

# Investigating the role of the interleukin-23/-17A axis in rheumatoid arthritis

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**Objective.** IL-23 is a pro-inflammatory cytokine proposed to be central to the development of autoimmune disease. We investigated whether IL-23, together with the downstream mediator IL-17A, was present and functional in RA in humans.

**Methods.** RA synovial cells were cultured in the presence or absence of antibodies directed against IL-23p19 or -23R and -17. IL-23, -12, -17, and their receptors, and IL-6, -1 $\beta$  and TNF- $\alpha$  were measured by ELISA and/or PCR.

**Results.** Small amounts of cell-associated IL-23 (median 110 pg/ml) were detected in RA synovial cultures, and found to be functional as IL-23R blockade resulting in a significant inhibition of TNF- $\alpha$  (57%), IL-1 $\beta$  (51%) and IL-6 (30%). However, there was a considerable variability between individual patient samples, and anti-IL-23p19 was found to be considerably less effective. IL-17A protein was detected in ~40% of the supernatants and IL-17A blockade, in IL-17A-producing cultures, resulted in a small but significant inhibition of TNF- $\alpha$  (38%), IL-1 $\beta$  (23%) and IL-6 (22%). Addition of recombinant IL-23 to cultures had a variable effect on the spontaneous production of endogenous IL-17A with enhancement observed in some but not all cultures, suggesting that either the low levels of endogenous IL-23 are sufficient to support cytokine production and/or that the relevant Th17 cells were not present.

**Conclusions.** These results suggest that although IL-23 may have pathogenic activity in a proportion of patients with late-stage RA, it is not abundantly produced in this inflammatory tissue, nor does it have a dominant role in all patient tissues analysed.

**KEY WORDS:** IL-23, IL-17, Rheumatoid arthritis, Anti-IL-23p19, Anti-IL-23R, Synovium, Cytokine.

## Introduction

IL-23 is a heterodimeric pro-inflammatory cytokine, secreted by activated dendritic cells (DCs) and macrophages [1], that binds to memory T cells, NK cells, macrophages and DCs. IL-23 is composed of the p19 and the p40 subunits that it shares with the Th1 cytokine IL-12. The IL-23 and -12 receptors are also heterodimers, which share the  $\beta$ 1 chain (IL-12R $\beta$ 1). The IL-12 receptor utilizes the  $\beta$ 2 chain (IL-12R $\beta$ 2), whereas the IL-23 receptor uses the unique IL-23 receptor subunit (IL-23R) [2]. IL-12 induces the development of Th cells that secrete IFN- $\gamma$ , whereas IL-23 preferentially stimulates the recently defined Th17 subset that produces IL-17A, -17F, -6, -22, TNF- $\alpha$  and GM-CSF [3–5]. Additionally, IL-23-activated macrophages produce IL-1 $\beta$  and TNF- $\alpha$  [6].

IL-12p40 knockout mice are resistant to autoimmune pathology [7, 8]. More recent studies have shown that mice lacking p19 subunit (lacking IL-23) and mice lacking the p40 subunit (lacking both IL-12 and -23) were protected from experimental autoimmune encephalomyelitis [6] and CIA, whereas mice lacking the p35 subunit (deficient in only IL-12) developed more severe disease [6, 9, 10], suggesting that IL-23 rather than -12 is pathogenic. Evidence that IL-23 may be important in human inflammatory diseases was shown in psoriasis, where the p19 and p40 subunits were expressed in affected, but not unaffected skin [11] and in inflamed tissue in Crohn's disease [12, 13]. The IL-23R gene had a highly significant association with Crohn's disease and

ulcerative colitis [14], although no associations with IL-23p19, -12R $\beta$ 1 or -12p40 were identified. Furthermore, recent genetic evidence suggests that IL-23R is also a significant determinant of the co-occurrence of IBD and psoriasis as well as PsA and AS [15–17]. However, studies on RA cohorts have not shown a strong association between the IL-23R haplotypes seen in other chronic inflammatory diseases [18, 19].

IL-17A is the signature pro-inflammatory cytokine produced by Th17 cells. In mice, Th17 differentiation is dependent upon TGF- $\beta$  and IL-6, whereas recent papers suggest that IL-23, pro-inflammatory cytokines and TGF- $\beta$  are all required for differentiation of mature human Th17 cells from naïve cord blood cells. This is in contrast to previous studies that used adult cells which suggested that IL-23 was sufficient for IL-17 induction [20, 21]. In human gut mucosa, inhibition of TGF- $\beta$  resulted in up-regulation of several cytokines including IL-17 [22], suggesting that IL-17 is at least partially regulated by TGF- $\beta$  in human cells. IL-17A has pleiotropic effects on many cell types where it induces up-regulation of NF $\kappa$ B, HLA class I and cytokines [23, 24]. Th17 cells are found in RA synovial tissue and IL-17A protein found in RA SF and SM culture supernatants, where it acts in synergy with IL-1 $\beta$  and TNF- $\alpha$  [25–29]. Th17 cells function as the major osteoclastogenic Th cell subset that links T-cell activation and bone destruction [30], although a recent paper [31] suggested that IL-23 may drive osteoclastogenesis in an IL-17-independent manner through the induction of RANK ligand. CIA is markedly suppressed in IL-17(-/-) mice [32], and treatment of CIA mice with an anti-IL-17 antibody after disease onset significantly reduced joint inflammation of cartilage and bone [33].

We explored the IL-23/-17A pro-inflammatory axis in human RA using synovial tissue obtained from joint replacement therapy, which, by definition, indicated that all patients had late-stage active disease. The message and protein levels of IL-23p19, -12p35, -12p40, -17A and their receptors were measured using dissociated cell cultures from these RA synovial tissues. The modulatory effect of neutralizing anti-IL-17A, -23p19 and -23R antibodies on spontaneous cytokine production was addressed. We show that small amounts of biologically active cell-associated

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IL-23 are present in RA synovial tissue and that IL-17A is found at even lower levels (which are biologically active). Blocking IL-23R or -17A had modest, but significant, inhibitory effects on pro-inflammatory cytokines, whereas blocking IL-23p19 was even less effective. Importantly, blockade of IL-23R resulted in total inhibition of IL-17 in those RA synovial cultures where IL-17 was detected. In contrast, addition of IL-23 to synovial cultures did not significantly augment the production of IL-17A. These results indicate that contrary to our expectation, IL-23 was not found abundantly in these RA synovial cultures, and that the small amounts of IL-23 present were 'cell-associated' and not secreted. Furthermore, although blocking IL-23R was efficacious in down-regulating the key inflammatory cytokines, the inhibition of the p19 subunit of IL-23 was less effective than the neutralizing IL-23R antibody. These findings are in keeping with both the small amounts of IL-23 present, and the 'cell-associated' nature of this cytokine in this tissue.

