Osteoarthritis and Cartilage 31 (2023) 1176–1188

Osteoarthritis and Cartilage

B-cell capacity for expansion and differentiation into plasma cells are altered in osteoarthritis

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A R T I C L E I N F O

Article history:
Received 25 October 2022
Accepted 4 March 2023

Keywords:
Osteoarthritis
B-cell differentiation
Synovial niche

A B S T R A C T

Objective: Autoantibody (autoAbs) production in osteoarthritis (OA), coupled with evidence of disturbed B-cell homoeostasis, suggest a potential role for B-cells in OA. B-cells can differentiate with T-cell help (T-dep) or using alternative Toll like recptor (TLR) co-stimulation (TLR-dep). We analysed the capacity for differentiation of B-cells in OA versus age-matched healthy controls (HCs) and compared the capacity of OA synovitis-derived stromal cells to provide support for plasma cell (PC) maturation.
Methods: B-cells were isolated from OA and HC. Standardised in vitro models of B-cell differentiation were used comparing T-dep (CD40 (cluster of differentiation-40/BCR (B-cell receptor)-ligation) versus TLR-dep (TLR7/BCR-activation). Differentiation marker expression was analysed by flow-cytometry; antibody secretion (immunnoglobulins IgM/IgA/IgG) by ELISA (enzyme-linked immunosorbent assay), gene expression by qPCR (quantitative polymerase chain reaction).
Results: Compared to HC, circulating OA B-cells showed an overall more mature phenotype. The gene expression profile of synovial OA B-cells resembled that of PCs. Circulating B-cells differentiated under both TLR-dep and T-dep, however OA B-cells executed differentiation faster in terms of change in surface marker and secreted more antibody at Day 6, while resulting in similar PC numbers at Day 13, with an altered phenotype at Day 13 in OA. The main difference was reduced early B-cells expansion in OA (notably in TLR-dep) and reduced cell death. Stromal cells support from OA-synovitis allowed better PC survival compared to bone marrow, with an additional population of cells and higher Ig-secretion.
Conclusion: Our findings suggest that OA B-cells present an altered capacity for proliferation and differentiation while remaining able to produce antibodies, notably in synovium. These findings may partly contribute to autoAbs development as recently observed in OA synovial fluids.

Introduction

Osteoarthritis (OA) is the commonest rheumatic disorder, associated with pain and loss of joint function.[1] Despite not being considered an adaptive immunity immune-mediated inflammatory disease, there are reports of autoantibodies[2] as well as alterations in the proportions of circulating immune B-cells[3,4] (naïve and memory)[5,6] identified with commonly used cell surface markers for lineage/differentiation (cluster of differentiation: CD19/CD20 and CD24/CD27/CD38/CD138/Immunoglubulin-D (IgD). This suggests that B-cells may play a role in OA pathogenesis, especially as the relative loss of antigen-inexperienced

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https://doi.org/10.1016/j.joca.2023.03.017

B-cells could result in increase in antibody producing plasmablasts/plasma-cells (PBs/PCs). Following antigen encounter and differentiation, antibody-secreting cells need support from a stromal microenvironment for survival and to attain their endpoint differentiation as PCs. This is described as a need for a 'PC niche.' Bone marrow (BM) stromal cells usually provides this by expressing the signals required for long-lived PC survival, notably IL-6 (interleukin)/TNF-α (tumour necrosif factor-α)/APRIL (B-cell proliferation-inducing ligand)/BAFF (B-cell activating factor), and CXCL12 (chemokine (CXC motif ligand 12) chemokine to retain PCs locally.[7]

In OA synovitis, synovial stromal cells (i.e., fibroblasts-like cells) can also produce high levels of APRIL/BAFF/TNF-α/IL-6 which may locally assist in B-cell maturation/differentiation and class immunoglobulin switching.[8] Both B-cells and PCs have been observed in OA synovial-tissue.[9,10] Furthermore, the synovium displays tissue architecture (TA) organised either with diffuse, or aggregates of T- or B-cell or ectopic germinal centre-like (GC) structures.[5] A cytokine/chemokine expression profile of synovial tissue suggested that CCL19 (chemokine (CC motif) ligand-19)/CCL21/IL-7/RANKL (Receptor activator of nuclear factor kappa-B ligand)/CXCL12 were able to support the development of ectopic lymphoid structures as well as aggregates of B-cells, both resulting in B-cell maturation.[11]

Here, we explore whether circulating B-cells in OA have an intact capacity for differentiation compared to age-matched healthy controls (HCs), using standardised in vitro differentiation assays and advanced flow cytometry techniques,[12,13] allowing a comparison of the outcome in a T-cell dependent or independent context. We also investigate whether OA synovial stromal cells can support PC survival as efficiently as BM-derived cells. We identified phenotypic changes in circulating OA B-cells with an accelerated capacity for differentiation and Ig-secretion but in the absence of an early proliferation response. In addition an OA synovial stromal cell culture appeared better at supporting PC survival than BM cells.

Patients and methods

Patients and samples

OA patients (n = 95) and HCs (n = 23) samples were obtained (Table 1, all available information). Ethics approval and informed consent were obtained from all participants (detailed in SUPP-samples chart). The diagnosis of OA was made by NICE clinical criteria and radiographs.[14] All patients used for all B-cell differentiation had knee OA while for the gene profiling of purified circulating B-cells (n = 62), patient with hands (n = 11) or hips (n = 17) OA were also included in addition to knee only patients (n = 34).

Blood, fresh tissue and frozen PBMC were used and details of processing are provided in SUPP-methods as previously described.[12,15–17]

In vitro B-cell differentiation assays and other experimental procedures

Assays have been extensively described[12,13] with schematic in SUPP-material.

A classic flow cytometry analysis recording % of positive cells and two multi-dimensional data display tools were used (visualisation of *t*-distributed stochastic neighbourhood embedding [viSNE, https://www.cytobank.org/]),[18] and an analysis using Spanning-tree Progression Analysis of Density-normalized Events (SPADE, https://www.cytobank.org/). Details of how the tools work are included in SUPP-methods.

