Interleukin-7: a potential factor supporting B-cell maturation in the rheumatoid arthritis synovium

F. Ponchel[1,2], S.M. Churchman[1,2], J.J. El-Jawhari[1,3,4], A.N. Burska[1], P. Chambers[1], E.M. Vital[1], Y.M. El-Sherbiny[1,3,4], E. Jones[1,2], P.G. Conaghan[1,2], V. Goeb[1], P. Emery[1,2]

[1]Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds; [2]NIHR Leeds Musculoskeletal Biomedical Research Centre, Chapel Allerton Hospital, Leeds, United Kingdom; [3]Department of Biosciences, School of Science and Technology, Nottingham Trent University, Nottingham, United Kingdom; [4]Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Abstract

Objective

The exact function of interleukin-7 (IL-7) in autoimmune diseases remains unclear although it is a recognised therapeutic target for cytokine blockade. Our objective was to investigate the regulation and downstream effect of IL-7 in diseased tissue from rheumatoid arthritis (RA) patients notably with respect to its function as bone turnover regulator and tissue architecture (TA) organiser.

Methods

Synovial tissues (fresh, frozen or fixed) were obtained from our tissue bank and distributed between experiments for live cell cultures, histology, immunohistochemistry or gene expression array by qPCR.

Results

IL-7 expression in synoviocyte cultures was up-regulated by pro-inflammatory cytokines, notably IL-6. Gene expression profiling segregated synovial biopsies based on the presence of B/plasma cells and ectopic TA. IL-7 gene expression was associated with that of several genes whose function was to support B-cell maturation in tissue with distinct B-cell aggregates (despite the lack of IL-7-Receptor expression on B-cells) as well as with ectopic germinal-like centres. IL-7 was associated with bone turnover regulation in biopsies with diffuse infiltration. A novel relationship between the IL-7 and IL-6 axis was also highlighted in human tissue.

Conclusion

Overall, IL-7 may contribute to the maintenance of the pro-inflammatory cycle perpetuating inflammation in RA synovium. We therefore propose a novel role for IL-7 as an orchestrator of TA with an impact on B-cell maturation in relation with IL-6.

Key words

IL-7, synovial tissue, tissue architecture, niche

Frederique Ponchel
Sarah M. Churchman
*Jehan J. El-Jawhari**
Agata N. Burska
Philip Chambers
Edward M. Vital
*Yasser M. El-Sherbiny**
Elena Jones
Philip G. Conaghan
*Vincent Goeb***
Paul Emery

**Current address:*
Biosciences,
School of Science and Technology
Nottingham Trent University, UK.

***Current address:*
Rheumatology Department,
University Hospital of Amiens, University of Picardie Jules Verne, Amiens, France.

Please address correspondence to:
Frederique Ponchel,
Head of Translational Research in Immune Mediated Inflammatory Diseases,
Leeds Institute of Rheumatic and Musculoskeletal Medicine,
Clinical Sciences Building,
St. James's University Hospital,
LS9 7TF Leeds, United Kingdom.
E-mail: mmefp@leeds.ac.uk
f.ponchel@leeds.ac.uk

Received on September 24, 2019; accepted in revised form on March 16, 2020.

Funding: this work has been partly supported by a European Union funded FP7-integrated project Masterswitch no. 223404 and the EU/IMI funded project BeTheCure no. 1151422.
S.M. Churchman was initially funded by Arthritis Research UK (award 17354) and is currently funded by NIHR-Leeds Musculoskeletal Biomedical Research Unit (LMBRU).
F. Ponchel received a personal ARUK career development fellowship (P0585).
V. Goeb received a travel fellowship from the French Society of Rheumatology.
P.G. Conaghan and P. Emery are supported in part by the National Institute for Health Research (NIHR) Leeds Musculoskeletal Biomedical Research Unit.
The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Competing interests: none declared.

Introduction

Interleukin-7 (IL-7) is now a recognised player in several autoimmune diseases (AID), HIV, idiopathic CD4 T-cell lymphopenia or genetic immunodeficiencies such as DiGeorge syndrome (for reviews see (1, 2)), through its essential activity in the maintenance of T-cell homeostasis from thymic development and T-cell maturation to peripheral expansion. IL-7 is also an essential adjuvant for immune reconstitution following high dose chemotherapy (1, 2).

IL-7 has been implicated in several auto-immune diseases (colitis (3), multiple sclerosis (4), Sjögren's syndrome (5), diabetes mellitus type-1 (6) and rheumatoid arthritis (7)). Therapeutic intervention by blocking its effects has been proposed (8-11) and IL-7 blockade has demonstrated great promises in animal models (10, 12-14). Importantly, in a transgenic mouse model of spontaneous arthritis development, where the disease is due to a dominant IL-6 signalling mutation (F759 mice) (15), the only difference identified was the over-expression of IL-7, which was sufficient to induce arthritis; its development being subsequently abolished by anti-IL-7R antibody therapy. In another transgenic model (IL-7/CIITA mouse), we showed that over-expression of IL-7 in stromal cells was sufficient to recruit lymphocytes into the joints and initiate inflammation (14). Despite the consensus on the potential of anti-IL-7 therapy in AID, the exact function of IL-7 at the disease site (here the synovium for arthritis) remains unclear.

In RA, reduced levels of circulating IL-7 (16, 17) probably underlies some of the dysfunction associated with circulating T-cell (lack of immune reconstitution following therapeutic lymphodepletion (11, 18, 19) immune regulation (18)). However, IL-7 is highly expressed in the joints of RA patients (11, 20, 21), where its role was more directly related to inflammation (21-23). Another function of IL-7 is its ability to initiate the development of ectopic (tertiary) lymphatic tissue (24, 25); which are present in the synovium (25, 26). Finally, IL-7 is also involved in bone damage, being a master regulator of bone turnover (for review see (1)), with older data suggesting a direct role in osteoclast differentiation via the induction of RANKL expression (27).

The aim of this study was therefore to extend our understanding of the role of IL-7 at the disease site in arthritis, the synovium; its relationship with other cytokines and its implication in cellular networks. We used culture expanded synoviocytes to investigate the regulation of IL-7 expression and synovial biopsies to identify potential cellular networks and/or pathways affected by IL-7 towards perpetuating inflammation, orchestrating TA and regulating bone turnover. Histology, immunohistochemistry and image analysis were used to classify tissue and assess IL-7 and its receptor's expression.

Materials and methods

Patients, biopsies and arthroscopic knee evaluation

Anonymised synovial tissue biopsies (see details in the Supplementary material) from patients with RA (according to ACR 1987 criteria) and osteoarthritis (OA) (Table I) were obtained from a tissue bank with informed consent and ethical approval (Leeds Teaching Hospitals NHS Trust Ethics Committee). These were biopsies of <1 mm in diameter. Limited clinical information was available on cohort-1 and none at all on cohort-2 except diagnosis (Table I). Biopsies in cohort-1 were snap frozen and embedded in OCT while for cohort-2, they were fixed with formalin and embedded in wax.