## Materials and methods

### *Human tissue and reagents*

Synovial tissue from patients with inflammatory arthritis undergoing joint replacement surgery was collected after written consent. Ethical approval was granted by the Riverside Research Ethics Committee. Synovial membrane samples were digested using collagenase and DNase as previously described [34]. The synovial cultures were incubated in Roswell Park Memorial Institute (RPMI)/glutamine (PAA Laboratories, Pasching, Austria) with 10% Fetal Calf Serum (FCS) (Biowest, Nuaille, France) and 1% penicillin/streptomycin (Sigma, Poole, UK) for 48 h at  $1 \times 10^6$ /ml in the presence or absence of  $10 \mu\text{g}/\text{ml}$  of anti-TNF- $\alpha$  monoclonal antibody (Infliximab, Centocor, Horsham, PA, USA), anti-IL-23p19, -23R, -17A and appropriate isotype control antibodies [mouse IgG1, rat IgG2a and rat IgG1 (BD Pharmingen, Oxford, UK), respectively]. In some experiments, recombinant IL-23 or -17A proteins were added. Unless otherwise stated, all antibodies and recombinant proteins were from Schering-Plough Biopharma (Palo Alto, CA, USA). Supernatants were collected for ELISA analysis, cells were lysed using real life technologies buffer (Qiagen, Valencia, CA, USA) and 1% 2-mercaptoethanol (Sigma) and analysed for mRNA by quantitative PCR. It is not feasible to use 'normal' synovium as a comparator, as there is very limited availability of such tissue (obtained after trauma surgery), and that tissue which is obtained contains very few cells [35].

### *Generation of antibodies*

Monoclonal antibodies against human IL-23R, -23p19 and -17A were developed at Schering-Plough Biopharma and were all shown to be specific.

### *IL-23 bioassay*

The murine B cell line (Ba/F3) transfected with IL-12R $\beta$ 1 and -23R was grown in RPMI containing 25 mM HEPES, glutamine (PAA Laboratories), 10% FCS, 1% penicillin/streptomycin, 10 ng/ml murine IL-3 (Schering-Plough Biopharma) and  $50 \mu\text{M}$  2-mercaptoethanol. Cells were plated at  $5 \times 10^3$  cells/well in 96-well flat-bottomed tissue culture plates (Falcon, Meylan, France) with mIL-3 (0.06 pg/ml to 10 ng/ml), human IL-23 (1 pg/ml to 200 ng/ml) or RA culture supernatants diluted 30-fold. Proliferation was tested with 5 ng/ml IL-23 and 0.0625– $10 \mu\text{g}/\text{ml}$  anti-IL-23p19 or -23R. [ $^3\text{H}$ ] thymidine (Amersham Biosciences, Amersham, UK) was added at  $1 \mu\text{Ci}/\text{well}$  for the final 6 h of the 48-h incubation. Plates were harvested (Tomtec cell harvester, Hamden, CT, USA) and proliferation was quantified using a Wallac Microbeta scintillation counter (Perkin Elmer, Beaconsfield, UK).

### *ELISA*

TNF- $\alpha$ , IL-6, -1 $\beta$  (BD Pharmingen) and IL-17A (R&D Systems, Abingdon, UK) in supernatants were measured by ELISA following the manufacturer's instructions. IL-23 protein (rather than the individual subunits) in the supernatants and lysates (lysed using 0.5% NP-40 in Tris-HCl followed by three freeze-thaw cycles) was measured using coating anti-IL-12p40 monoclonal antibody (Diacclone, Besancon, France) and rat anti-human IL-23p19 monoclonal antibody 12F12 (Schering-Plough Biopharma) for detection. The limit of detection of the TNF- $\alpha$  ELISA was  $>10 \text{ pg}/\text{ml}$ ; IL-6  $>13 \text{ pg}/\text{ml}$ ; IL-1 $\beta$   $>13 \text{ pg}/\text{ml}$ ; IL-23  $>50 \text{ pg}/\text{ml}$ ; and IL-17A  $>10 \text{ pg}/\text{ml}$ .

### *Real-time quantitative PCR*

Total RNA was isolated using the RNeasy method (Qiagen), and  $5 \mu\text{g}$  was treated with DNase (Roche Molecular Biochemicals, Indianapolis, IN, USA) reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). Primers were designed using Primer Express (PE Biosystems, Foster City, CA, USA), or purchased from Applied Biosystems (Foster City, CA, USA). Real-time quantitative PCR on 10 ng of cDNA was done using either of two methods; two gene-specific unlabelled primers were utilized at 400 nM in an Applied Biosystems SYBR green real-time quantitative PCR assay utilizing an ABI 7000, 7300 or 7900 instrument; two unlabelled primers at 900 nM each were used with 250 nM of FAM-labelled probe (Applied Biosystems) in a TAQMAN<sup>TM</sup> real-time quantitative PCR reaction on an ABI 7000, 7300 or 7700 instrument. The absence of genomic DNA contamination was confirmed using primers that recognize the CD4 genomic promoter. Ubiquitin levels were used for normalization by the  $\Delta-\Delta C_t$  method: using the mean cycle threshold values, the equation  $1.8^e (C_t \text{ ubiquitin} - C_t \text{ gene of interest}) \times 10^4$  was used to obtain the normalized values.

### *Immunohistochemistry*

Cryostat sections of  $5 \mu\text{M}$  of synovial tissue were cut, fixed in acetone and endogenous peroxidase activity was blocked with hydrogen peroxide in methanol. Normal horse serum (10%) (Serotec, Oxford, UK) in Tris-buffered saline (TBS) was used to block for 10 min, and non-neutralizing anti-IL-23p19 antibody added at  $3 \mu\text{g}/\text{ml}$  or non-neutralizing anti-IL-23R (both from Schering-Plough Biopharma) added at  $2.06 \mu\text{g}/\text{ml}$  for 1 h. Sections were washed in TBS and biotinylated secondary antibodies (Vector Labs, Peterborough, UK) were added, and stained with avidin-biotin complex (Vector Labs) with diaminobenzidine added for 5 min. Slides were counterstained with haematoxylin, dehydrated, cleared and mounted.

### *Phenotyping of RA synovial mononuclear cell population*

Cells were stained for CD3, -14, -45 and HLA-DR (Becton-Dickinson, Franklin Lakes, NJ, USA), and results were expressed as per cent positive cells. IL-23R and -12R $\beta$ 1 chains were stained using the non-neutralizing biotinylated IL-23R antibody (Schering-Plough Biopharma) or biotinylated IL-12R $\beta$ 1 antibody (R&D Systems) and detected with streptavidin PE (BD Pharmingen). IL-23p19 antibodies are not currently available for flow cytometric staining. Cells were acquired and analysed using the LSR1 flow cytometer with CellQuest software (Becton-Dickinson) and Flowjo for analysis (Treestar, Ashland, OR, USA).