ELISA and real time qPCR were performed as previously described.[12]

Fresh blood	**HC**	**OA**
n	7	9
Age*	63 (58–70)	64 (58–75)
F/M	4/3	6/3
Frozen PBMCs	**HC**	**OA**
n	16	62
Age*	63 (61–67)	66 (59–76)
F/M	5/4	35/27
BMI*	20.9–27.5	20.7–42.8
Arthroscopic biopsies	**Anonymised tissue**	
n	32	
Age*	55 (44–67)	
F/M	21/11	
Ar-VAS*	61.5 (38–79)	
Synovitis score (n = 13)	2–8	
TA (n) Diff	14	
Agg	12	
GC	6	
Knee replacement	**OA**	
n	11	
Age *	70 (61–85)	
F/M	7/4	
Synovitis score (n = 6)	0–5	
TA (n = 6) Diff	2	
Agg	3	
GC	1	

Samples from OA patients (n = 95, blood and tissue) and healthy control (n = 23, blood) were obtained from our tissue bank (TB), all fully anonymised with all available information described; or were collected as fresh blood specifically for the B-cell differentiation assays. HC donors were selected based on responses to a health questionnaire allowing the exclusion of anyone with any symptoms of painful joints.

n, number; yrs, years old; F, female; M, male; BMI, Body Mass Index; Diff, Diffuse structure; Agg, Aggregate structure; GC, GC-like structure; Ar-VAS, arthroscopy Visual Analogue Scale reflecting joint inflammation. Note that synovitis scores were only available in only 6/11 of the knee replacement samples. Data are displayed as Ranges in groups with small numbers and with median (IQR indicated by a *) for larger groups.

Table 1 Osteoarthritis and Cartilage

Demographic and clinical data for HCs and OA patients.

Data analysis

Statistical analysis was performed using SPSS Statistics 26 (IBM). Gene expression data between three groups are presented as boxplot; longitudinal data (cells number, % of positive cells, gene expression) as median and upper/lower range (ULR); ELISA data as histograms. Due to small number of participants normality was not achieved and non-parametric test were used. Man Witney U (MWU) test was used to compare two groups for circulating subsets (no correction was applied as no difference was observed) and for % of positive cells. Analysis of covariance (ANCOVA) was used to compare gene expression between three groups, correcting for age/sex. Two-way mixed analysis of variace (ANOVA) (with Dunn's test correction) was used to compare OA and HC groups (fixed effect) over time for cell numbers, % of positive cells, ELISA and gene expression (random effects). Correlations between circulating memory cells and PCs generated at Day 13 were assessed using Spearman coefficient rho. Paired *T*-tests were used to evaluate cell number/Ig-secretion at Day 13 from the same culture divided between two types of stromal support at Day 6 however, as n = 4 data are indicative. p-value < 0.050 was considered significant.

Results

B-cells in health and in OA

Seven HCs and six OA patients were age matched (1:1) as closely as possible (±or - 2 year) for the B-cell differentiation assays (Table 1), while other participants were used for gene expression studies.

Prior to testing the capacity for B-cell differentiation in OA, we investigated differences in the initial pool of circulating B-cells (using markers for lineage/differentiation and genes expression).

Frequency of B-cells subsets is dysregulated in OA

We previously reported a shift in the balance of the naïve/memory subsets in OA.[5] We compared frequencies of naïve (CD27–CD38+), memory (CD27+CD38+), B-reg (CD24highCD38+) and EPB (CD24+CD38high) subsets in HCs (n = 7) and OA patients (n = 6) (Fig. 1a). We observed less naïve B-cells in OA (not significant), no difference in memory, B-reg and a trend for more PBs.

The phenotype of circulating B-cells is altered in OA

The phenotype of B-cells was examined. On SPADE-trees, positioning of nodes in subsets (Fig. 1b, particularly large nodes) suggested a shift in the distribution of cells within differentiation subsets. Naïve B-cells shifted from a position at the upper branch of the SPADE-tree in health to one closer to memory cells in OA. Memory B-cells in HCs showed clearly separated nodes from naïve B-cells but were more widely distributed in OA. Levels of expression were plotted using viSNE-maps (Fig. 1c). Clear changes in expression levels were observed in OA in sub-population of cells (circled) with higher levels of CD138/CD24/CD27 and lower IgD. Looking at nodes in naïve and memory subsets (Suppl-Fig. 1) a more mature phenotype for both was confirmed in OA. A classic flow cytometry analysis quantifying positive cells (% on Fig. 1c) failed to identify differences in frequencies/except for CD138 (Suppl-Fig. 2/3, Panel b, $p = 0.014$), demonstrating the utility of the more advanced flow methodologies. There was no difference in % of IgM+ (immunoglobulin-M) B-cells in OA, while IgA+ (immunoglobulin-A) and IgG+ (immunoglobulin-G) B-cells were more frequent (Suppl-Fig. 1a/b) and also expressed higher levels of IgA and IgG.

In summary, these observations identified a change in the phenotype of circulating B-cell subsets in OA, with a shift towards a more advanced state of differentiation.

OA synovial B-cells show a PC gene expression profile compared to circulating B-cells

B-cell differentiation includes phases of proliferation, cell death and maturation regulated by several differentiation factors and survival genes. We measured expression of nine genes regulating apoptosis/differentiation in purified circulating B-cell from HCs (n = 16) and OA patients (n = 62) as well as in B-cells sorted from OA synovial tissue digests (n = 11) (Fig. 1d). The age/gender between the groups was not different ($p = 0.479$ and $p = 0.705$ respectively) despite older patients in the synovial tissue group. We used ANCOVA to exclude confounding effects of age/gender which were not significant. We then used ANOVA to compare the three groups and all genes but PRDM1 (PR domain zinc finger protein-1)/*Blimp1 (lymphocyte-induced maturation protein-1)*, showed significant differences. However, this was mainly due to the OA synovial tissue group, while circulating B-cells from HCs and OA patients showed comparable data although small shifts were seen (higher median expression) for the pro-apoptotic *BAD* (BCL2 associated agonist of cell death) (1.8-fold) and differentiation factor *XBP-1* (X-box binding protein-1) (1.5-fold). OA circulating B-cells show no particular profile based on OA joint involvement (hand/hip/knee) detailed in Supp-material.

The gene expression profile of synovial OA B-cells was different compared to circulating B-cells, showing marginal 2/3-fold reduced pro-apoptotic *BAD, BAX (Bcl-2-associated X protein)* and anti-apoptotic *MCL1* (*induced myeloid leukemia cell differentiation protein-1)* genes and increased anti-apoptotic gene *BCL2L1* (Bcl-2-like protein-1) but significant higher *BCL2* (*B-cell lymphoma-2)* (20-fold, $p = 0.0003$). Therefore, the overall balance of pro/anti-apoptotic factors in synovial B-cells appeared to be dysregulated with no clear pro-apoptotic profile, while possibly less prone to cells death.

