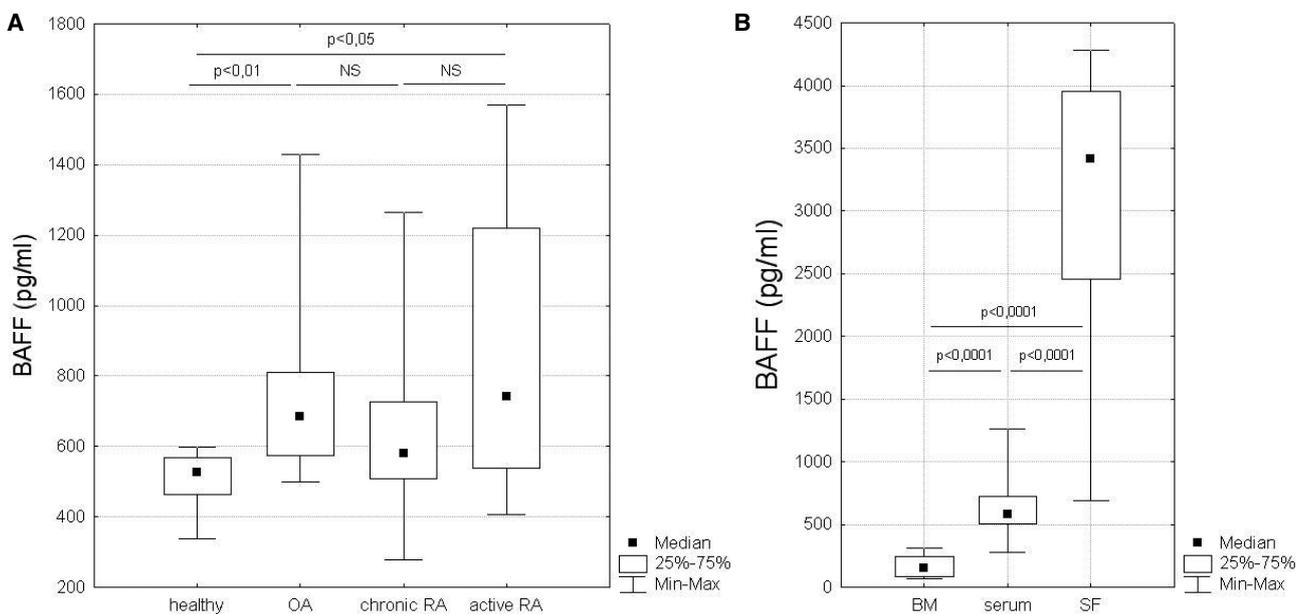


Fig. 3. BAFF protein determined by ELISA in A) sera of healthy controls (median: 527.3; quartiles 462.1–568.0 pg/ml), OA (685.0; 574.8–809.3 pg/ml), chronic RA (578.6; 507.7–726.4 pg/ml) and active RA (741.1; 537.4–1220.0 pg/ml) patients. BAFF concentration is significantly elevated in sera of patients with OA ($P < 0.01$) and exacerbated RA ($P < 0.05$). B) BAFF protein determined by ELISA is significantly elevated in active RA synovial fluid (SF) (3416.5; 2453.0–3958.0 pg/ml) and in RA serum (S) (606.1; 528.9–980.4 pg/ml) as compared to RA bone marrow (BM) (149.5; 87.2–242.7 pg/ml), ($P < 0.0001$).

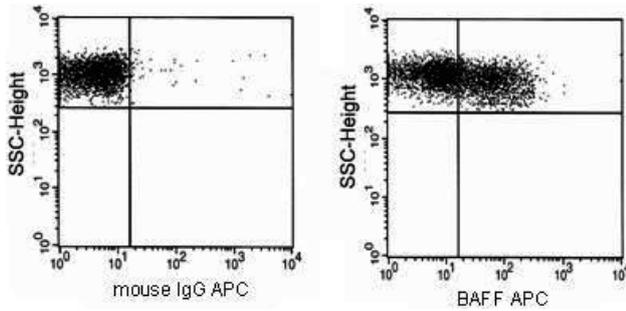


Fig. 4. BAFF protein expression by RA MSCs. BAFF protein is expressed intracellularly in gated RA MSCs as identified by FSC/SSC parameters and BAFF-specific antibody after permeabilization of MSCs.