## Results

Synovial tissue was obtained from a cohort of 20 patients with a diagnosis of RA (ACR 1987) and from three further patients; two with juvenile idiopathic arthritis (JIA) and one with PsA. Mean duration of disease was 18 years indicating advanced-stage disease. Patients required joint replacement, or corrective surgery

TABLE 1. Patient demographics showing age, sex and duration of disease

Patient	Age, years	Sex	Duration, years	RhF	ESR	Joint	DMARD	TNF- $\alpha$ blockers
1	58	F	19	NA	160	MCP	Yes	No
2	68	M	8	NA	19	Elbow	Yes	No
3	70	F	23	NA	NA	MCP	Yes	Yes
4	63	F	19	NA	25	MCP	No	No
5	46	F	17	NA	NA	Wrist	No	No
6	59	F	18	NA	NA	Wrist	Yes	No
7	35	F	16	Positive	23	Hand	Yes	No
8	36	M	13	Positive	NA	Hand	Yes	No
9	73	F	NA	NA	13	Knee	No	No
10	67	F	NA	Positive	21	Knee	Yes	No
11	54	M	12	Positive	18	Hip	No	No
12	39	F	20	Positive	9	Knee	Yes	No
13	72	F	29	Positive	62	Knee	No	No
14	59	M	30	Positive	6	Knee	Yes	No
15 <sup>a</sup>	65	M	7	Negative	34	Knee	No	No
16	50	M	13	Positive	14	Hip	Yes	No
17 <sup>b</sup>	51	M	48	Negative	15	Knee	No	No
18	75	F	10	Positive	5	Knee	No	No
19	58	F	9	Negative	8	Hip	No	No
20	55	M	5	Negative	15	Hip	Yes	No
21	53	F	9	Positive	5	Knee	Yes	No
22 <sup>b</sup>	41	F	34	NA	41	Wrist	No	No
23	58	F	20	Positive	48	Hand	Yes	No

All patients had RA except <sup>a</sup>patient with PsA, <sup>b</sup>patients with JIA. Where available, RF status and ESR were obtained. 160 refers to CRP level, Joint indicates source of synovial material; DMARD indicates more than one drug for >12 months; TNF- $\alpha$  blockers refer to anti-TNF- $\alpha$  biological therapy >12 months. NA: not available.

despite 57% of the patients receiving drug treatment with more than one DMARD. Inflammatory markers were generally low, with ESR values (where available) <20 mm/h in the majority of patients; although three RA patients (Donors 1, 13 and 23) had elevated ESR (>20 mm/h) or CRP (>12 mg/dl) levels at the time of surgery, suggesting more active disease (Table 1).

#### IL-23 and -17A cytokine and receptor mRNA and protein levels in RA synovium

Expression levels of each of the subunits of IL-23, -12, -17A and their receptors in the synovial cultures were determined by real-time quantitative PCR (Table 1). Results are expressed as median (interquartile range) relative to ubiquitin. IL-23p19 message was found at high levels [87.3 (6.8–1627)], whereas p40 message was very low [0.1 (0–2)]. IL-23 protein was unexpectedly not detected in the synovial culture supernatants by ELISA (sensitivity of 50 pg/ml). In contrast, all cell lysates produced detectable levels of IL-23 [110 (57–229) pg/ml] (Fig. 1A) indicating that it is produced by synovial cells, but not secreted at high levels (unlike other macrophage-derived cytokines). This does not exclude the possibility that small amounts of IL-23 (<50 pg/ml) are secreted and/or that IL-23 is predominantly cell bound.

GM-CSF-differentiated macrophages produced 400–600 pg/ml IL-23 in lysates, but only ~50 pg/ml was secreted into the supernatants (data not shown). The secreted IL-23 protein increased (up to 300 pg/ml) upon toll-like receptor (TLR) ligation with LPS, and/or R848 with >600 pg/ml in the lysates. This suggests that ~10-fold more IL-23 protein is found in cell lysates compared with secreted IL-23 (again, suggesting that the IL-23 is surface bound), and in comparison with other macrophage-derived cytokines (e.g. TNF- $\alpha$ ), picogram but not nanogram amounts of IL-23 are produced.

IL-17A message has been detected in some, but not all, synovial membrane biopsies [26, 36]. In our synovial cultures, IL-17A message was expressed at very low levels [0.03 (0–1.1)] and was undetectable in 5 of 18 samples (Table 1). Lack of IL-17A mRNA was also reflected in low levels of IL-17A protein in culture supernatants as determined by ELISA (detection limit >10 pg/ml). Protein was detectable in 6 of 16 samples [10 (0–100) pg/ml] (Fig. 1A). Low levels of IL-17A were also present in synovial lysates from five of seven samples examined [15.2 (3.3–23.1) pg/ml]. Somewhat surprisingly, in two cultures where protein was

detected in supernatants and lysates, IL-17A mRNA was undetectable, suggesting that IL-17A transcripts, like some other T-cell cytokines, could be targeted for rapid degradation. IL-27 is involved in suppressing Th17 development [37], and low levels of IL-27 message (Table 2) were detected in all donors tested [2.1 (0.8–11.8)].

IL-23 receptor message was more variable with low levels of IL-23R [0.58 (0–3.6)] and moderate levels of IL-12R $\beta$ 1 [9.6 (0.79–35.2)]. High expression of the IL-17A receptors was observed [152.6 (19.1–348.4) for IL-17RA and 105.9 (10.3–508.8) for IL-17RC] in synovial cells. No correlations were found between percentage of CD3, -14 or -45 cells detected by FACS and any of the cytokine subunits or receptor subunits detected by mRNA (Spearman test).

The presence and relative abundance of IL-23, and to a lesser extent IL-23R, in RA synovial tissue was confirmed by staining of tissue sections. Abundant IL-23p19 was observed in cells in both the lining layer and interstitium (Fig. 1B) consistent with the presence of macrophages, whereas IL-23R was less evident by this methodology and was examined further by flow cytometry in seven donors (for representative donor see Fig. 1C).

IL-23R and -12R $\beta$ 1 were expressed in freshly isolated SM cells from all seven donors examined. CD14+ macrophages (mean positive cells 74  $\pm$  10%) and CD4+ lymphocytes (36  $\pm$  28%) expressed IL-23R, with lower expression of IL-12R $\beta$ 1 (CD14+, 27  $\pm$  16%; CD4+, 9  $\pm$  7%) (Fig. 1C). The majority of lymphocyte staining for IL-23R was on CD45RO+ cells (52  $\pm$  28%) with fewer (13  $\pm$  11%) CD45RO+ lymphocytes positive for IL-12R $\beta$ 1+, suggesting that this might be a limiting factor on CD45RO cells and that perhaps up to 13% of memory lymphocytes have the potential to produce IL-17, since a functional IL-23 receptor is now known to be a marker for IL-17-producing Th17 cells in humans [20]. There are few CD45RA cells in RA SMs (mean 17% compared with 33% CD45RO), and the expression of IL-23R on these cells was extremely variable [35  $\pm$  30% (interquartile range 5–84%)], but an even lower proportion (15  $\pm$  11%) expressed IL-12R $\beta$ 1. RA synovial fibroblasts expressed no detectable IL-23 or -23R message or -23 protein (in supernatants or lysed cells). Synovial culture supernatant contains bioactive IL-23 protein.