There was significant lower expression of *IKZF3* (Ikaros family zinc finger protein-3)/Aiolos (regulator of lymphocyte development[19]) and *AICDA* (Activation-induced cytidine deaminase) (somatic hypermutation/class-switch[20]), and increased *XBP-1* (Ig secretion[21]) in synovial B-cells, but no change for *PRDM1*/BLIMP1 (PC differentiation[22]). Such changes in profile of synovial B-cells resemble patterns previously described in mature antibody secreting PCs.[23]

OA-specific alteration in B-cell capacity for differentiation

OA B-cells do not proliferate well in response to activation

We performed in vitro assays comparing the capacity for differentiation of healthy and OA B-cells. Activation results in early expansion of B-cells up to Day 6, followed by their death in both assays. The initial expansion of B-cells after stimulation in T-dep was reduced in OA resulting in lower total cell numbers at Day 6 (Fig. 2a, mean 13.9-fold live + dead cells) compared to HCs (97.6-fold, $p = 0.073$), with a reduced increase in live cells ($p = 0.056$) and less massive cell death. In TLR-dep, early massive expansion of B-cells was observed in HCs (488.6-fold live+dead cells) but was also minimal in OA (4.3-fold, $p = 0.008$). This was associated by massive cell death in HCs (~500 fold) compared to OA (~4 fold, $p < 0.001$), resulting in a 2.0-fold live cell number in HCs compare to Day 0,[12,13] where a reduction was observed in OA (0.45-fold).

In both assays, surviving cell numbers observed at Day 13, showed ~3.5-fold less live cells for T-dep in OA (0.20×10^6 cells) compared to HCs (0.71×10^6 cells, $p = 0.101$) while more similar numbers were observed in TLR-dep (0.078×10^6 and 0.093×10^6 cells respectively). The number of identifiable dead cells were similar in both assays and groups due to a lot of events no longer being accountable as debris.

The frequency of memory B-cells at Day 0 has a positive effect on the number of PCs generated at Day 13 in the TLR-dep assay only.[24] This was observed here for HCs (Fig. 2b, Rho = 0.714, $p = 0.003$), but in OA, this relationship was lost.

The main difference observed was therefore the loss of OA B-cell capacity for early expansion in both assays and the lack of cell death resulting from proliferation after activation in OA.

Differentiation in OA is achieved by Day 6 while at Day 13 in HCs

By Day 6 in T-dep, most naïve B-cells in HCs had not responded to stimulation (Fig. 3, top), while a fraction of memory B-cells remained at their initial Day 0 position, as expected.[12,13] In contrast, in OA, most B-cells had been activated and had already reached the PB/PC stages. In TLR-dep, such differences were not observed and both naïve and memory B-cells already moved to PB/PC subsets at Day 6 in HCs and OA (Fig. 3) however, they remained in EPB in HCs and reached LPB/PC in OA. Dense and sparse clusters of cells on the density viSNE maps, can be seen in both assays with some clearly missing in HC or OA (highlighted in coloured contour lines). By Day 13, differentiation had similarly reached PB/PC subsets in HC and OA for T-dep but cells advanced to PC subset in