Tissue culture of synoviocytes and cytokine stimulation of IL-7 expression

Primary cultures of synovial fibroblast like cells were previously established (28). At passage 3, cells were treated for 16 hours with several cytokines and IL-7 expression was measured by qPCR (as described in Suppl. material).

Bisulphite sequencing

Genomic DNA (500ng) was bisulphite converted and the IL-7 gene promoter sequenced (details in Suppl. material and Table S1).

Gene expression

Shavings from frozen biopsies for card-

1/cohort-1 and from the fixed biopsies characterised by histology and IHC for card-2/cohort-2 were processed for gene expression (as described in Suppl. material). Two custom designed TaqMan low density arrays (card-1 and card-2, Suppl. material, Tables S2 and S3) were used. Gene expression relative to *GAPDH* was calculated using the $2^{-\Delta Ct}$ method. Datasets could not be combined due to batch effect associated with the use of two different gene sets (48 and 96 gene arrays). Hierarchical clustering for the generation of heat map was performed using the Cluster 3 and TreeView softwares.

H&E staining

Histology analysis was performed according to standard methods (details in Suppl. material). Synovitis scores were established based on the method described by Kren and colleagues (29).

Immunohistochemistry (IHC)

IHC analysis was performed according to standard methods (details in Suppl. material) for T-cells (CD3 marker), B-cells (CD19 or CD20), macrophages (CD68), IL-7 and IL-7R. CD21 IHC was attempted but did not yielded satisfactory staining on the fixed synovial tissue biopsies despite clear positive/negative staining on control tissue (tonsil).

Automated lymphocyte infiltration scoring

Slides were examined using the Nuance v.3.0.1.2 software (Caliper, PerkinElmer). The software enabled a series of images to be captured and automatically merged into a single image covering the whole piece of tissue. The inForm software (Perkin Elmer) was then used for quantifying of CD3, CD20 or CD68 staining as % of cells (details in Suppl. material). The percentage of cells positive for IL-7 was also determined.

Quantification of IL-7 expression using multispectral image analysis

The level of IL-7 was further analysed as a percentage of the total surface (nuclear, cytoplasmic and extra-cellular) to account for total IL-7 expression (details in Suppl. material).

Table I. Synovial biopsy cohorts (median, (range)).

	Cohort 1	Cohort 2	
	RA	RA	OA
n	42	17	12
Age (years)	58 (28-79)	53 (27-69)	60 (50-78)
Sex (F/M)	34/8	10/7	8/4
arVAS	35 (3-71)	77 (14-100)	44 (5-70)
Histology synovitis	NA	6.5 (3-8)	3 (2-7.5)
CRP (mg/l)	12.5 (<5-126)	11 (<5-185)	NA
DAS	5.8 (4.3-7.5)	NA	NA
RF (positive/n)	37/42	NA	NA
Duration (years)	NA	NA	NA
Drug	All failed MTX	All on MTX*	NA

Data are displayed as median (range). Both cohorts were recruited as anonymised biopsies from our tissue bank. Cohort-1 included samples from patients who failed synthetic DMARDs and were about to receive a biologics (2005-07). Samples form cohort-2 were collected at a time when biologics were not yet available (1997/99), with altogether limited data (age, sex and diagnosis, CRP although only available on 9/17 of the RA patients).
*possibly in combination with other DMARDs; NA: not available.

Statistical analysis

Linear variables were not normally distributed amongst groups; therefore non-parametric tests were used throughout. Spearman's rank correlation coefficient was used to correlate two variables. Mann-Whitney U-test for was used to compare two independent groups. Pearson correlations were used to investigate relationship between gene levels of expression. SPSS v.19 software was used.

Results

IL-7 expression in synovial cell cultures

We previously demonstrated a direct relationship between IL-7 expression in fibroblast-like cell cultures derived from RA biopsies (passage 3) and *in vivo* levels of inflammation measured in the joint during arthroscopic visual inspection (arVAS) (displayed in Fig. 1A for reference (32)). We selected 6 synovial cell cultures and stimulated cells with cytokines (Fig. 1B). In cultures from lower arVAS biopsies, IL-1-beta, IL-6, IFN-gamma and TNF-alpha increased the expression of IL-7, while IL-10 and TGF-beta had limited effect. In contrast, in cultures from high arVAS, no clear effect could be detected for any cytokines with the exception of TGF-beta showing reduction.

An *in silico* DNA methylation analysis of the IL-7 gene was performed (Fig. 1C) revealing two CpG rich regions. Ninety-one CpG positions were analysed over both regions (Fig. 1D). No difference were observed suggesting that differential DNA accessibility of the IL-7 promoter/gene may not be directly responsible for the differences in the regulation of IL-7 expression.

Exploratory gene expression profiling: card-1 (42 RA biopsies)

An exploratory gene expression profiling card was designed and tested on 42 RA frozen biopsies. These were obtained from long-lasting RA patients, who had failed synthetic DMARDs and were about to receive a Biologic (Table I). Expression of the CD4 (T-cell) transcript was detected in all samples (with up to 225 fold difference) as well as those for CD31 (endothelial cells) and CD55 (fibroblasts). Expression of CD19 (B-cells) was very heterogeneous with 16/42 samples with below detection levels. We observed similar pattern when staining for CD19 by IHC, with only ~50% of biopsies not showing any positive B-cells (data not shown). CD20 (another B-cell marker) was very widely distributed (>1000 fold difference) with very low levels in 3 biopsies and below-detection in 9 (indicated in yellow in Fig. 2). In contrast, the CD138 transcript, representative of plasma cells was detected in most samples (38/44). CD8 expression (CD8 T-cells) was also below detection in 10 samples.

Unsupervised clustering analysis generated a hierarchy separating out 2 groups of biopsies (Fig. 2). Gene expression segregated tissues in 2 groups of sam-