Despite the inability to detect IL-23 in supernatants using ELISA, we tested for bioactive IL-23 by using the supernatants

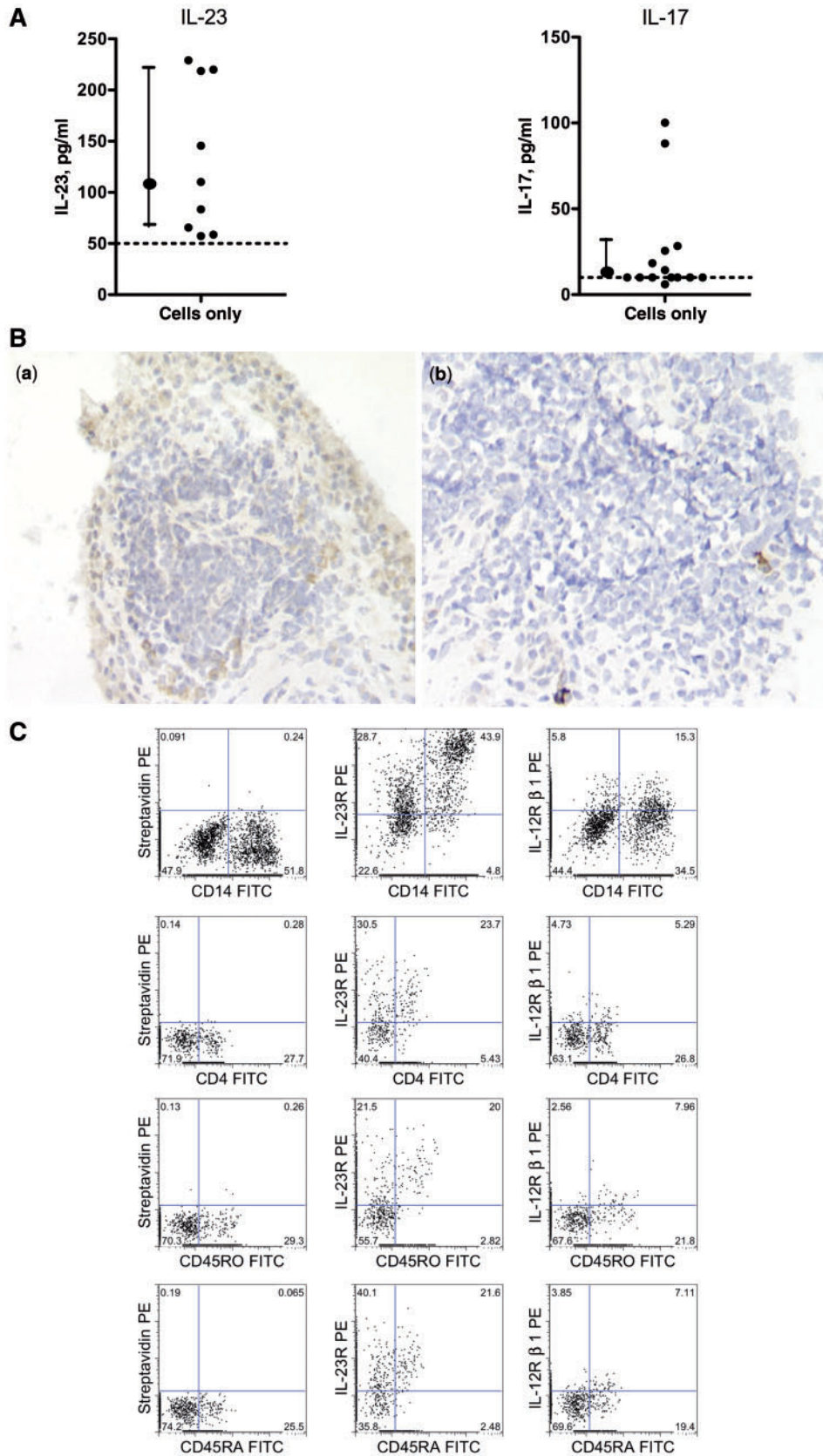


FIG. 1. Expression of IL-23, -23R and -17 in RA SMs. Mixed synovial cells were cultured for 48 h before lysates were tested for IL-23 heterodimer levels, or supernatants tested for IL-17A by ELISA as described in 'Materials and methods' section (A). Results are expressed as mean of three replicates. Median and interquartile ranges are indicated. Dotted line indicates limit of sensitivity of ELISA. RA synovial tissue sections were stained using non-neutralizing antibodies directed against the p19 subunit, or the IL-23R as indicated in 'Materials and methods' section (B). Freshly isolated RA SM cells ( $n=7$ ) were stained for IL-23R and -12R $\beta$ 1 and cell surface markers, then analysed by flow cytometry as described in 'Materials and methods' section (C). The CD4, -45RA and -45RO populations were analysed from the lymphocyte gate as determined by forward and side scatter, whereas the CD14 population was determined from the macrophage gate. Per cent of positive cells in each quadrant is indicated. Data shown are representative plots from one (Donor 4) of seven patients.

to induce proliferation of IL-23R/-12Rβ1-transfected BaF/3 cells. Increasing concentrations of IL-23 protein added to the BaF/3 cells result in proliferative responses. IL-23 of 10.2 pg/ml induced 3209 ± 250 c.p.m. proliferation, whereas unstimulated cells measured 2039 ± 110 c.p.m. and the limit of detection was 2260 c.p.m. (0.7 pg/ml). Two of seven supernatants induced proliferation above the limit of detection equivalent to a mean of 47.5 pg/ml after allowing for dilution factors (2406 ± 74 c.p.m.). This is consistent with small but functional amounts

of IL-23 produced by the synovial cells, but notably were only found in two of the RA synovial cell supernatants. This induced proliferation was reduced to below background levels after addition of a neutralizing anti-IL-23p19 antibody (1986 ± 17 c.p.m.). DC and GM-CSF-differentiated macrophage supernatants also demonstrated IL-23-specific bioactivity, but only after stimulation with TLR ligands (data not shown).