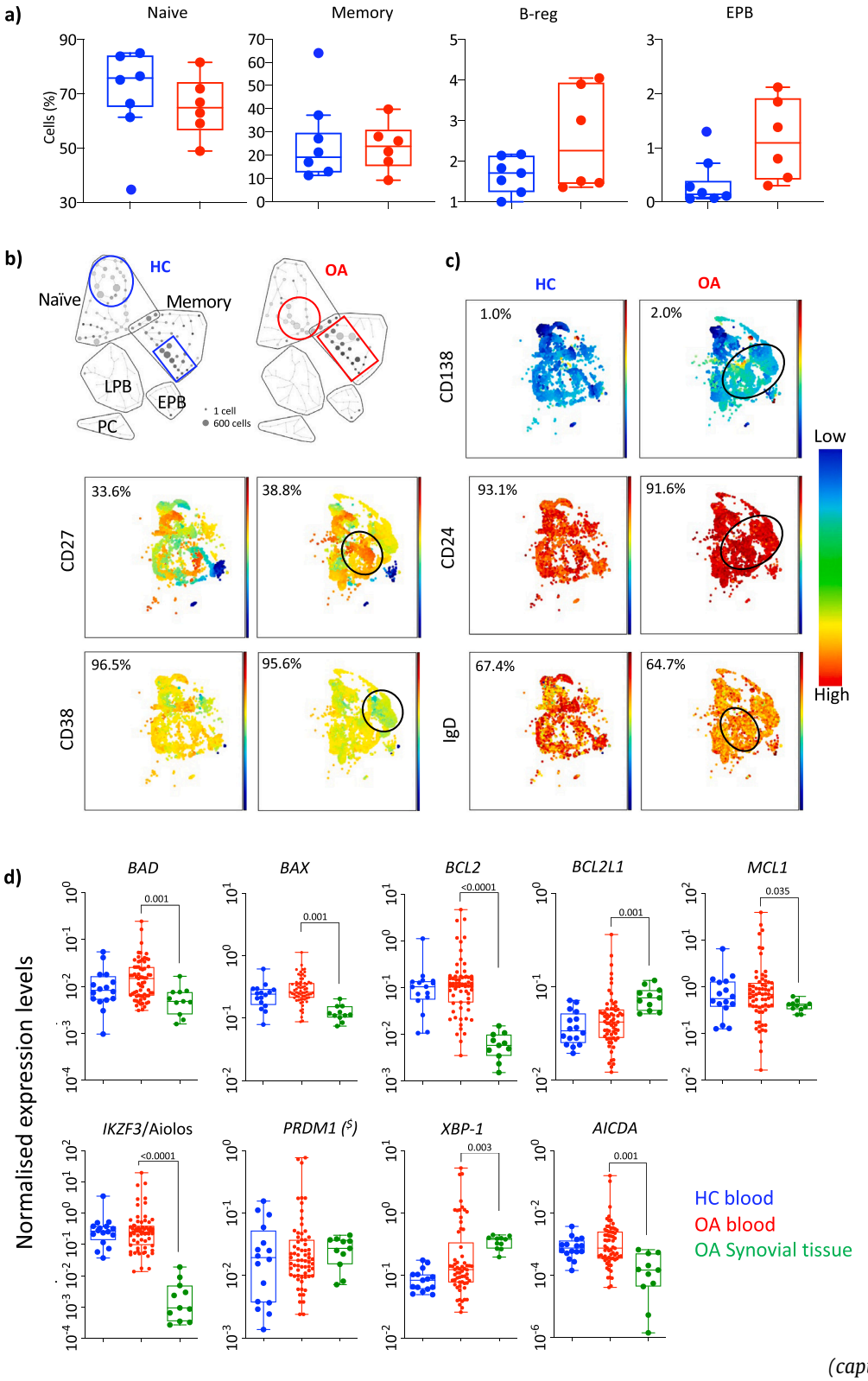

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Fig. 1

Osteoarthritis and Cartilage

B-cells in health and in OA. (a) Frequency of circulating B-cell subsets analysed using classic multi-marker flow cytometry analysis: naïve (CD19+CD27-CD38 +), memory (CD19+CD27+CD38+), B-reg (CD19+CD24highCD38+) and EPB (CD19 +CD24 +CD38high) subsets in the HCs (n = 7) and OA patients (n = 6) used in the differentiation assays. (b) and (c) The visualisation tools viSNE and SPADE were used to analyse data. The tools integrate all data (> 30.000 cells from each participants and at each time points) and uses continuous levels of expression of five markers to define clusters of cells and grouped them in nodes. It then create a representative maps (all events grouped by similarities in their profile) or trees (of nodes of cells related to each other by their profiles) to visualise data and colour code levels of expression of individual marker for each cells/node. Displays then show maps or trees representing patients or HCs for each time point. SPADE trees (b): cellular hierarchy of clusters of cells (nodes), grouped in subsets (naïve, memory, EPB/LPB early/late plasmablasts; PC plasma cells by manual annotation). The position of nodes in naïve (circle) and memory (square) B-cells in OA indicates a closer phenotype than in HCs. viSNE maps (c): display expression levels (graded blue to red for low to high levels). Clear changes in expression can be seen in OA, notably dense groups of cells absent in HCs (circled in OA) with higher CD138 (loss of dark blue cells, increase in light blue cells), lower IgD (lighter orange), and higher CD24 (darker red), and smaller populations for CD38 (light green) and CD27 (more orange cells, less blue cells). Positivity (median % of total B-cells) using a classic flow cytometry analysis are indicated in the left-top corner showing significant difference only for CD138 ($p < 0.05$). (d) Expression profile (by qPCR, displayed as boxplot, median line, IQR-box, extremes whiskers) of genes associated with the regulation of apoptosis and B-cell differentiation in purified B-cells (magnetic bead cell sorting) from the blood of HCs (n = 16) and OA patients (n = 62) as well as from knee synovial tissue digests (n = 11). ANCOVA analysis confirmed that age and gender were not affecting results. *p*-Value calculated by ANOVA were all significant ($p < 0.001$) with the exception of PRDM1 ($). Circulating and synovial OA B-cells were different by post-hoc testing (*p*-value displayed), but no difference was observed between circulating B-cells from HCs or OA patients.

OA while only in LPB in HCs. Furthermore, more large nodes were observed in PC subset. There were almost no naïve B-cells remaining in OA, but some were still present in HCs. Again different dense/sparse clusters of cells could be observed in HC/OA.

The phenotype of B-cells considerably change by Day 6 during both assays. In T-dep, there was an overall higher expression of CD27/CD138 in OA (Fig. 3). Less up-regulation of CD38 was observed in OA and less down-regulation of CD24 and IgD. A small group of cells with very low CD27 was still present in HCs in T-dep (arrow) but not in OA. This resulted in significant differences in CD27+/CD38+/CD138+ cell frequencies (Suppl-Fig. 2c). In TLR-dep, similar changes were seen with a notable lack of reduction in CD24 expression in OA and significant differences in CD38+/CD138+/IgD + cells (Suppl-Fig. 3c). Altogether, differences between assays in HCs could still be seen at Day 6 (for example cells with lower CD27 expressing in T-dep absent in TLR-dep) but were not as noticeable in OA. Progressing to Day 13, markers demonstrated a more mature pattern in OA in both T-dep and TLR-dep (particularly for CD27/CD138). Expression of CD38 did not increase as much in OA (particularly in the two regions of the viSNE maps with high cell density). The reduction in expression of IgD was less pronounced in OA. Levels of CD24 were highly variable in HC (very low/high levels) with same scattered patterns in OA. Positive cells were also significantly different for CD38+, consistently reaching > 99% in both T-dep and TLR-dep in OA while significantly less in HCs (Suppl-Fig. 2c/3c).

OA patient B-cells therefore appeared to have an intact capacity to undergo differentiation, although the PC stage of differentiation was already achieved by day-6, with pronounced changes in level of expression of PC markers (CD27/CD138) and a final differentiation phenotype that did not reproduce the T-dep/TLR-dep specific differences observed in HCs.

Early antibody secretion in OA

As a result of differentiation, antibody class switch and secretion is initiated. We compared levels of antibody secreted (Fig. 4). IgM secreted in high amount from Day 6 (increasing at Day 13 in HCs), were higher in OA ($p = 0.009$), while maximum levels were already reached and did not increase further. In agreement with a faster differentiation, IgA levels were higher in OA at Day 6, significantly in TLR-dep ($p = 0.014$). Levels increased in HCs at Day 13 ($p < 0.0001$), but remained similar in OA. IgG levels were higher at Day 6 ($p = 0.035$, in TLR-dep) and keep increasing in HC at Day 13 ($p = 0.005$ and $p < 0.0001$) in both assays, while remaining similar in OA in T-dep but increasing further in TLR-dep ($p = 0.03$). In summary, the main differences observed was that B-cells in OA were able to secrete Ig earlier from Day 6 at maximum capacity with less difference at Day 13.