natants and to identify MSCs as the source of BAFF, cultured MSCs were examined for the expression of BAFF mRNA by RT-PCR and expression was assessed semi-quantitatively. The identity of the RT-PCR product was confirmed by sequencing analysis. Unstimulated MSCs from both, RA and OA patients, were found to express similar levels of BAFF mRNA. This, however, did not correspond to the observation that MSCs from RA patients were more effective in supporting the viability of B cells. Therefore, we examined cultured double-stained CD105⁺ MSCs for the expression of BAFF protein by conjugation with specific antibodies in flow cytometry. We confirmed intracellular expression of BAFF protein in MSCs from both RA (Fig. 4) and OA patients, with no significant differences in the quantity of BAFF expressed between the two groups. Only very low expression of BAFF could be detected on the surface of cultured MSCs. Since the effect of MSCs on B-cell survival might have been due to soluble BAFF that was enzymatically released from the surface of MSCs, we examined MSC culture supernatants by ELISA in two patients (1 RA, 1 OA) and were able to detect soluble BAFF.

To account for the different magnitude of B-cell survival between unstimulated MSCs from RA and OA patients, we speculated that similarly to FLS, BAFF expression may be affected by inflammatory cytokines that are commonly found to be elevated in the sera and BM of RA patients. The cytokine-rich milieu of RA BM might have primed our MSCs to produce higher levels of BAFF protein. We examined the effect of TNF- α 10 ng/ml, IFN- γ 10 ng/ml, IL-15 10 ng/ml and their combinations on the expression of BAFF mRNA and protein by cultured MSCs. In semi-quantitative assessment of GAPDH-adjusted BAFF mRNA net fluorescence intensity, expression of BAFF was extremely variable between individuals and only minor effects of cytokines could be observed. Interestingly, soluble BAFF protein levels detected by ELISA in cultures of two patients (1 RA, 1 OA) were increased by TNF- α and IFN- γ and a cumulative effect of these two cytokines in the induction of BAFF release by MSCs could be observed; however, only in the RA patient. As opposed to

RA MSCs, the release of BAFF from MSCs of the OA patient seemed to remain unaffected by cytokines. Since BAFF release may not necessarily reflect its production, we examined the effect of TNF- α on intracellular expression of BAFF by flow cytometry and found that TNF- α was not able to further up-regulate intracellular BAFF expression in MSCs.

To establish the link between BAFF production and B-cell survival, we set up cultures of B cells and MSCs and examined the effect of BAFF blockage on the survival of B cells. BAFF transmits its effects via three receptors; BCMA and TACI mainly expressed by immature B cells and BAFF-R, which is acquired at the T2 stage and is the principal BAFF receptor of circulating B cells (Schiemann et al., 2001; Hsu et al., 2002; Ng et al., 2004). To account for this receptor heterogeneity, we used anti-BAFF and anti-BAFF-R antibodies to interfere with BAFF signal transmission. Although B cell/MSC co-culture in the presence of anti-BAFF antibodies resulted in a median 8.6 % decrease in viability (50.2; 60.1 \pm 12.5; 41.5–69.1 %) as compared to parallel experiments (59.0; 54.96 \pm 18.1; 52.8–68.2 %), this lay within the interindividual variance of viability of B cells co-cultured with MSCs observed in previous experiments (63.9; 62.8 \pm 12.3; 52.8–74.4 %) and thus was not significant ($P = 0.36$). What's more, the resulting viability after blocking remained significantly improved as compared to B cells cultured without MSC support ($P < 0.05$). Hence, although blocking of BAFF action did have a minor effect, it was not able to completely abrogate the observed viability-promoting effect of MSCs on B cells. This demonstrates that other molecular pathways have to participate on the promotion of B-cell viability in this setting.

Discussion

Several lines of evidence demonstrate that subchondral bone marrow may function as a secondary lymphoid organ that interacts with the overlying inflamed synovium of rheumatoid joints (Marinova-Mutafchieva et al., 2002; McQueen et al., 2003, 2006; Ostergaard et al., 2003; Jimenez-Boj et al., 2005). MSCs derived from bone marrow are known to have important functions in bone marrow physiology with implications for the entire immune system and, in RA, are the source of fibroblast-like synoviocytes. In the last decade, B lymphocytes have acquired a central position in the pathophysiology of RA and BM has been shown to be not only responsible for their generation, but also for the development of B-cell tolerance and the maintenance of the peripheral B-cell population by generation of survival signals such as BAFF.

MSCs of both healthy donors and paediatric SLE patients have been demonstrated to support the proliferation and differentiation of naïve B cells into immunoglobulin-secreting cells in the absence of B-cell receptor stimulation (Traggiai et al., 2008). As for peripheral, more advanced stages of B cells, mainly inhibitory ef-

fects of MSCs on activated B cells have been described, so far. However, in our study, we observed improved survival of purified peripheral B cells upon co-culture with MSCs *in vitro*. This finding is in line with our previous observation of a better viability of isolated BM-derived B cells *in vitro*, as compared to their peripheral counterparts (our unpublished observation), and is analogous to recently published observations by other authors (Tabera et al., 2008). Also, it parallels a similar observation in quiescent T cells (Benvenuto et al., 2007).