*Effect of anti-IL-23R and -23 antibodies*

IL-23R and -23p19 blocking antibodies were added to the RA synovial cultures to examine whether the predominantly 'cell-associated' IL-23 protein contributed to the inflammatory cytokine cascade (Tables 3 and 4, Fig. 2). Significant inhibition in TNF-α message ( $P < 0.02$ ) from median 35–16 U was observed (Table 3), reflected in 57% inhibition ( $P < 0.001$ ) of mean TNF-α protein levels (Table 4) following treatment with anti-IL-23R in comparison to a mock treatment control ('cells only') (Fig. 2). Similar inhibition in IL-1β message (Table 3) was observed in anti-IL-23R-treated cultures with 1748 U compared with 9655 ( $P < 0.008$ ) in the mock controls. This too was reflected in mean protein levels with a 51% inhibition ( $P < 0.005$ ) (Table 4, Fig. 2). IL-6 message levels were inhibited from 38 218–24 698 U [not significant (NS)] reflected in a smaller (30%) but still significant ( $P < 0.02$ ) inhibition of IL-6 protein (Table 4, Fig. 2). We also included a rat IgG2a isotype control (for the rat anti-IL-23R) in addition to the mock control, and in these experiments significant

TABLE 2. Cytokine and cytokine receptor mRNA expression in freshly isolated mononuclear cells from rheumatoid synovium

Cytokine	Median (interquartile range)
IL-23p19, n=20	87.3 (6.8–1627)
IL-23R, n=16	0.6 (0–3.6)
IL-12p40, n=13	0.1 (0–2.0)
IL-12p35, n=17	4.7 (0.1–24)
IL-12Rβ1, n=17	9.6 (0.8–35.2)
IL-12Rβ2, n=17	3.8 (0–135.4)
IL-17A, n=18	0.03 (0–1.1)
IL-17RA, n=17	153 (19.1–349)
IL-17RC, n=14	106 (10.3–509)
IL-27, n=7	2.1 (0.8–11.8)

Cytokine mRNA expression of IL-23p19 and -23 receptor, IL-12p35, common chain p40, IL-12 receptor (β2) and common receptor chain (β1), IL-27 and -17A together with IL-17 receptor units A and C were assessed by Taqman PCR as described in 'Materials and methods' section in freshly isolated synovial membrane mononuclear cells from patients with RA. Results are expressed relative to ubiquitin.

TABLE 3. Modulatory effect of blockade of IL-23 receptor or IL-23p19 upon pro-inflammatory cytokine mRNA expression in cultured RA mononuclear synovial membrane cells

	Cells only, median (interquartile range)	rtIgG2a isotype, median (interquartile range)	anti-IL-23 receptor, median (interquartile range)	P-value		mSgG1 isotype, median (interquartile range)	Anti-IL-23p19, median (interquartile range)	P-values	
				Cells	IgG2a			Cells	IgG1
TNF-α	35 (7–328)	59 (7–230)	16 (2–104)	0.02	0.008	52 (5–91)	42 (1–132)	NS	NS
IL-1β	9655 (748–69 350)	14 802 (669–52 746)	1748 (172–17 835)	0.008	0.02	3304 (767–20 854)	9457 (424–30 105)	NS	NS
IL-6	38 218 (1913–144 868)	28 562 (2579–98 435)	24 698 (7–80 739)	NS	NS	71 841 (14 718–10 835)	24 308 (2749–103 525)	NS	NS
IL-23p19	80 (7–684)	68 (4–971)	18 (5–456)	NS	NS	52 (5–1308)	63 (1–119)	NS	NS

Effect of anti-IL-23R or -23p19 neutralizing antibodies on pro-inflammatory cytokine mRNA levels (units relative to ubiquitin) compared with cells only (n=8, except p19 where n=5). Cells were harvested 48 h after culture, and mRNA was prepared as described in 'Materials and methods' section. P-values were calculated using a Wilcoxon signed rank test and compared with either cells only or with the appropriate isotype control.

TABLE 4. Modulatory effect of IL-23 receptor or p19 blockade upon spontaneous cytokine production in RA synovial membrane cultures

Donor	TNF-α, pg/ml			IL-1β, pg/ml			IL-6, ng/ml		
	Cells only	Anti-IL-23R	Anti-IL23p19	Cells only	Anti-IL-23R	Anti-IL23p19	Cells only	Anti-IL-23R	Anti-IL23p19
1	408 (47)	250 (5)	367 (54)	13 (4)	12 (10)	10 (1)	96 (10)	55 (17)	69 (4)
2	782 (13)	526 (56)	647 (110)	55 (2)	13 (1)	27 (1)	347 (12)	10 (1)	ND
3	680 (2)	567 (10)	593 (25)	116 (4)	72 (1)	89 (2)	235 (19)	222 (22)	196 (22)
4	6373 (740)	ND	4331 (238)	279 (28)	ND	179 (12)	128 (14)	ND	110 (16)
5	432 (6)	381 (24)	534 (48)	170 (4)	106 (12)	121 (13)	645 (71)	818 (32)	590 (45)
6	122 (51)	115 (29)	ND	94 (17)	34 (14)	58 (21)	192 (14)	132 (35)	85 (29)
7	966 (140)	613 (31)	983 (68)	141 (23)	68 (8)	92 (26)	62 (5)	56 (4)	64 (11)
8	1759 (198)	ND	50 (23)	117 (23)	ND	106 (23)	987 (145)	ND	661 (24)
13	1713 (244)	20 (1)	847 (122)	216 (70)	20 (1)	136 (13)	79 (3)	78 (4)	ND
14	UN	UN	UN	105 (13)	20 (1)	ND	UN	UN	UN
15	UN	ND	UN	83 (28)	43 (14)	ND	888 (321)	66 (7)	ND
16	2163 (128)	1596 (165)	2046 (86)	247 (72)	63 (10)	232 (67)	1028 (127)	832 (112)	757 (97)
17	UN	UN	UN	26 (9)	20 (1)	20 (1)	584 (16)	80 (17)	529 (117)
18	560 (92)	ND	533 (50)	129 (35)	ND	78 (21)	223 (7)	ND	198 (17)
19	1159 (20)	817 (5)	801 (13)	28 (7)	14 (0.4)	15 (8)	944 (109)	768 (3)	689 (120)
20	1993 (556)	1243 (156)	1866 (688)	909 (34)	593 (70)	921 (89)	1067 (323)	1012 (43)	887 (139)
21	493 (50)	474 (38)	192 (159)	31 (12)	26 (8)	ND	868 (67)	620 (52)	615 (128)
n	14	11	13	17	17	17	16	13	13
Mean	1400 ± 1572	600 ± 471	1053 ± 1145	162 ± 207	79 ± 151	149 ± 231	524 ± 389	365 ± 378	419 ± 303
Median	875	526	648	116	30	91	466	132	529
Interquartile range	122–6373	20–1596	50–4331	13–909	12–593	10–921	63–1067	10–101	265–887
P-value		0.001	0.0005		0.0001	0.0009		0.005	0.002

Rheumatoid synovial mononuclear cells were set up in triplicate in culture (anti-IL-23R or -23p19). Supernatants were harvested after 48 h and cytokine levels determined by ELISA as described in 'Materials and methods' section. Results are expressed as mean (s.d.), median and interquartile range from 17 samples. Statistical analysis was performed using Wilcoxon signed rank or paired t-test as appropriate after testing for Gaussian distribution of data using a Kolmogorov–Smirnov test. ND: data not available; UN: cytokine undetectable.