A better anti- apoptosis gene expression balance in OA over differentiation

Gene expression was evaluated over time (Fig. 5 shows expression levels, Suppl-Fig. 4 fold-change over baseline). Of note, at Day 0, only *BCL2*/*BCL2L1* showed higher levels in the OA ($p = 0.05$). Patterns over time followed changes expected for all genes (two-way mixed ANOVA, all $p < 0.010$) but for BAD/BAX where changes were minimal. At Day 6/13, the levels of expression of the anti-apoptotic genes (*BCL2L1*/*MCL1* and *BCL2* less-markedly) was higher in OA in T-dep and TLR-dep ($p < 0.050$). Pro-apoptotic genes showed limited difference. At Day 13, expression of *BCL2* ($p = 0.008$) and *BCL2L1* ($p = 0.001$) were higher in OA in TLR-dep. This suggests an overall better anti- apoptosis balance in OA at Day 6 in T-dep and over time in TLR-dep. The differentiation genes *XBP-1*, *Aiolos*, *Blimp1* were not significantly different at Day 0 and over T-dep differentiation in OA, but all three genes were consistently more expressed (Blimp1, $p < 0.05$) at Day 6 in both assay and over time in TLR-dep (Day 13, *XBP-1*, $p = 0.016$, *Blimp1* $p = 0.05$). *AICDA* increased markedly at Day 6 and returned to baseline at Day 13 in both HC and OA.

Synovial TA and B-cell gene expression profile

We compared the expression of 22 genes in 32 biopsies presenting with a wide range of synovitis features (summarised in Table 1), local inflammation measured during arthroscopic inspection (ar-VAS 20-83), immune cell infiltration (synovitis scores[25]) and different TAs. Genes were chosen to gain insight into the capacity of the synovium to support B-cells in the presence of T-cells (i.e., in a GC-like reaction) or independently (i.e., aggregates of B-cells).

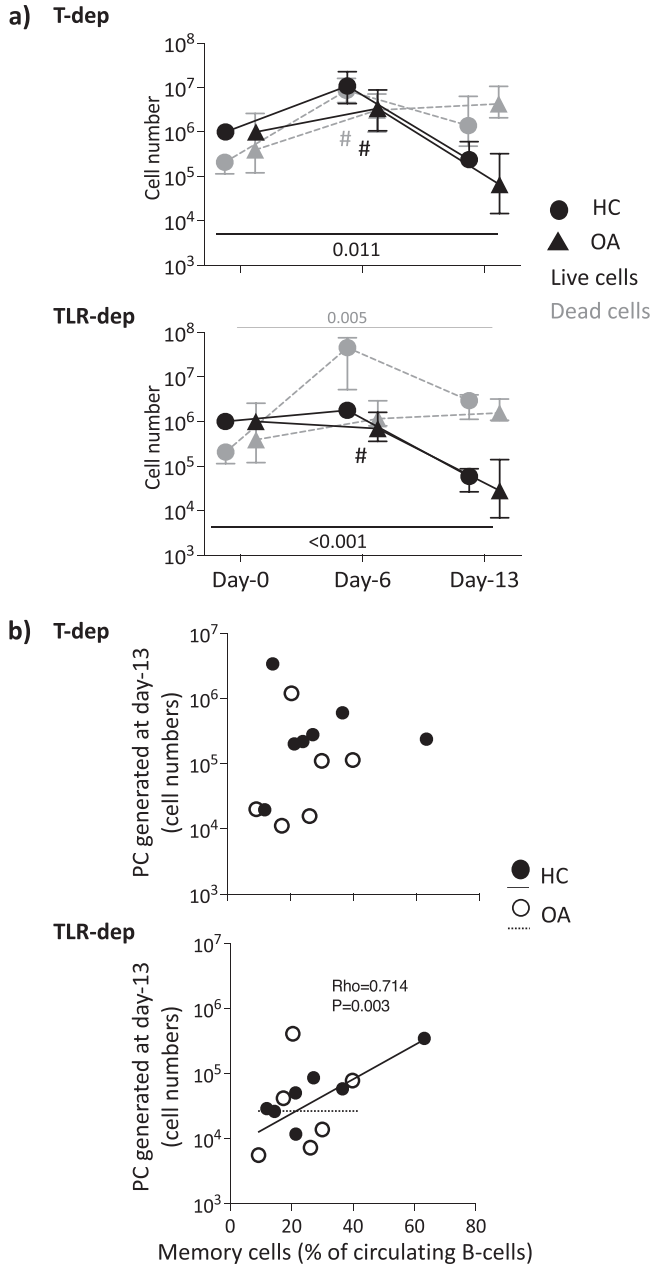


Fig. 2 Osteoarthritis and Cartilage

OA B-cells do not proliferate well in response to activation. (a) Number (median/ULR) of live and dead B-cells during T-dep and TLR-dep differentiation assays in HC (n = 7) and OA (n = 6). *p-Value* for the overall assays between groups were calculated by two-way mixed ANOVA (bars) and post hoc testing between HC and OA displayed next to the group at individual time points. $^{\#}p < 0.100$. (b) The Relationship between % memory B-cells in circulating B-cells at Day 0 and number of PC generated at Day 13 in TLR-dep is verified in HCs (n = 7, Spearman, rho = 0.714 (95% CI 0.436–0.884), $p = 0.003$) displayed as a black fitted-line for illustration, but is lost in OA (n = 6, flat dotted-line). This relationship was not expected in T-dep and was not observed in either OA or HC.

Gene expression differences were observed comparing synovitis with different TA (Fig. 6, age/gender did not affect gene expression). The B-cell lineage marker (CD20) was expressed at similar levels independently of TA, suggesting similar number of B-cells in most tissues. In contrast, PC markers (CD38/CD138) were higher in complex-TA and CD138 was highest in Aggregates-tissues. Expression of the immunoglobulin genes (*IGKC*-constant chain, IgD/IgM/IgG) was increased with TA complexity, also reflected by the high expression of *Aiolos*/*XBP-1*, and survival factors (*BAFFR*) and B-cell activation marker CD79, while chemokine (CXCL12/CXCL13/CCL21) and survival factors (*APRIL/BAFF*) expressed by synovial stromal cells were not different.

These data suggest that effective B-cell maturation can be supported in complex-TA, with similar capacity for Ig-secretion/isotype-switch. Expression of CD138 was clearly increased in aggregate-tissue while CD38 was highest in GC, suggesting difference between the states of differentiation of B-cells in these two TAs.

Capacity of the OA-Synovium to support B-cell differentiation

We then evaluated the capacity of a stromal cell culture derived from OA knee synovitis, to support late B-cell differentiation/survival (Day 6 to Day 13 of the assay) in paired cultures using BM-derived stromal cells as comparator.

We performed IHC on 11 synovial tissues (CD20/CD3/CD68/CD138 markers (Suppl-Fig. 5) to assess synovitis and selected a tissue to grow stromal-cells from, showing features of synovitis (synovitis score 4) with large aggregates of T-/B-cells, a few dispersed PCs ($CD138^{+}$) and small GC-like structures.