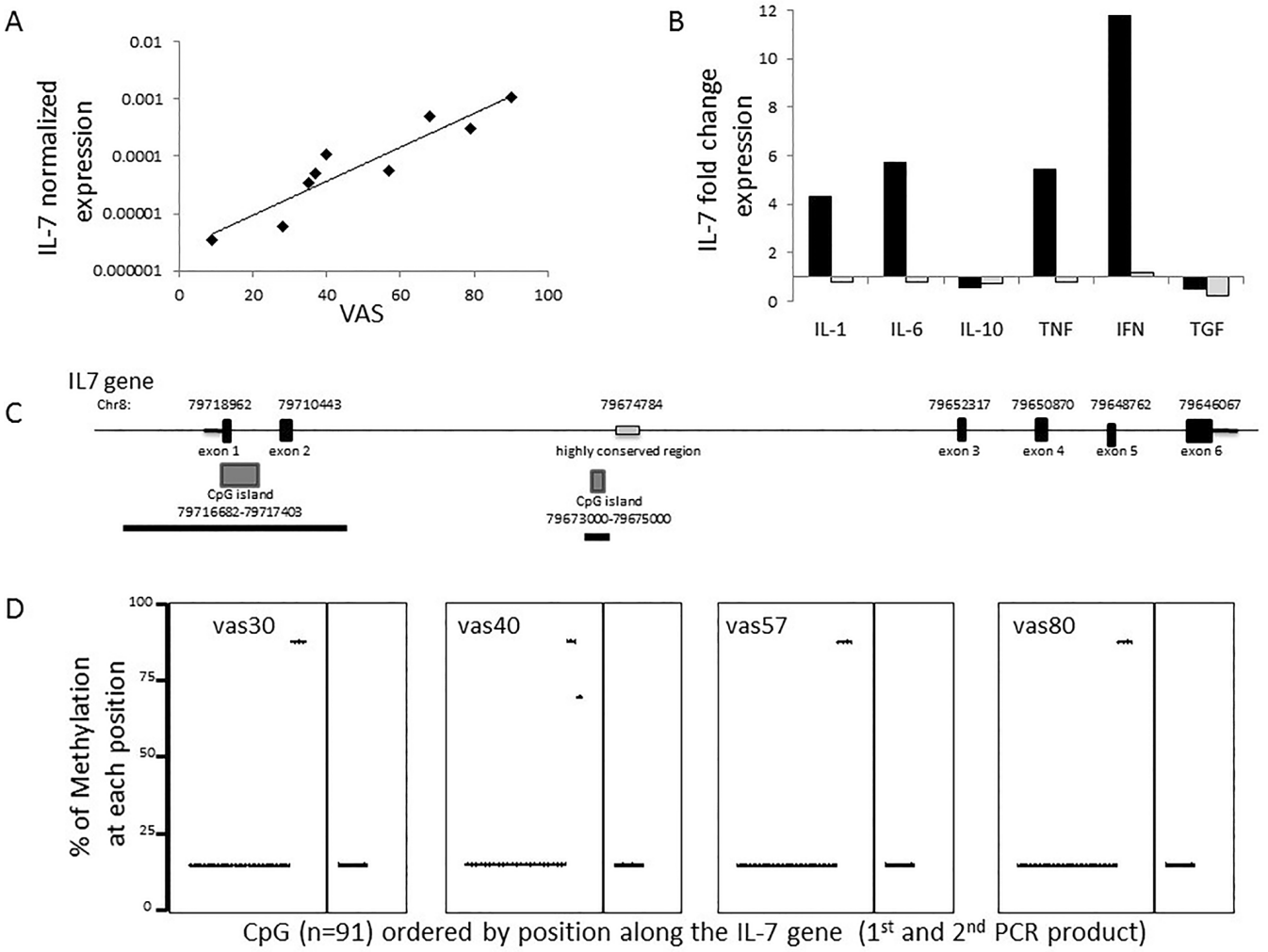

Fig. 1. IL-7 Expression and regulation in synovial cells.
A: Reference data: IL-7 expression was measured by qPCR in synovial stromal cells at passage 3 in tissue culture (32). Expression was directly related to *in vivo* local levels of inflammation in the joint (n=9, rho=0.939, $p<0.0001$).
B: Cultures form 3 low arVAS (black bars, VAS 9; 28; 37) and 3 high arVAS (grey bars, VAS 68; 79; 90) biopsies were stimulated with different cytokines: IL-1-beta, IL-6, IL-10, TNF-alpha, IFN-gamma and TGF-beta, followed by measurement of IL-7 expression by qPCR. IL-1-beta, IL-6, IFN-gamma and TNF-alpha increased the expression of IL-7 (4.8 to 11.8 fold) in low VAS biopsy. IL-10 and TGF-beta had limited effect (reduction by 0.58 to 0.73 fold). No clear effect could be detected with any cytokines (0.77 to 1.17 fold), in cultures from high arVAS biopsies except with TGF-beta showing a 4 fold reduction.
C: Structure of the IL-7 gene: in silico DNA methylation analysis of the IL-7 gene was performed using the Human Genome Browser (https://genome.ucsc.edu/). Analysis showed the position of exons (black box), 2 CpG islands (grey box, in the promoter and middle of the gene next to highly conserved region) and the PCR product generated for sequencing (black thick line).
D: Results of the sequencing the IL-7 gene. DNA was extracted from 4 biopsy cultures (arVAS ranging from 30 to 80) and analysed by direct sequencing following bisulphite DNA conversion. Low levels of methylation (about 18%) at the beginning of the promoter island (first box/1st PCR product) and in intron-2 (second box/2nd PCR product) were observed, but high methylation levels was seen toward the end of the 1st PCR product (first box, about 85%). No significant difference could be observed between 4 samples (but for 3 out of 91 CpG in one1 biopsy, VAS 40).

ples. It appears that the main difference between the 2 groups of biopsies was the presence/absence of B-cell and associated genes. The first group was segregated based on transcripts associated with B-cell biology: lineage CD19, CD20, CD138, immunoglobulin IgM, IgG1, Ig-constant chain, B-cell survival (PCNA, TACI, CXCL-13, IL-6) and several cytokines IL-1-beta, IL-6, IL-10, TNF-alpha, defining a group of "B-cell containing tissues". In contrast the second biopsy group was discriminate by transcripts associated with a stromal cell origin (CD55), the regulation of synovial infiltration (CXCL12 chemokine), growth factors (EGF, FGF, VEGF), further referred to as the "stromal gene group", although CD4, CD68 and CD31 clustered with this group. On the gene assembly, IL-7 expression was associated with the B-cell gene group, suggesting that a novel function of IL-7 in the synovium may be related to B-cells.

Tissue characterisation: 29 biopsies (RA and OA)

We obtained fixed tissues from a second cohort including RA (n=17) and osteoarthritis (OA, n=12) patients (Table I - anonymised samples from a biopsy collection collected between 1995-99). Histology and IHC were performed on these biopsies (notably using CD20 instead of CD19 for B-cells). Synovitis scores ranged from 2-8 (Table-S4) and TA classification was confirmed by