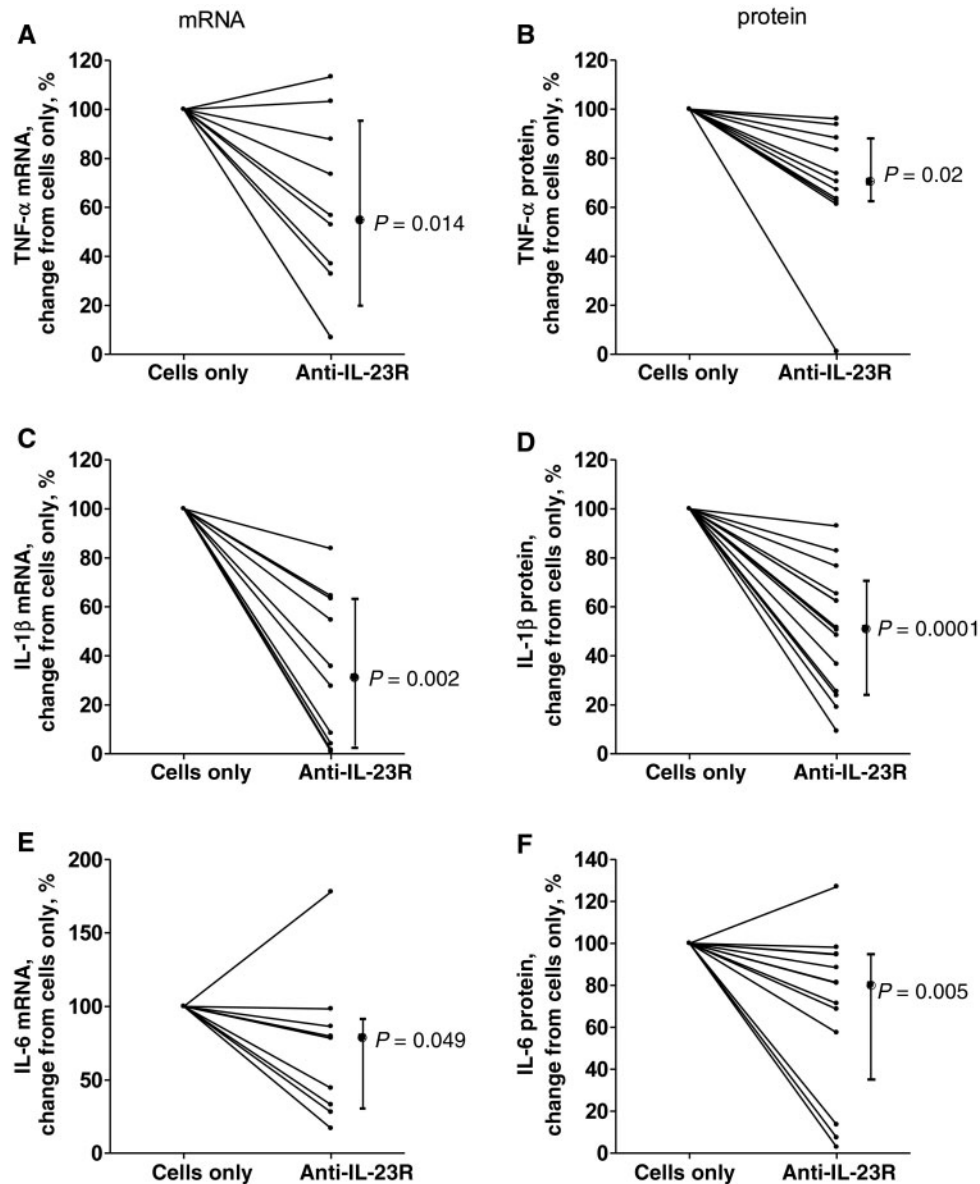


FIG. 2. Cytokine production and mRNA expression in RA SM mononuclear cell cultures: effect of IL-23 blockade. Mixed RA synovial cell cultures were set up in triplicate and either left untreated or with 10  $\mu\text{g/ml}$  anti-IL-23R monoclonal antibody for 48 h. Pro-inflammatory cytokines [(A and B) TNF- $\alpha$ ; (C and D) IL-1 $\beta$ ; (E and F) IL-6] in culture supernatant were quantified by ELISA (B, D, F) and cytokine mRNA by Taqman PCR (A, C, E), both as described in 'Materials and methods' section. Data are normalized to 100% for each of the cells only levels, and per cent change from this is plotted. Median and interquartile range of per cent change is indicated,  $P$ -values were calculated using Wilcoxon signed rank test.

inhibition of TNF- $\alpha$  and IL-1 message and protein was still observed, with 73% inhibition of TNF- $\alpha$  mRNA ( $P=0.02$ ), 88% inhibition of IL-1 $\beta$  mRNA ( $P=0.02$ ) (Table 3) and 51% inhibition of TNF- $\alpha$  protein ( $P=0.003$ ) with 44% inhibition of IL-1 $\beta$  protein ( $P=0.007$ ) (data not shown). IL-6 levels, although reduced, did not reach statistical significance when compared with the isotype control (Table 3 and data not shown).

*In vitro* treatment of the synovial cultures with a neutralizing anti-IL-23p19 was ineffective in this culture system. No significant changes in message levels of TNF- $\alpha$ , IL-1 $\beta$  or -6 were observed compared with mock treatment or mouse IgG1 isotype controls (Table 3). Small, but significant, changes in protein levels (Table 4) were observed with 25% inhibition of TNF- $\alpha$  protein ( $P=0.0005$ ), 17% inhibition of IL-6 protein production ( $P=0.001$ ) and 11% inhibition of IL-1 $\beta$  ( $P=0.001$ ) (Table 4) when compared with mock treatment. When these data were compared against isotype control-treated cultures (mouse IgG1)

in the four cultures where we had sufficient cells to perform this control, the modest changes in cytokine levels were even less and did not reach significance (data not shown).

To investigate whether IL-23 had any impact on its own production, anti-IL-23R or -23p19 were added to the cultures and IL-23 message assessed. Anti-IL-23R addition caused a significant decrease in IL-23p19 mRNA expression (mean 43%;  $P=0.03$ ) from 60 to 18 U (Table 3). Anti-IL-23p19 addition, however, did not significantly change IL-23p19 message.

IL-17A (Fig. 1) was detectable in 6 of 16 supernatants (interquartile range of cells only 0–100 pg/ml), and was markedly inhibited either by addition of the anti-IL-23R (interquartile range 0–15 pg/ml) but to a lesser extent by addition of the anti-IL-23p19 (interquartile range 0–74 pg/ml) (data not shown).