At day-13, more live cells were observed with OA-syn (Suppl-Fig. 6), with mean 2.4-fold more PCs in T-dep and 1.86-fold in TLR-dep compared to BM. Paired statistics suggested significance although only indicative (n = 4). Cells were mostly in the LPB/PCs with BM-support but were in the PC-subset with OA-syn (with larger nodes) in both assays (Fig. 7a/Suppl-Fig. 7). Most of the differentiation markers presented similar expression patterns, however with OA-syn there were differences in dense population of cells not developed with BM-support in T-dep assay. OA synovial stromal cells were therefore able to fully support the maturation of PCs, showing consistently more live-PCs with an additional sub-population of mature cells.

Ig-secretion in paired samples showed increase in IgM levels (in 3/4 pairs) (Fig. 7b). IgA levels were also higher (4/4 pairs) in T-dep (not TLR-dep) and IgG levels were also increased, particularly in T-dep but also in TLR-dep.

Discussion

This is the first report of a phenotypic and functional capacity analysis of B-cells in OA. Circulating B-cells displayed a more mature phenotype and synovial B-cells showed a PC-like gene expression profile. B-cells retained the capacity to mature into PCs (with/without T-cell help), with accelerated speed of differentiation and earlier antibody secretion, but lost their ability to proliferate at the early stage of the response. PCs generated in the TLR-dep assay were equally numerous while there was a reduction in viable cells generated in T-dep. TA complexity in synovitis was associated with expression of PC genes and notably Ig-chain. OA synovitis-derived stromal cells were better at supporting B-cell survival, generating PCs with a phenotype close, but not identical, to that of cells differentiated using BM support.

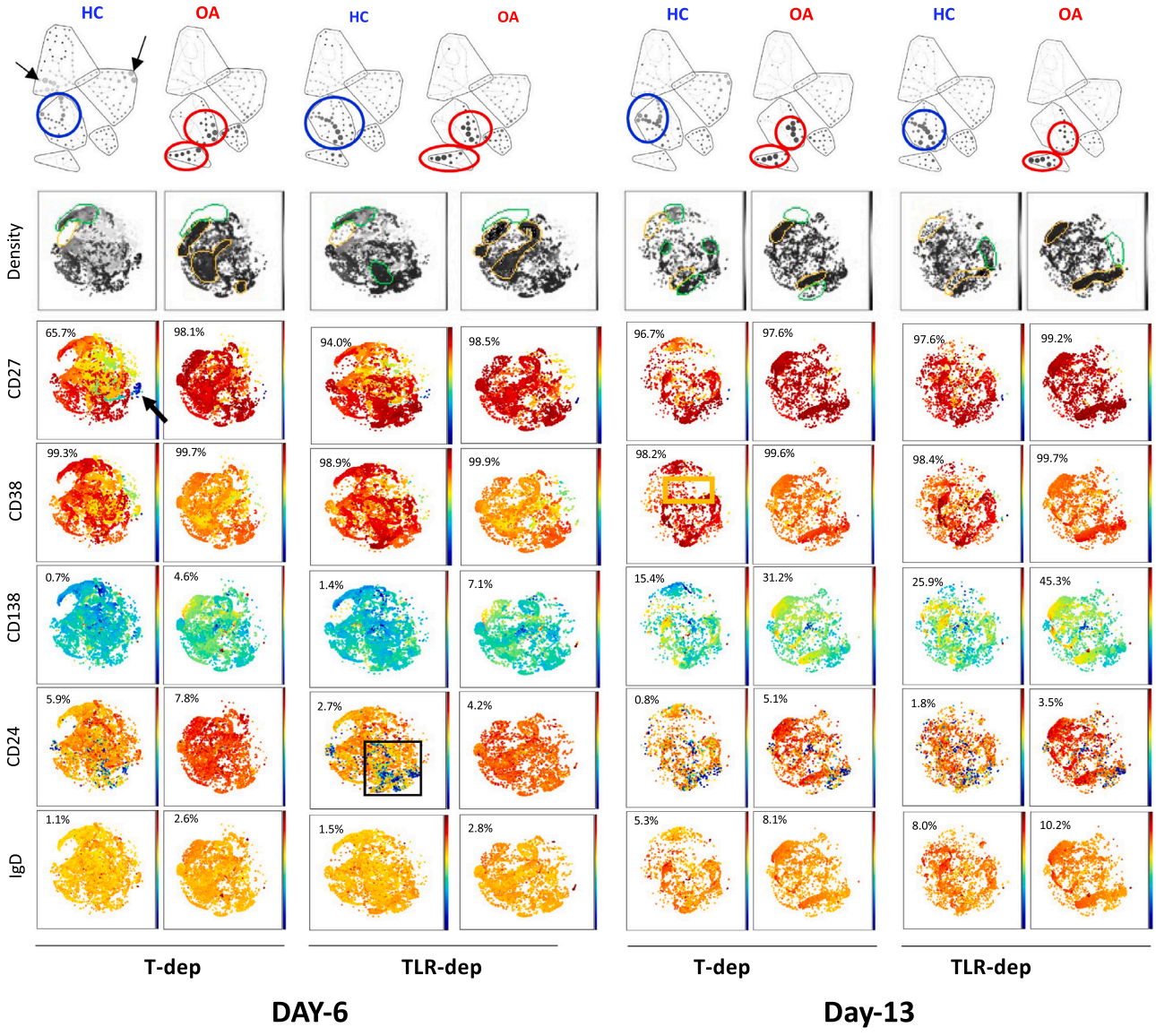


Fig. 3

Osteoarthritis and Cartilage

Further differentiation in OA is achieved by Day 6 while at Day 13 in HCs. SPADE trees and viSNE maps were created in for HC (n = 7) and OA (n = 6) over the T-dep and TLR-dep assay at Day 6 and Day 13. Positivity (median % of total B-cells) is indicated in the left-top corners of maps and detailed in Suppl-Fig. 2/3. The position of nodes in SPADE trees in naïve, memory and PB (circle) in HC and in OA are very different at Day 6, notably in T-dep, while closer in TLR-dep. At Day 13, most cells (large nodes) remain in the LPB subset in HC but are in the PC subset in OA. On the viSNE maps, the grey-shaded map at the top highlight change in density of clusters of cells with noticeable differences in dense sub-populations of cells highlighted (present/absent in HC or OA). On expression maps (coloured), higher expression of CD27 and CD38 was achieved at Day 6 and 13 in OA notably in the dense sub-populations. CD24 levels remained higher in OA at Day 6, while low levels (blue) were still observed in scattered cells (square) in HC under TLR-dep more noticeably. CD24 expression was then more heterogeneous (high/low) at Day 13 with similar patterns. Levels of CD138 and IgD showed less marked differences. Positivity using a classic flow cytometry analysis (median % of total + B-cells) is indicated in the left-top corners of maps and detailed in Suppl-Fig. 2/3.