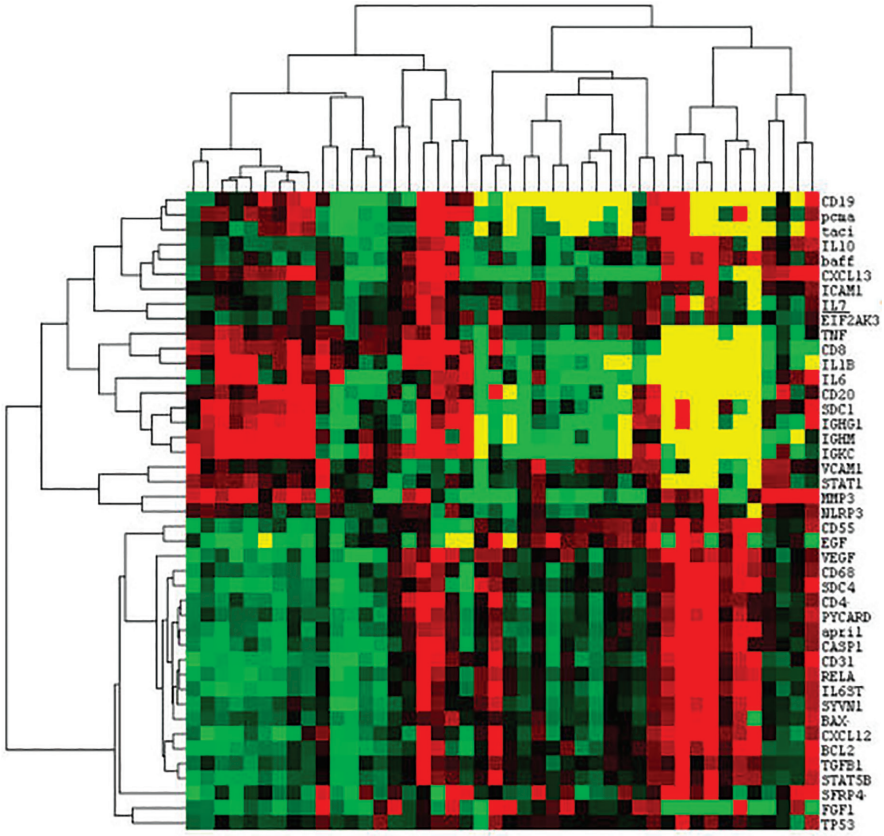

Fig. 2. Hierarchical clustering of 42 biopsies from RA patients.
Unsupervised hierarchical clustering analysis generated a hierarchy of 2 groups of biopsies. IL-17 and MMP-1 genes were removed as expression was only detected in 4-5 samples, leaving 43 genes in the final analysis (and 3 house-keeping genes).

IHC for the CD3/CD20/CD68 markers, and showed diffuse infiltration (isolated cells positive for either CD3 or CD20, n=11), presence of aggregates (isolated groups of ~100 cells either CD3 or CD20, n=11) and formation of putative germinal centre-like (GCL) structures (positive CD3 and CD20 staining organised in 2 distinct zones, n=7), despite not being able to detect CD21 staining. TA structures are illustrated in Supplementary Figure S1. There was no clear TA association with RA or OA samples, although in RA samples there was tendency towards more complex TA.

We used digital image analysis to quantify infiltration of each cell-type into the lining and stromal layers separately. T- and B-cells were mostly detected in the stromal layer in direct relation to synovitis score and arVAS (Suppl. Table S4). In contrast $CD68^+$ macrophages were mostly located within the lining layer, with no relationship with either score. Increased T- and B-cell infiltration also correlated with increasing TA complexity whereas CD68 cell quantification remained unrelated to TA.

Gene expression profiling in relation with TA: card-2 (29 RA/OA biopsies)

We designed a second array to cover additional genes (details in Suppl. Table S3). We first examined gene expression between RA and OA and found no significant difference (after correction for multiple testing), however trends for higher expression of CCL21, BAFFR and VGF in RA were observed ($p<0.040$ without correction).

CD20/B-cell (MSA-1/2) was below detection levels in 4/29 biopsies. All other lineage markers (CD4, CD8, CD38, CD68, CD55, CD31, CD11c) were detectable in all 29 samples with the exception of CD138 detectable in 28/29 biopsies.

Cluster analysis generated a two-group hierarchy (Fig. 3). The first group of biopsies (n=12) contained mainly diffuse infiltration samples (8/12 biopsies) and the second aggregates and GCL (n=17) based on IHC classification ($p=0.010$). There was no disease segregation for RA and OA samples ($p=0.556$) and there was no difference in inflammation levels (arVAS) between the two groups ($p=0.245$), although higher synovitis scores ($p=0.030$) were associated with the aggregate/GCL cluster as expected. As most samples showed detectable lineage markers for all cell types, it appeared that the main difference between groups was again the level of infiltrating B-cells, although in this 2nd series, it was more specifically plasma B-cells (CD138) rather than B-cells (CD20). CD8 and dendritic cells (ITGAX/CD11c) clustered with plasma B-cells but no other lineages. Genes involved in B-cell maturation (XBP-1, BLIMP-1/PDRM1, AIOLOS/IKZF3) and progression towards plasma B-cells (all Ig-subtypes but not IgD) were associated with this cluster therefore renamed "plasma-cell cluster". This particular group of genes also showed genes associated with B-cell biology (CD5, CD79A, CD80, CD86, CD40L), whereas most of the non-B-cell related genes appeared excluded from the gene dendrogram. IL-7 was again included in this gene cluster and its expression was higher in this group of tissue ($p=0.027$). In the second group of biopsies associated predominantly with diffuse infiltration, there was no clear group of genes with higher/lower expression. However, using 2-group comparison (Mann-Whitney U-test), COMP and OPG (both involved in bone turnover), CD55 (fibroblast lineage) and SUMO1 (transcriptional regulation, apoptosis, protein stability) were expressed at higher levels (all $p<0.05$) in these biopsies compared to those from the plasma B-cell group, which suggests a more stromal drive with increased bone turnover in these samples.

IL-7 gene expression in relation to TA

We further focused our analysis on the second gene array. The expression of IL-7 (detected in all samples) clustered with the plasma B-cell biopsy group. To determine how IL-7 could influence the tissue microenvironment and regulate the development of TA, we investi-