Hence, the BM milieu may provide mature B cells with signals that improve their viability, and according to our data, are derived from MSCs. Though this may be a physiologic mechanism of essential importance to early stages of B-cell maturation, under pathologic conditions, as those in RA, this may contribute to the pathogenesis of the inflammatory process. MSCs have been shown to migrate via vascularized canals from BM to the inflamed synovial tissue even at early stages of RA (Marinova-Mutafchieva et al., 2002) and the invasion of the pannus into the bone marrow space is a common histological finding at late stages of subchondral bone destruction (Jimenez-Boj et al., 2005). B cells are the main cellular component of synovial B-cell/T-cell aggregates and GCs as well as large mononuclear cell aggregates mainly composed of mature B cells that have been observed at sites of synovial invasion into the BM space (Jimenez-Boj et al., 2005). The former are important sites of B-cell affinity maturation and memory B-cell, plasma cell and possibly auto-antigen specific B-cell generation (MacLennan et al., 1997). The latter represent a sub-compartment with a unique cellular composition distinct from inflamed synovial tissue and thus are a pool of mature B cells in the direct neighbourhood of arthritis. Hence, direct contact between MSCs and mature peripheral B cells is probable at both early and late stages of the articular inflammatory process. MSCs might participate in the maintenance of B cells (including auto-antigen-specific B cells) as well as B cell-containing complex structures and thus contribute to the perpetuation and chronicity of the inflammatory process. This is supported by our observation that RA MSCs were significantly more effective in providing survival signals to B cells than OA MSCs; hence excessive survival signals provided by MSCs might contribute to the onset, progression and perpetuation of RA.

MSCs are considered to be universal suppressors of the immune system by some authors (Maccario et al., 2005), making them an interesting therapeutic option for autoimmune diseases. In mice, MSC application ameliorates experimental autoimmune encephalitis (Zappia et al., 2005), increases survival of HLA mismatched allogenic haematopoietic stem cell transplants (Chung et al., 2004), improves severe graft-versus-host disease (Le Blanc et al., 2004) and skin allograft survival, and facilitates melanoma growth (Djouad et al., 2003). MSCs inhibit the proliferation, activation and cytokine produc-

tion of alloantigen- and non-specific mitogen-stimulated T cells in mixed lymphocyte cultures (Maccario et al., 2005), induce reversible T-cell anergy (Krampera et al., 2003; Klyushnenkova et al., 2005), and a similar effect has been observed in stimulated B cells. In the light of our observation, an application of MSCs as immunosuppressive cell therapy for RA and other B cell-mediated autoimmune diseases, however, has to be assessed critically and possible consequences on the maintenance of auto-reactive B cells may have to be considered.

A point of concern in our study is a significant difference in the age of RA and OA patients that reflects the degenerative character of OA. Since OA patients were older, a declining ability of MSCs to support B-cell survival with donor age would produce similar results as we have observed in our study. In fact, proliferation, differentiation, migration and cytokine release (VEGF, SCF, FLT3-L, IL-6, SDF-1) of aged MSCs are significantly impaired (Xin et al., 2009). To the best of our knowledge, there are no studies examining the effect of donor age on immunologic functions of MSCs on immune cells. Increasing age, however, is a well-known risk factor for autoimmune diseases. Thus, if age has an effect on the observed mechanism, its enhancement with age in OA MSCs and not RA MSCs might be expected. On the other hand, in the light of a decline of MSC functions with age, the presence of a significantly pronounced B-cell viability effect in MSCs of younger RA donors supports our hypothesis that an active mechanism rather than passive loss of immunosuppressive properties may be responsible for the improved viability of B cells in this setting.

BAFF is a recently discovered survival (Do et al., 2000) and co-stimulation factor for B cells that has been shown to regulate autoreactive B-cell depletion by rescuing self-reactive B cells from cell death (Mackay et al., 1999; Khare et al., 2000; Levine et al., 2000; Lesley et al., 2004). Although it has been implicated in autoimmunity due to its potential to break immune tolerance, its role in RA has not been clarified. In collagen-induced arthritis, however, administration of TACI-Fc reduces Ig production and prevents progression of the disease (Gross et al., 2001). We suspected that BAFF from MSCs could be responsible for the improved viability of B cells co-cultured with MSCs. In fact, we were able to detect soluble BAFF in BM supernatants as well as culture supernatants of MSCs by ELISA. The latter finding, the detection of BAFF mRNA by RT-PCR and the flow-cytometric detection in MSCs, confirm that BAFF is produced by BM-derived MSCs. Hence, although we and others (Cheema et al., 2001) have found highest levels of BAFF in synovial fluid of patients with active RA, demonstrating that the inflamed synovium may be the main site of BAFF release, our data suggest that also MSCs from human BM may contribute to elevated BAFF levels in sera of RA patients (Pers et al., 2005).