Circulating B-cell numbers in OA were not different to those of HCs, while B-cell subsets relationships with age were lost[5,12] with reduction in naïve B-cells compensated by a gain in memory B-cells. We reproduced this trend here in the current study, despite the small sample size. Thanks to advanced flow cytometry data analytics, B-cells displayed further phenotypic alterations and present with a more mature phenotype. Differences in gene expression between circulating B-cells were not observed, maybe as total B-cells were analysed while subsets may have allowed to identify more subtle changes. In contrast synovial B-cells gene expression profiles were clearly different. The overall balance of pro/anti apoptotic factors was perturbed (low *BCL2*/high *MCL1)* resembling similar reduction/increased observed at the end of differentiation. Expression profiles of the differentiation factors *AIOLOS*/*XBP-1*/*AICDA* showed

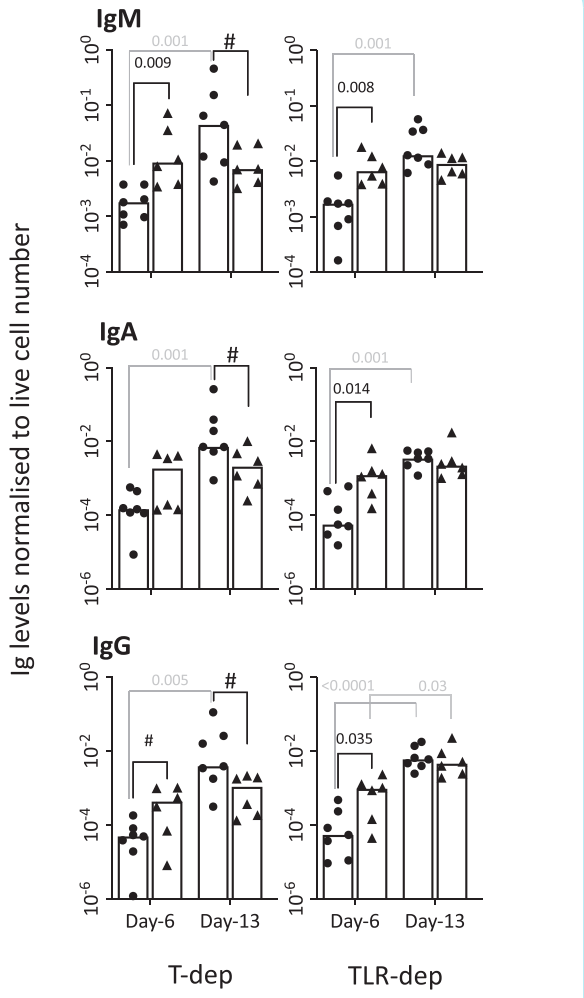


Fig. 4 Osteoarthritis and Cartilage

Antibody secretion in OA is higher at Day 6 in both differentiation assay. Levels of secreted IgM, IgA and IgG (displayed as individual value in histograms), were measured by ELISA from tissue culture supernatants collected at Day 6 and 13 during the T-dep and TLR-dep assays from HC (n = 7) and OA (n = 6), and normalised to the number of cells in the cultures. *p*-Value were calculated by two way mixed-ANOVA (not significant) and post hoc test with *p*-values next to each comparison in black between groups and in grey for time. # $p < 0.100$.

reduction/increase also matching changes observed during differentiation. Altogether, this suggested that the population of B-cell in synovitis resembled LPB/PC, also, being relatively homogenous considering the limited spread of the data distribution.

Overall, the differentiation of B-cells in OA appears to be progressing well in both assays, while faster in OA compared to HCs and with a major difference in the initial early proliferation response. The overall more mature phenotype of B-cells may contribute to the accelerated differentiation, although maybe at the cost of proliferative capacities. Despite OA memory B-cells having a more advanced state of differentiation, the relationship between circulating memory B-cells and PCs generated at Day 13 was unexpectedly lost in OA, which may be related to the deficit in early proliferation. Changes in marker expression were more marked in OA notably for CD138 at Day 6. The higher expression of *MCL1* (and *BCL2/BCL2L1*) in OA throughout both assays, may then contribute to the better survival of PCs.[26] CD138 plays a direct role in PC survival, reduces premature apoptosis inducing a higher expression of *MCL1* and facilitates IL-6/APRIL mediated survival.[27] Both genes were expressed at higher levels in OA over time in both assays. *MCL1* is produced in response to BAFF/APRIL signalling and is particularly important at time when long-lived cells are differentiating.[28] The increase in *MCL1* was more pronounced (Day 6) in OA in both differentiation pathway and may contribute to the reduced amount of cell death between Day 6/13. Previous studies associated high levels of IL-6/APRIL/BAFF with synovitis, enabling differentiation/survival of B-cells.[7] We indeed observed higher expression of *BAFF*, *APRIL* (and *CXCL12*/*CXCL13*) in the OA-syn stromal cells compared to the BM cells (data not shown), which may contribute to this observation.

The differentiation assays suggested early increased capacity to produce IgA and IgG in OA at Day 6, in both assays. Expression of Ig genes in biopsies was increased with TA complexity, while levels of IgM were low compared to IgG, suggesting a full PC differentiation status of synovial B-cells, with isotype switch. This was observed in tissues with B-cell aggregate TA, with higher expression of PC markers (CD138 notably), suggesting that the maturation of B-cells may not only rely on T-cell help in the synovium (i.e., CG-like structures) as previously suggested.[29–32] Higher levels of IgA/IgG were observed using OA-syn stroma (particularly in TLR-dep). These data align well with recent evidence showing the presence of high levels of IgG autoantibodies in OA synovial fluids (not in the blood[2]), suggesting that B-cells can differentiate in OA joints, possibly independently of T-cell help, to produce immunoglobulin locally. The signals needed for B-cells to engage in non T-cell-dependent pathway involve pattern recognition receptors (Toll-like, TLRs or NOD-like receptors) enhancing BCR-mediated activation in the absence of T-cell help.[33] There is no data on circulating B-cell expression of the NOD-like receptors in OA but synovial fibroblasts express NOD1, components of the inflammasome-1/3[34–36] and changes in patterns of TLRs expression.[37–40]

The major limitation of our study was the relatively small number of samples used for in vitro assays, where the heterogeneity of live material is widely accepted notably when using highly standardised assays/methodologies. Due to this, corrections for looking at multiple markers, genes or Ig-secretion were not attempted but time versus group evaluation was performed. Despite this, quantitative differences were identified for most assays (cell counts, classic flow analysis, gene expression, ELISA) while changes in phenotype and distribution of cells between nodes in various subsets were more qualitative but recapitulated millions of events, therefore providing good confidence in data being representative of changes in phenotypes. In comparisons using more samples, age/gender correction did not affect the results although analysis was limited due to other unavailable confounding factors (BMI for example).