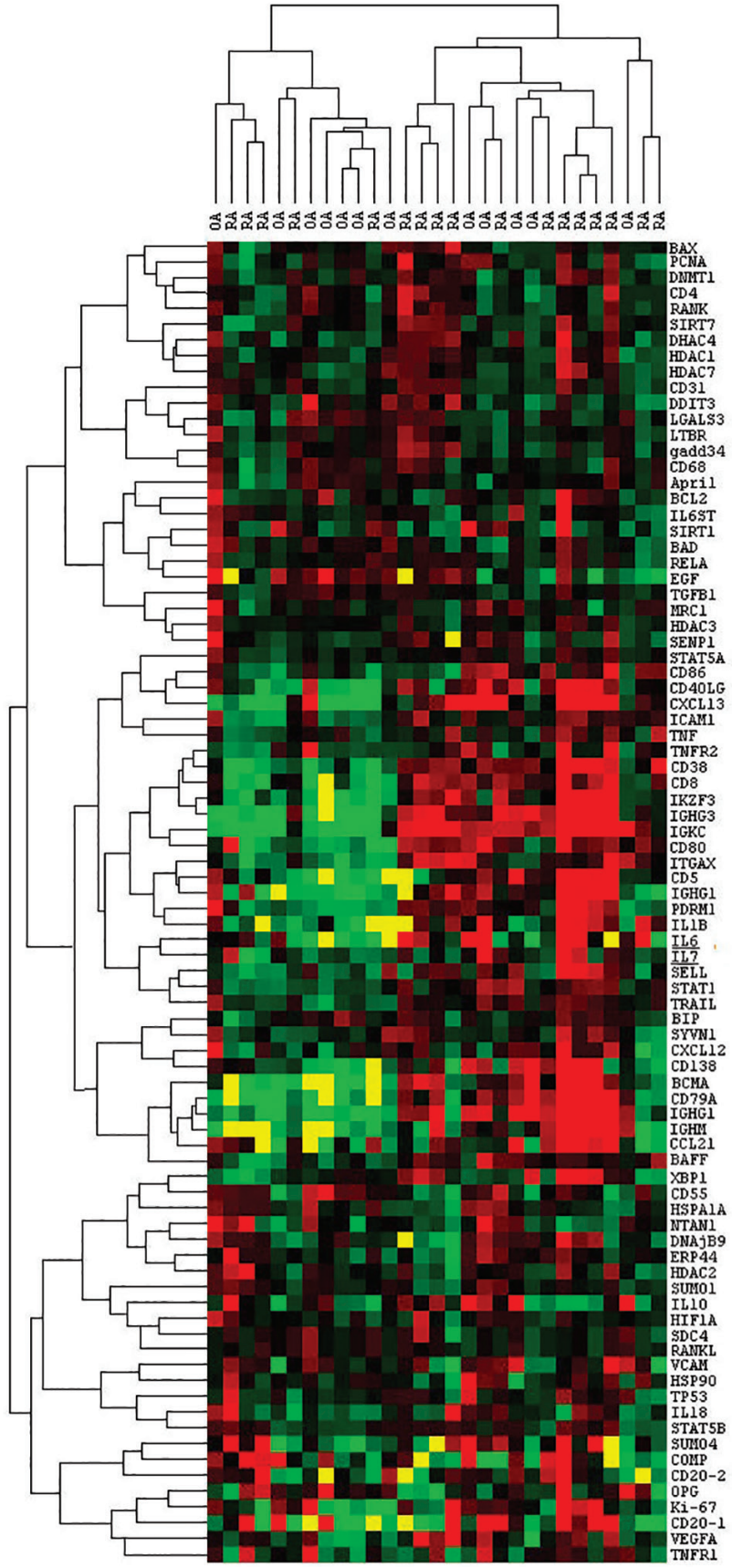

Fig. 3. Hierarchical clustering of 29 biopsies from RA (n=17) and OA (n=12) patients. 11 genes were removed as expression was only detected in a few samples, leaving 82 genes in the final analysis (and 3 house-keeping genes).

gated gene expression relationships in the 3 TA groups separately. Results can only be treated as indicative (considering factor of correlation rho >±0.600) as the number of samples and associations tested would not allow for true significance.

In the putative GCL TA group (n=7), STAT5a expression (but not STAT5b) was particularly strongly associated with that of IL-7 (Table II), suggesting signalling in these biopsies alongside a strong association with CD4. There was no association between IL-7 and the fibroblast marker (CD55) but a strong association with macrophage (CD68) suggesting that the expression of IL-7 may also derive from these cells in CGL biopsies as previously hypothesised(21-23). Strong relationships with genes involved in building GCL structure (ICAM, CD31, LTR) and for a plasma cells niche formation (APRIL) were observed, although correlation factors for other GCL building genes remained low (rh <0.600, but with significant *p*-values). Further associations were seen with genes involved in DNA-biochemistry (SIRT7, HDAC-3, GADD34 and SUMO-4) and cell proliferation (PCNA) or apoptosis (P53, BAX).

In the aggregate TA biopsies (n=11), the expression of IL-7 was the highest. An association with STAT5a also supported signalling. Association with CD38 suggested a relationship with plasma B-cell development, in agreement with the cluster data. High expression of B-cell maturation genes (IgG1, IgKC, AIOLOS, BLIMP1) was also observed as well as with professional antigen presentation functions (CD80, CD40L) and evidence of proliferation (KI67). The expression of BAFF-R(33) was also associated with IL-7. The expression of RAG1 (removed from the cluster analysis as only detected in a 7 biopsies), was detected in 5 tissues in this group (the other 2 being GCL)). Finally, IL-7 showed a unique relationship with the IL-6 gene, not seen in the other groups, but also clearly highlighted on the cluster heat map (Fig. 3), as well as with CXCL13, a chemokine involved in B-cell migration

In the diffuse infiltration TA biopsies

Table II. Association with the expression of IL-7.

	Diffuse infiltration (n=11)			Aggregates (n=11)			GCL structures (n=7)		
	gene	rho	*p*	gene	rho	*p*	gene	rho	*p*
Lineage									
CD4, CD8. CD20, CD138,							CD4	0.929	0.001
CD31, CD55, CD68							CD31	0.751	0.026
							CD68	0.929	0.001
IL-7 regulation and signalling cascade									
IFN, TGF, STAT5A, STAT5B				STAT5A	0.600	0.029	STAT5A TGF-b	0.929	>0.0001
								0.964	0.001
GLC formation									
I-CAM, V-CAM, LTR, TNF-R1,2,				TNFR1	0.673	0.012	I-CAM	0.679	0.047
CD4, CD20, CD138, CD31, CD68							LTR	0.607	0.074
							CD4	0.929	0.001
							CD31	0.750	0.026
Apoptosis									
P53, BAX, BAD, BCL-2							TP53	0.607	0.074
							BAX	0.964	>0.0001
BAFF/APRIL system									
BAFF, APRIL, BAFFR, TACI,	BAFF	0.745	0.007	BAFFR	0.745	0.007	APRIL	0.714	0.036
BCMA,	TACI	0.821	0.012						
B-cell maturation									
CD5, CD38, CD79A, AIOLOS,	CD5	0.773	0.003	CD38	0.736	0.005			
BLIMP-1, XBP1, ITGAX,	AIOLOS	0.648	0.043	AIOLOS	0.636	0.018			
all Ig classes,	BLIMP1	0.794	0.003	BLIMP1	0.673	0.012			
				IGHG1	0.782	0.004			
				IGKC	0.600	0.026			
APC functions									
CD40L, CD80/86, CTLA4, CD206	CD80	0.636	0.018	CD40LG	0.664	0.013			
				CD80	0.700	0.008			
Heat shock/stress									
BIP, HSP40, HSP90, HSPA1A				HSP40	0.727	0.006	HSP40	-0.679	0.047
							HSP90	0.750	0.026
							HSPA1A	-0.750	0.026
Cytokine/chemokine									
CCL21, CXCL12, CXCL13,	STAT1	0.661	0.019	CXCL13	0.655	0.014			
TNF, IL-1b, IL-6, IL-10, IL-18,				IL1b	0.697	0.013			
IL-6ST, STAT-1				IL6	0.633	0.034			
Bone damage									
COMP, TRAIL, OPG RANK,	COMP	-0.718	0.006	TRAIL	0.764	0.003			
RANKL	OPG	0.745	0.007						
	TRAIL	0.745	0.013						
Growth factors and proliferation									
FGF1, EGF, VEGF, KI67, PCNA,				KI67	0.773	0.003	PCNA	0.643	0.06
				VEGFA	0.636	0.018			
DNA regulation									
HDACs, GADD34, GADD153,							HDAC3	0.821	0.012
PADIs, SIRTs, SUMOs, DNMT1							GADD34	0.857	0.007
							SIRT7	0.964	>0.0001
							SUMO4	-0.657	0.078

(n=11), the expression of IL-7 was the lowest with no significant relationship with CD4 and STAT5a, although potential relationships with B-cell survival (BAFF, TACI) and maturation (CD5, AIOLOS, BLIMP1) were still observed. A possible effect on bone damage/turnover was detected by association with the OPG/TRAIL axis and COMP (Table II). Other associations were seen for transcripts related to antigen presenting cell function (CD80) and possibly the activation of macrophages (CD206, rho=0.576, *p*=0.03).