To date, myeloid cells are considered to be the main source of BAFF, although reports on non-myeloid cells producing BAFF have been published (Moreaux et al.,

2005). BAFF produced by bone marrow stromal cells was shown to be an important factor for the survival and localization of multiple myeloma cells to BM (Tai et al., 2006). The contamination of our MSC cultures by haematopoietic cells was low, with a maximum of 10 % cells being CD45⁺. Also, stem cell characteristics of MSC, used in our laboratory have been demonstrated in a previous study (Warnawin et al., 2005). By flow-cytometric analysis of double-stained CD105⁺BAFF⁺ (intra-cellular staining for BAFF) MSCs, we were able to demonstrate that MSCs produce BAFF protein. Hence, MSCs may correspond to radio-resistant stromal cells that were identified as a major source of BAFF required for the maintenance of the normal peripheral B-cell pool in a murine chimera model (Gorelik et al., 2003) and might contribute to the maintenance of synovial GCs (Yan et al., 2000). Nonetheless, only little BAFF expression was detected on the MSC surface. Therefore, soluble BAFF that we could detect in culture supernatants might be responsible for the observed effect.

Blocking signal transmission via the BAFF pathway with specific antibodies to BAFF and BAFF-R reduced B-cell survival mainly when anti-BAFF was used. A possible explanation for this observation could be that a weak effect may be transmitted by soluble BAFF via BCMA and TACI receptors and not via BAFF-R. These, however are expressed in particular at early stages of B-cell maturation, and this B-cell population remains largely unaffected in BAFF-transgenic as well as BAFF-deficient mice (Mackay et al., 1999; Gross et al., 2001; Schiemann et al., 2001). Nevertheless, BCMA signalling was found to be essential for long-term bone marrow plasma cell survival in a mouse model (O'Connor et al., 2004).

Blocking BAFF signalling resulted in an up to 11.4% reduction of B-cell viability to a level that is comparable to the improved viability of B cells resulting from co-culture with RA MSCs as compared to OA MSCs. Although due to a small number of experiments this reduction was statistically insignificant, this suggests that BAFF may be responsible for the superior ability of MSCs from RA patients to support B-cell survival. In spite of that, we were unable to find significant differences between RA and OA MSCs in BAFF production neither at mRNA nor at the protein level as evaluated by RT-PCR, ELISA and flow cytometry, respectively. Also, none of the three cytokines (TNF- α , IFN- γ , IL-15) used for stimulation of MSCs increased BAFF production. Interestingly and in line with the above hypothesis, the only significant increase in BAFF production in a stimulation experiment was observed in MSCs originating from a RA patient with the combination of two cytokines (TNF- α , IFN- γ).

Thus, in our setting, BAFF does not play a major role in the maintenance of B-cell survival *in vitro*. This is supported by the observed minor reduction of B-cell viability upon BAFF blockage. However, the consistently superior ability of RA MSCs to improve B-cell viability, the magnitude of the reduction after BAFF blockage and the increased production of BAFF upon stimulation with

cytokines in only a RA patient suggest that under pathologic conditions, soluble BAFF released by cytokine-stimulated or cytokine-primed RA MSCs may improve the B-cell viability-promoting properties of RA MSCs that originate from a cytokine-rich milieu.

In conclusion, MSCs are able to support survival of unstimulated peripheral B cells *in vitro*. This feature seems to be constitutive to all MSCs, and hence use of MSCs as cell therapy in B cell-mediated autoimmune diseases needs to be critically evaluated. The observed effect is more prominent in RA MSCs, possibly due to cytokine priming in RA bone marrow. Although MSCs produce BAFF mRNA and protein, this may not be the main factor responsible for the support of B-cell survival upon co-culture with MSCs. Further analyses to identify a survival factor different from BAFF produced by MSCs are required. Possible candidates may be IL-6, IL-7 (Tang et al., 1997), IL-15 and SDF-1, which may be produced by stromal cells and may deliver activation and survival signals to B cells. BAFF produced by MSCs, however, may be responsible for the better B-cell viability-promoting properties of RA MSCs; nevertheless, further experiments are required to prove this hypothesis.

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