TNF- $\alpha$  has been reported to inhibit IL-12 and -23 production by mouse macrophages and DCs [38]. However, addition of neutralizing anti-TNF- $\alpha$  antibody to the synovial cultures ( $n=8$ ) had

TABLE 5. Modulatory effect of IL-17 blockade upon spontaneous cytokine production in RA SM mononuclear cell cultures

Patient	TNF- $\alpha$ , pg/ml		IL-1 $\beta$ , pg/ml		IL-6, ng/ml	
	Cells only	Anti-IL-17	Cells only	Anti-IL-17	Cells only	Anti-IL-17
1	646 (11)	628 (54)	22 (3)	24 (13)	27 (1)	14 (7)
4	2300 (238)	1586 (73)	160 (23)	85 (5)	291 (47)	217 (23)
5	1784 (155)	204 (110)	1320 (105)	903 (68)	2334 (157)	1894 (354)
6	361 (64)	264 (8)	38 (5)	25 (6)	530 (36)	461 (11)
7	2801 (125)	2445 (27)	210 (47)	165 (31)	643 (25)	465 (2)
8	1759 (198)	201 (208)	117 (23)	106 (45)	987 (164)	561 (130)
16	2163 (128)	2035 (271)	247 (72)	240 (98)	1028 (224)	718 (160)
18	560 (92)	370 (6)	129 (35)	55 (9)	223 (35)	190 (25)
20	67 (24)	10 (1)	342 (69)	402 (56)	589 (37)	715 (64)
23	488 (77)	334 (24)	74 (15)	31 (10)	235 (2.1)	165 (13)
Mean	1293 $\pm$ 971	808 $\pm$ 876	266 $\pm$ 383	204 $\pm$ 273	689 $\pm$ 664	540 $\pm$ 532
Median	1202	352	144	95	559	464
Interquartile range	67–2801	10–2445	22–1320	24–903	27–2335	14–1894
P-value		0.002		0.049		0.03

Rheumatoid synovial mononuclear cells were set up in triplicate in culture  $\pm$  anti-IL-17. Supernatants were harvested after 48 h and cytokine levels were determined by ELISA as described in 'Materials and methods' section. Results are expressed as mean (s.d.), median and interquartile range from 12 samples. Statistical analysis was performed using Wilcoxon signed rank test or paired *t*-test as appropriate after testing for Gaussian distribution of data using a KS test.

no significant impact on IL-23 protein in cell lysates (data not shown). Although IL-23p19 mRNA was decreased in each patient following addition of anti-TNF- $\alpha$ , the inhibition did not achieve statistical significance ( $P=0.056$  by Wilcoxon signed rank test) (data not shown).

IL-23R and -23p19 neutralizing antibodies were not 'toxic' and did not promote cell death as determined by MTT assays (100 untreated *vs* 102% anti-IL-23R treated,  $n=3$ ;  $P=0.75$  by Wilcoxon signed rank test) and propidium iodide (PI) staining by flow cytometric analysis (cells only: 8% PI positive; anti-IL-23R: 11% PI positive; anti-IL-23p19: 10% propidium iodide (PI) positive; data representative of three experiments).

#### Effect of neutralizing IL-17A antibody

A neutralizing IL-17A antibody was added to the RA synovial cultures ( $n=10$ ) in order to determine the effect of IL-17A upon TNF- $\alpha$ , IL-1 $\beta$  and -6 production (Table 5). IL-17A had previously been detected in the supernatant of 5 of these 12 donors (mean 21 pg/ml) and in the lysate of four out of six of these donors (mean 17 pg/ml). Two donors expressed no detectable IL-17 in lysates or supernatants. Blockade of IL-17A overall resulted in inhibition of TNF- $\alpha$ , IL-1 $\beta$  and -6 by a mean of 38 ( $P=0.002$ ), 23 ( $P=0.049$ ) and 22% ( $P=0.03$ ) by Wilcoxon signed rank test in comparison with mock treatment, respectively. Due to the lack of tissue availability, isotype controls (rat IgG1) were used in only six of these samples (data not shown). In these six donors, significant inhibition of TNF- $\alpha$  (mean 33%;  $P=0.03$ ) and IL-1 $\beta$  (mean 35%;  $P=0.03$ ) was shown by Wilcoxon signed rank test, but inhibition of IL-6 (2%) was not significant.

#### Effect of addition of exogenous recombinant IL-23

IL-23 promotes expansion of Th17 lymphocytes that produce IL-17A, -17F, -22, -6 and TNF- $\alpha$ , following antigen stimulation [3]. To examine whether low endogenous IL-23 was limiting IL-17 production, exogenous IL-23 (interquartile range from 0 to 200 ng/ml) was added to synovial cultures for 48 h and cytokines in the supernatants determined by ELISA. Recombinant IL-23 induced TNF- $\alpha$  production in 3 of 13 synovial cultures (Patients 4, 6 and 22; Table 1) and IL-17A in one of these three specimens and in a further two samples (Patients 1, 4 and 8; Table 1) (data not shown). There were no significant differences in the percentage of CD3+, -14+ or HLA-DR+ cells from the five subjects that responded to IL-23 compared with those in which there was no response.

## Discussion

We explored the role of endogenous pro-inflammatory cytokine IL-23 in RA using *ex vivo* synovial cell cultures from patients undergoing joint replacement surgery. The tissue was predominantly from patients with advanced disease, but was generally less 'inflammatory' than RA synovial joint tissue used in earlier studies from our group in the 1990s [34, 39–42]. This reduced inflammatory phenotype is characterized by fewer inflammatory cells with markedly lower spontaneous production of TNF- $\alpha$  in some specimens, and has been observed by several researchers in the field (unpublished data). This change is generally thought to be due to earlier therapeutic intervention with DMARDs (particularly MTX) and biological therapies [43], although only one of our donors (Donor 3) had received anti-TNF- $\alpha$  therapy (Table 1).

IL-23 protein was not detected in the supernatants from these RA synovial cell cultures (limit of detection  $\sim$ 50 pg/ml). However, reasonable IL-23 levels [mean (median) 130 (110) pg/ml] were detected in cell lysates, suggesting that the IL-23 measured was either cell-surface bound or intracellular. Owing to lack of availability of flow cytometry antibodies, this possibility was not addressed in the current study. However, we suspect that this IL-23 was bioavailable, since IL-23R blockade significantly inhibited the spontaneous production of IL-1 $\beta$ , TNF- $\alpha$  and, to a lesser extent, IL-6. IL-23p19 blockade was less effective than the IL-23R antibody in these cultures. The greater potency of receptor *vs* ligand blockade was not expected but has been observed previously, notably with IFN- $\gamma$  in this RA synovial system (F.M. Brennan, unpublished data), and possibly reflects the superiority of blocking the receptor where the ligand is not abundant and/or is cell bound. The modulatory effect of the IL-23R antibody on cytokine production was not due to non-specific effects, nor increased apoptosis or cell death as determined by MTT assay and annexin V analysis. Furthermore, the observation that the IL-23R antibody was more efficient than the p19 antibody at blocking TNF- $\alpha$  and IL-1 in these synovial cell cultures is not due to affinity, as both antibodies had a similar affinity (pM) for their respective ligands and both inhibited IL-23-mediated proliferation of transfected BAF cells, IFN production by human splenocytes and STAT3 phosphorylation.