IL-7 protein expression in synovial tissue is associated with both inflammation and TA complexity

We stained biopsies (n=29) with an anti-IL-7 antibody and quantified its expression using spectral image analysis, first as the percentage of positive cells and second as the area of diffusion (total IL-7). IL-7 was detected in both lining and stromal layers at relatively similar levels but with large variation between samples. There was an association between percentage of IL-7 positive cells in the stromal layer and arVAS (Fig. 4A, rho=0.621, $p<0.0001$) as well as with the synovitis score (rho=0.576, $p=0.002$). No significant relationship was detected in the lining layer. Similar results were obtained using total IL-7 expression (Fig. 4B, rho=0.615, $p=0.0001$ with arVAS and rho=0.553, $p=0.002$ with synovitis score). An increase in the number of positive cells was also observed with increasing TA complexity (Fig. 4C). Higher total IL-7 expression, was also associated with aggregates and putative GCL TAs (Fig. 4D).

Finally, we investigated the expression of IL-7, IL-7R and T- and B-cells using IHC on sequential sections. We selected biopsies showing defined aggregates of B- or T-cells and those with putative GCL structures (Fig. S2). In tissue with discrete B- or T-cell aggregates, IL-7 was observed surrounding both types of aggregates but the IL-7R was only observed in regions positively stained for T-cells. In tissue with putative GCL structures, IL-7 was more closely associated with the T-cell zone although, also surrounding the B-cell zone. IL-7R was again only observed in regions positively stained for T-cells.

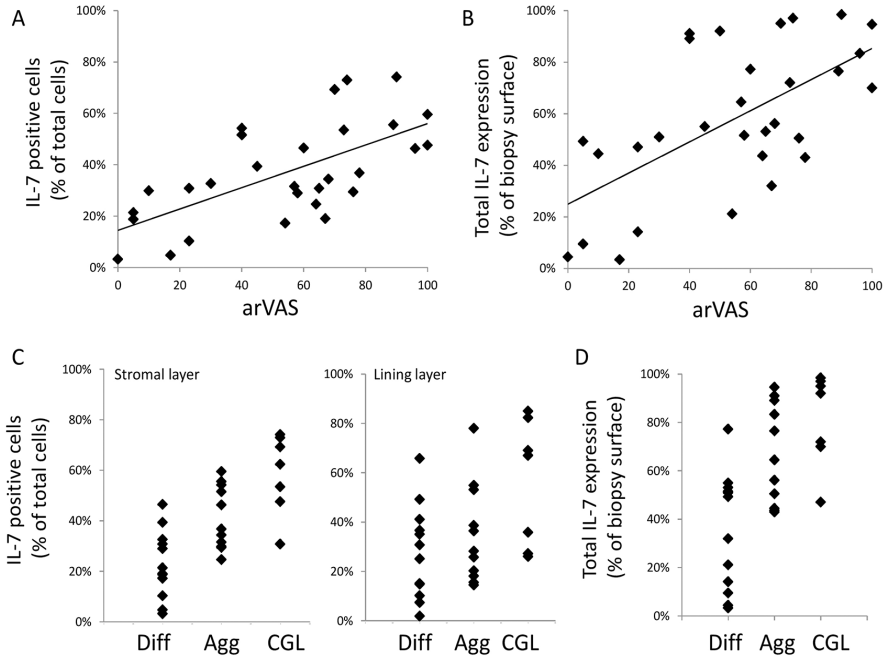

Fig. 4. IL-7 protein expression in 29 fixed synovial tissue blocks from RA (n=17) and OA (n=12) patients (cohort-2).
A: Correlation between positive cells expressing IL-7 and arVAS (rho=0.615, $p<0.0001$).
B: Correlation between total IL-7 expression and arVAS (rho=0.621, $p<0.00001$).
C: Increasing number of positive cells for IL-7 with increasing TA complexity in stromal layer (left) and lining layer (right).
D: Increasing total IL-7 expression with increasing TA complexity.

Discussion

Analysis of the RA primary disease site, the synovial membrane, confirmed that levels of IL-7 protein were clearly associated with increasing inflammation and TA complexity. The cellular composition of the synovial membrane was highly variable, with T- and B-cell infiltration directly reflecting local levels of inflammation. The TA of the synovial membrane was also highly variable and gene expression analysis suggested that IL-7 was implicated in both aggregate and putative GCL TAs and surprisingly more closely related to plasma cells than T-cells despite the lack of expression of the IL-7R on B-cells. Our data therefore suggest a niche effect mediated by IL-7 for the survival of plasma cells. The detection of RAG1 expression mainly in tissue with B-cell aggregates also strongly supports the concept that B-cell maturation may take place in these aggregates, notably considering that IL-7 is a direct regulator of gene rearrangement and RAG1 expression (34-37), and was associated with TACI, also known as a marker of T-cell independent B-cell maturation (38-40). This effect does not directly affect the B-cells as they do not express the IL-7R, but appears to affect the surrounding area of the B-cell aggregate/ putative GCL structures suggesting local modification of the microenvironment. How IL-7 is implicated in the remodelling of the microenvironment required to support the aggregates remains less clear, however, IL-7 has been implicated as a tissue inducer in GCL development (34). With its expression being regulated by the local cytokine networks, the inflammatory milieu may contribute to this mechanism and different combinations of cytokines may result in alternative TA outcome.

Despite IFN-gamma being the best-known positive regulator of IL-7 expression (30), no relationship with IL-7 was observed in synovial biopsies (nor with TNF-alpha) suggesting that other cytokine(s) may be playing that major regulatory role in the synovium. Furthermore, if the cellular source of IL-7 appears to be large fibroblast-like cells in the stromal layer, it also appears that macrophages in the lining layer may contribute to the overall IL-7 expression. IL-1-beta and TNF-alpha are known to increase the production of IL-7 by synovial stromal cells (20), and in turn, IL-7 up-regulates the production of TNF-alpha, IFN-gamma and most importantly the osteoclastogenic cytokines RANKL and M-CSF, leading to osteoclast maturation and ultimately to bone destruction (1, 27). We previously reported that IL-7 gene expression was maintained in passage 3 synovial cell cultures in direct relation to levels of inflammation experienced *in vivo* (14, 40). We could not

identify a region of the IL-7 promoter where a methylation status could account for this observation, although the expression of IL-7 could only be further increased by IL-1-beta, IL-6, IFN-gamma or TNF-alpha in *ex vivo* cultures with low basal levels of IL-7 expression (*i.e.* low arVAS). These data remain inconclusive in terms of how imprinting of the *in vivo* effect of the inflammation experienced by the RA patient is maintained on passage-3 culture expanded cells. However, combined with the gene expression studies they suggest that a cytokine network is likely to be implicated in the fine tuning of IL-7 levels and the effects it has on the TA. The gene grouping in Figure 4 suggests a potentially important role for IL-6 as the genes most closely related to IL-7.

IL-7 is known to drive the formation of GCL structures. GCL structures have been associated with evidence of B-cell proliferation and affinity maturation in RA(41) as well as in Sjögren's syndrome more recently (42). Indeed, the development of tertiary lymphoid structures requires the interaction of several cell types including tissue inducers (CD3/CD4/IL-7R) and stromal cell tissue organisers (VCAM1/ICAM1). The role of IL-7 signalling is critical for the initial clustering of cells and the production of lymphotoxin-alpha by tissue inducers (43). Lymphotoxin-alpha then induces two signalling cascades resulting in recruitment of endothelial cells (CD31) and lymphocytes (CD4) to join the evolving TA structure. In our study, IL-7 protein expression was clearly associated with fibroblast-like cells co-localising with structures surrounding both the T/B-cell zone of putative GCL structures as well as separate B- and T-cell aggregates, also suggesting a role for IL-7 in driving this mechanism of tissue remodelling in RA (26). We could not detect relationships between TA and positivity for autoantibody to citrullinated peptide-(ACPA) in these patients or the expression of the PAD enzyme (PADI-1, 4) responsible for the citrullination of local antigens. Nonetheless, the role of IL-7 in building a niche for B-cell maturation appears likely, first considering the strong relationship with STAT5a expression (although this is not a direct proof of STAT5a-phosphorylation), second the strong relationship between IL-7 and CD138 in both aggregate and putative GCL TA, and third, the further association between IL-7 and the recruitment of CD4 T-cells and CD31 endothelial cells in tissues containing GCL structures.

Importantly, IL-6 signalling knock-in (F759 mice model) showed an IL-7-dependent specific phenotype (15) and this regulatory network appears reproduced here in human synovial biopsies for the first time, IL-6 being the closest related gene to IL-7. The IL-6R signalling subunit (gp130) expression was itself related to plasma cells (CD38 rho=0.638 $p<0.0001$ and CD138 rho=0.939, $p<0.0001$). Although expression levels did not significantly differ between TA groups, IL-7's relationship with IL-6, IL-1-beta, TNF-R and CCL21 expression essentially differentiated the plasma cell group (Table II). All signalling through IL-7 was therefore highly associated with plasma cell development, but similar associations between IL-6 expression and IL-1-beta or the TNF-Rs were not directly observed (low rho values). Taken together, these results suggest that plasma cell maturation is occurring in aggregate TA biopsies and this represents a potential novel role for IL-7 possibly through interaction with the IL-6 axis, independently of T-cell help.

In view of the recent association of serum levels of CXCL13 and ICAM with different synovial phenotypes and response to anti-TNF in RA (44), we looked at these two molecules in our gene expression dataset. IL-7 was directly related to expression of ICAM however only in GCL TA while it was also only related to CXCL13 in aggregates TA (Table II). These data are closely reproducing the association observed by Denis and colleagues (44) considering that GCL TA represents the myeloid tissues and aggregates TA the lymphoid phenotype. The association with IL-6 was, however, specific of the lymphoid/aggregate TA and may explain the unique relationship that drive TA towards one or the other phenotype, also providing a mechanism behind the unique signature with response to anti-IL-6 for the lymphoid phenotype reported by Denis and colleagues (44) while the myeloid phenotype associated with anti-TNF response.

In conclusion, we propose that IL-7 may be able to orchestrate the synovial tissue architecture with impact on B-cell maturation (both in aggregate and GCL TAs) while a role towards bone turnover is more likely in diffuse infiltration TA. Blocking IL-7 may therefore be of therapeutic value in RA, as recently supported by animal model in several autoimmune diseases (14). The relationship between IL-7 and the IL-6 axis is novel but is also consistent with data from an IL-6R mice model where the specific production of IL-7 was the only identified change associated with the development of arthritis (15). Therefore, the finely tuned effect that IL-7 exerts on T-cells in the circulation where its levels are tightly controlled is likely to be corrupted into a more sinister role in the synovial tissue, where IL-7 may contribute to a loop perpetuating inflammation and propagating disease (23). In addition, our data suggest a possible role in driving the development of different TA phenotypes with their impact on patient's outcome: on one hand, a TNF-alpha/IL-1-beta independent but IL-6 related pathway resulting in tissue remodelling towards supporting B-cell maturation (lymphoid phenotype) while on the other, the development of more classic GCL structures (myeloid phenotype).

Acknowledgements

The authors would like to thank Richard Cuthbert, Mike Shires and Sarah Field for technical support.

References

1. LEE SK, SURH CD: Role of interleukin-7 in bone and T-cell homeostasis. *Immunol Rev* 2005; 208: 169-80.
2. FRY TJ, MACKALL CL: Interleukin-7: from bench to clinic. *Blood* 2002; 99: 3892-904.
3. OKADA E, YAMAZAKI M, TANABE M *et al*.: IL-7 exacerbates chronic colitis with expansion of memory IL-7Rhigh $CD4^+$ mucosal T cells in mice. *Am J Physiol Gastroint Liver Physiol* 2005; 288: 745-54.
4. KREFT K, VERBRAAK E, WIERENGA-WOLF A *et al*.: Decreased systemic IL-7 and soluble IL-7Rα in multiple sclerosis patients. *Genes Immun* 2012; 13: 587-92.
5. JIN JO, KAWAI T, CHA S, YU Q: Interleukin-7 enhances Th1 response to promote the devel-