It was somewhat surprising that IL-23, which is produced by macrophages and DCs, was not more abundant in these RA synovial cultures. However, low levels of the IL-23 heterodimer (assessed by immunohistochemistry) in synovial tissue samples from patients with RA were also reported recently [44], and in another recent report [45], which despite indicating a strong association of systemic IL-23 in RA with disease activity,

closer inspection of the data revealed that SF levels of IL-23 in these patients was also very low (mean 20 pg/ml). It is possible that homeostatic mechanisms can effectively control IL-23 production, and reports (somewhat paradoxically) have described that TNF- $\alpha$  negatively regulates IL-23 (and IL-12) in mouse antigen-presenting cells during an inflammatory response [38]. Anti-TNF- $\alpha$  addition to the RA synovial cell cultures, however, did not increase IL-23 cell-associated levels; whereas a reduction (non-significant) in p19 mRNA levels was observed. It is not known how much IL-23 is necessary to initiate and maintain a response in inflamed human tissue. Since IL-23 can drive a highly pathogenic response, and is an important key cytokine for Th17 differentiation and proliferation in human cells [20, 21], it is likely that its expression is tightly regulated and may never reach levels similar to those seen for other inflammatory cytokines.

Based on our current understanding of IL-23 biology, the expectation was that the pathogenic effects of IL-23 would be mediated by its downstream effector IL-17A [3, 4, 46, 47]. Small amounts of IL-17A protein were found in ~40% of the culture supernatants [mean (median) 23 (10) pg/ml] (levels consistent with previous published studies [25, 26]). Other cytokines, such as IL-27, may inhibit Th17 differentiation [48], and in our study, IL-27 message was found at low but detectable levels [2.1 (0.8–11.8)], raising the possibility that it may exert homeostatic control over Th17 differentiation in this tissue. In our study, blockade of IL-17A significantly inhibited the spontaneous production of TNF- $\alpha$ , IL-1 $\beta$  and -6 in the synovial cell culture, but was less effective than IL-23R blockade indicating that IL-23 pathogenic effects were not induced exclusively via IL-17A. Since IL-17A exerts its pathology in synergy with macrophage-derived cytokines including TNF- $\alpha$  and IL-1 $\beta$  [28, 49], it is not surprising that IL-17A is not always dominant in RA synovium and likely that the relative contributions of IL-17A, TNF- $\alpha$ , IL-1 $\beta$  or -6 are variable between patients. Furthermore, other unique Th17 cell products, such as IL-17F, -21 and -22, may be involved in the inflammatory processes controlled by IL-23.

Addition of IL-23, however, to the cultures did not significantly enhance spontaneous production of endogenous IL-17A. A full dose interquartile range (from 0 to 200 mg/ml) of IL-23 was tested. Since there are few naive T cells in rheumatoid SMs and cells are only cultured for a 48 h, it is unlikely that significant 'new' Th17 differentiation occurs in response to IL-23 addition. The majority of lymphocyte staining for the IL-23R was on memory CD45RO T cells. A much lower percentage of CD45RO cells stained for IL-12R $\beta$ 1 suggesting that this might be a limiting factor for response to exogenous IL-23 in these cultures. It is possible that in these RA tissues, CD45RO cells have selectively lost the expression of IL-12R $\beta$ 1, as a mechanism to make them less responsive to IL-23 (and IL-12). However, addition of IL-23 to these cultures also did enhance spontaneous production of endogenous TNF- $\alpha$  (only in 3 out of 13 donors). In this case, production of TNF- $\alpha$  was not limited to the presence of Th17 cells. It is likely (as observed previously) that in this inflammatory tissue, the production of pro-inflammatory cytokines is already effectively maximal, and that further attempts to stimulate cytokine production with exogenous stimulation results in only modest effects increases in cytokine production.

This study demonstrates that small amounts of cell-associated IL-23 are present in rheumatoid tissue and that IL-23 regulates IL-17A and macrophage-derived cytokines, IL-6, -1 $\beta$  and TNF- $\alpha$ . Neither IL-23 nor IL-17A appear as dominant in human RA as might have been predicted from mouse knockout studies [9, 32], although it is possible that the IL-23/Th17 immune axis plays a more dominant role during the onset of disease or in an acute flare of disease. Of particular note was the observation that in the synovial cell cultures from Donor 13 which had active disease at the time of surgery (Table 1) blockade of IL-23R totally inhibited the TNF- $\alpha$  and IL-1 protein levels. Furthermore, it is not clear why the IL-23 receptor blockade was more efficacious than

p19 inhibition in our study, as in three different bioassays (IL-23-driven BAF cell proliferation, IL-23 stimulation of IFN- $\gamma$  from human splenocytes and IL-23-induced STAT3 phosphorylation) their efficacy was similar. It is possible that the neutralizing antibody which we used (which was generated against soluble IL-23) was unable to effectively neutralize the majority of IL-23 in our cultures that were cell surface-associated. Irrespective of the mechanism, blockade of IL-23 bioactivity (via the receptor) in these synovial cultures resulted in a similar degree of inhibition of IL-1 $\beta$  and -6 production as that observed previously with the humanized chimeric anti-TNF- $\alpha$  antibody, cA2 [50]. However, although it is not unreasonable to speculate that blockade of IL-23 in human RA might be efficacious, the necessity to block the IL-23 receptor (neutralization of IL-23p19 was ineffective) clearly has limitations.

Clinical trials of anti-IL-23 or -17A in humans have not yet been reported, but trials of anti-p40 in Crohn's disease and psoriasis were shown to be efficacious [51–53]. Of particular interest are the recent findings that SNPs in the IL-12B and -23R gene and haplotypes are associated with the risk of psoriasis [54–56] and other inflammatory diseases including Crohn's disease [14], ulcerative colitis [57], AS [58] and PsA [59]. However, despite these observations, several independent studies on patients with RA have indicated that they do not appear to play a major role in RA risk [60, 61], suggesting that these variants in the IL-12/-23 pathway may not differentially influence RA pathogenesis. In our study, there were additional tissues from two patients with JIA and one with PsA, and in both these samples (data not shown), blockade of IL-23R or -17A resulted in a similar modest, but significant, inhibition of TNF- $\alpha$ , IL-1 $\beta$  and -6. Whether the involvement of IL-23 and -17A in murine arthritis translates effectively to human arthritis remains an open question that can only be answered by clinical trials in humans.

#### Rheumatology key messages

- Small amounts of cell-associated IL-23 are found in RA synovial tissue.
- IL-23 regulates endogenous IL-17 production in RA synovial tissue explants.
- IL-23 is a potential therapeutic target in RA.

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