opment of Sjögren's syndrome-like autoimmune exocrinopathy. *Arthritis Rheum* 2013; 65: 2132-42.
6. MONTI P, BRIGATTI C, KRASMANN M, ZIEGLER AG, BONIFACIO E: Concentration and activity of the soluble form of the Interleukin-7 Receptor alpha in type I diabetes identifies an interplay between hyperglycemia and immune function. *Diabetes* 2013; 62: 2500-8.
7. HARTGRING SAY, BIJLSMA JWJ, LAFEBER F, VAN ROON JAG: Interleukin-7 induced immunopathology in arthritis. *Ann Rheum Dis* 2006; 65: 69-74.
8. WILLIS CR, SEAMONS A, MAXWELL J *et al*.: Interleukin-7 receptor blockade suppresses adaptive and innate inflammatory responses in experimental colitis. *J Inflamm* 2012; 9:39.
9. LEE LF, LOGRONIO K, TU GH *et al*.: Anti-L-7 receptor-alpha reverses established type 1 diabetes in nonobese diabetic mice by modulating effector T-cell function. *Proc Natl Acad Sci USA* 2012; 109: 12674-9.
10. HARTGRING SAY, WILLIS CR, ALCORN D *et al*.: Blockade of the interleukin-7 receptor inhibits collagen-induced arthritis and is associated with reduction of T cell activity and proinflammatory mediators. *Arthritis Rheum* 2010; 62: 2716-25.
11. CHURCHMAN SM, PONCHEL F: Interleukin-7 in rheumatoid arthritis. *Rheumatology* 2008; 47: 753-9.
12. CHEN Z, KIM S-J, CHAMBERLAIN ND *et al*.: The novel role of IL-7 ligation to IL-7 receptor in myeloid cells of rheumatoid arthritis and collagen-induced arthritis. *J Immunol* 2013; 190: 5256-66.
13. HILLEN MR, HARTGRING SA, WILLIS CR *et al*.: The additive inflammatory in vivo and in vitro effects of IL-7 and TSLP in arthritis underscore the therapeutic rationale for dual blockade. *PloS One* 2015; 10.
14. PONCHEL F, BURSKA A, MYRTHIANOU E, G. G: IL-7 in Rheumatoid Arthritis: Pathogenesis, Biomarker and Rationale for Anti-IL-7 Therapy. *In*: BERHARDT LV (Ed.) Advances in Medicine and Biology: Nova Science Publishers 2014: 31-53.
15. SAWA S, KAMIMURA D, JIN GH *et al*.: Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4(+) T cells. *J Exp Med* 2006; 203: 1459-70.
16. GOËB V, AEGERTER P, PARMAR R *et al*.: Progression to rheumatoid arthritis in early inflammatory arthritis is associated with low IL-7 serum levels. *Ann Rheum Dis* 2013; 72: 1032-6.
17. BURSKA A, NEILAN J, CHISMAN R *et al*.: Serum IL-7 as diagnostic biomarker for rheumatoid arthritis, validation with EULAR 2010 classification criteria. *Clin Exp Rheumatol* 2018; 36: 115-20.
18. CHURCHMAN SM, EL-JAWHARI JJ, BURSKA AN *et al*.: Modulation of peripheral T-cell function by interleukin-7 in rheumatoid arthritis. *Arthritis Res Ther* 2014; 16: 511.
19. PONCHEL F, VERBURG R, BINGHAM S *et al*.: IL-7 deficiency and therapy-induced lymphopenia in Rheumatoid Arthritis. *Arthritis Res Ther* 2005; 7: 80-92.
20. HARADA S, YAMAMURA M, OKAMOTO H *et al*.: Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 1508-16.
21. VAN ROON J, VERWEIJ M, WENTING-VAN WIJK M, JACOBS K, BIJLSMA J, LAFEBERFLORIS P: Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of $CD4^+$ T cells and macrophages. *Arthritis Rheum* 2005; 52: 1700-10.
22. NATSUMEDA M, NISHIYA K, OTA Z: Stimulation by interleukin-7 of mononuclear-cells in peripheral-blood, synovial-fluid and synovial tissue from patients with rheumatoid-arthritis. *Acta Med Okayama* 1993; 47: 391-7.
23. VAN ROON JAG, GLAUDEMANS K, BIJLSMA JWJ, LAFEBER F: Interleukin 7 stimulates tumour necrosis factor alpha and Th1 cytokine production in joints of patients with rheumatoid arthritis. *Ann Rheum Dis* 2003; 62: 113-9.
24. MEBIUS RE: Organogenesis of lymphoid tissues. *Nat Rev Immunol* 2003; 3: 292-303.
25. MEIER D, BORNMANN C, CHAPPAZ S *et al*.: Ectopic lymphoid-organ development occurs through interleukin 7-mediated enhanced survival of lymphoid-tissue-inducer cells. *Immunity* 2007; 26: 643-54.
26. TIMMER T, BALTUS B, VONDENHOFF M *et al*.: Inflammation and ectopic lymphoid structures in rheumatoid arthritis synovial tissues dissected by genomics technology identification of the interleukin-7 signaling pathway in tissues with lymphoid neogenesis. *Arthritis Rheum* 2007; 56: 2492-502.
27. TOYOSAKI-MAEDA T, TAKANO H, TOMITA T *et al*.: Differentiation of monocytes into multinucleated giant bone-resorbing cells: two-step differentiation induced by nurse-like cells and cytokines. *Arthritis Res* 2001; 3: 306-10.
28. JONES E, ENGLISH A, CHURCHMAN S *et al*.: Large-scale extraction and characterization of CD271+ multipotential stromal cells from trabecular bone in health and osteoarthritis: Implications for bone regeneration strategies based on uncultured or minimally cultured multipotential stromal cells. *Arthritis Rheum* 2010; 62: 1944-54.
29. KRENN V, MORAWIETZ L, BURMESTER GR *et al*.: Synovitis score: discrimination between chronic low-grade and high-grade synovitis. *Histopathology* 2006; 49: 358-64.
30. ARIIZUMI K, MENG Y, BERGSTRESSER PR, TAKASHIMA A: IFN-gamma-dependent IL-7 gene regulation in keratinocytes. *J Immunol* 1995; 154: 6031-9.
31. TANG J, NUCCIE BL, RITTERMAN I, LIESVELD JL, ABBOUD CN, RYAN DH: TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. *J Immunol* 1997; 159: 117-25.
32. PONCHEL F, CUTHBERT RJ, GOËB V: IL-7 and lymphopenia. *Clinica Chimica Acta* 2011; 412: 7-16.
33. VINCENT FB, MORAND EF, SCHNEIDER P, MACKAY F: The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol* 2014; 10: 365-73.
34. HIKIDA M, NAKAYAMA Y, YAMASHITA Y, KUMAZAWA Y, NISHIKAWA S-I, OHMORI H: Expression of recombination activating genes in germinal center B cells: involvement of interleukin 7 (IL-7) and the IL-7 receptor. *J Exp Med* 1998; 188: 365-72.
35. CORCORAN AE, RIDDELL A, KROOSHOOP D, VENKITARAMAN AR: Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. *Nature* 1998; 391: 904-7.
36. KIKUCHI K, LAI AY, HSU C-L, KONDO M: IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med* 2005; 201: 1197-203.
37. YE S-K, AGATA Y, LEE H-C *et al*.: The IL-7 receptor controls the accessibility of the TCRγ locus by Stat5 and histone acetylation. *Immunity* 2001; 15: 813-23.
38. MACKAY F, SCHNEIDER P: TACI, an enigmatic BAFF/APRIL receptor, with new unappreciated biochemical and biological properties. *Cytokine Growth Factor Rev* 2008; 19: 263-76.
39. BOMBARDIERI M, KAM N-W, BRENTANO F *et al*.: A BAFF/APRIL-dependent TLR3-stimulated pathway enhances the capacity of rheumatoid synovial fibroblasts to induce AID expression and Ig class-switching in B cells. *Ann Rheum Dis* 2011; 70: 1857-65.
40. GOËB V, WALSH C, REECE R, EMERY P, PONCHEL F: Potential role of arthroscopy in the management of inflammatory arthritis. *Clin Exp Rheumatol* 2012; 30: 429-35.
41. WAGNER UG, KURTIN PJ, WAHNER A *et al*.: The role of $CD8^{(+)}$ $CD40L^{(+)}$ T cells in the formation of germinal centers in rheumatoid synovitis. *J Immunol* 1998; 161: 6390-7.
42. VIRONE A, PASCAUD J, RIVIERE E *et al*.: O017 Il-7 in primary Sjögren syndrome (PSS) is secreted by salivary gland epithelial cells after ifn stimulation and is associated with B-cell activation. *Ann Rheum Dis* 2018; 77: A9-A.
43. YOSHIDA H, NAITO A, INOUE J-I *et al*.: Different cytokines induce surface lymphotoxin-[alpha][beta] on IL-7 receptor-[alpha] cells that differentially engender lymph nodes and Peyer's patches. *Immunity* 2002; 17: 823-33.
44. DENNIS G, HOLWEG CT, KUMMERFELD SK *et al*.: Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics. *Arthritis Res Ther* 2014; 16: R90.