In conclusion, the capacity of B-cells to develop into PCs in OA is impacted both quantitatively due to the lack of early proliferative response of OA B-cells while compensated by less apoptosis, and qualitatively with changes in differentiation profiles and further progression into the PC subset. These differences do not affect the capacity of OA B-cells to produce antibodies. Furthermore, IgA and

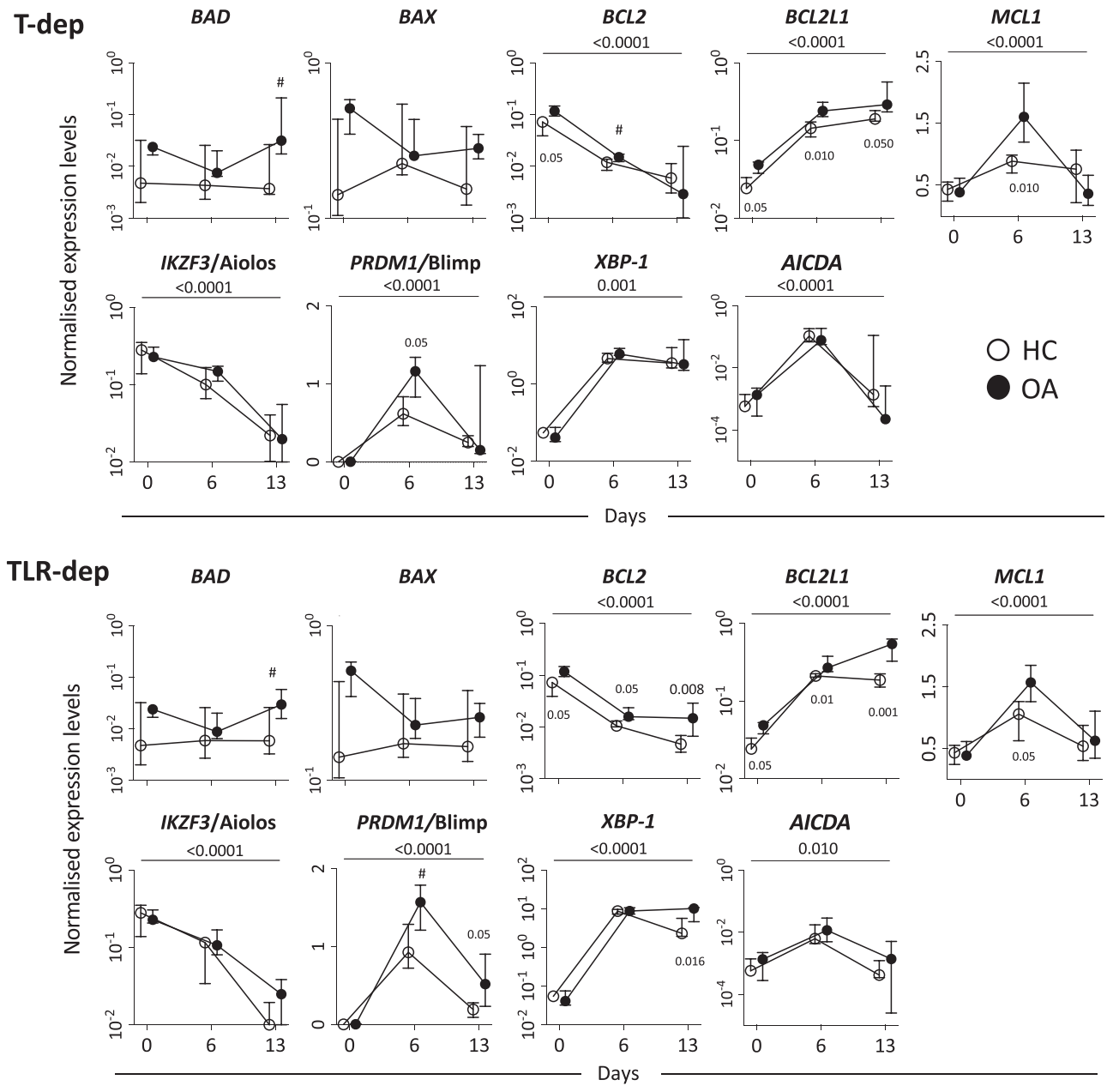


Fig. 5

Osteoarthritis and Cartilage

The expression of anti-apoptosis genes in OA is higher over time in both differentiation assays. Expression levels measured by qPCR (median; ULR) for nine genes associated with the regulation of apoptosis (pro-apoptosis *BAD*, *BAX* and anti-apoptosis *BCL2, BCL2L1* and *MCL1*) or differentiation (*XBP1, PRDM1/Blimp*, *IKZ3/Aiolos* and *AICDA*) in HC (open dots, n = 7) and OA (black dots, n = 6). Data expressed as fold change compared to baseline are displayed in Suppl-Fig. 4 for information. *p*-Value for the overall assays were calculated by two way mixed-ANOVA (bar at the top of the graph) and post hoc test next to each time point. $^{\#}$ $p < 0.100$.

IgM production was enhanced by the provision of OA-synovial cell support, both in the presence/absence of T-cell help. The similar pattern of gene expression observed in tissue with CG like structure (suggesting the provision of T-help) and B-cell aggregates (suggesting a T-cell independent situation) therefore suggests that synovitis is able to provide both type of signalling to B-cells, potentially allowing for antigen driven local antibody development. This is supportive of a mechanism that may explain the increased prevalence of autoantibodies observed in OA synovial fluid, as recently reported.[2]

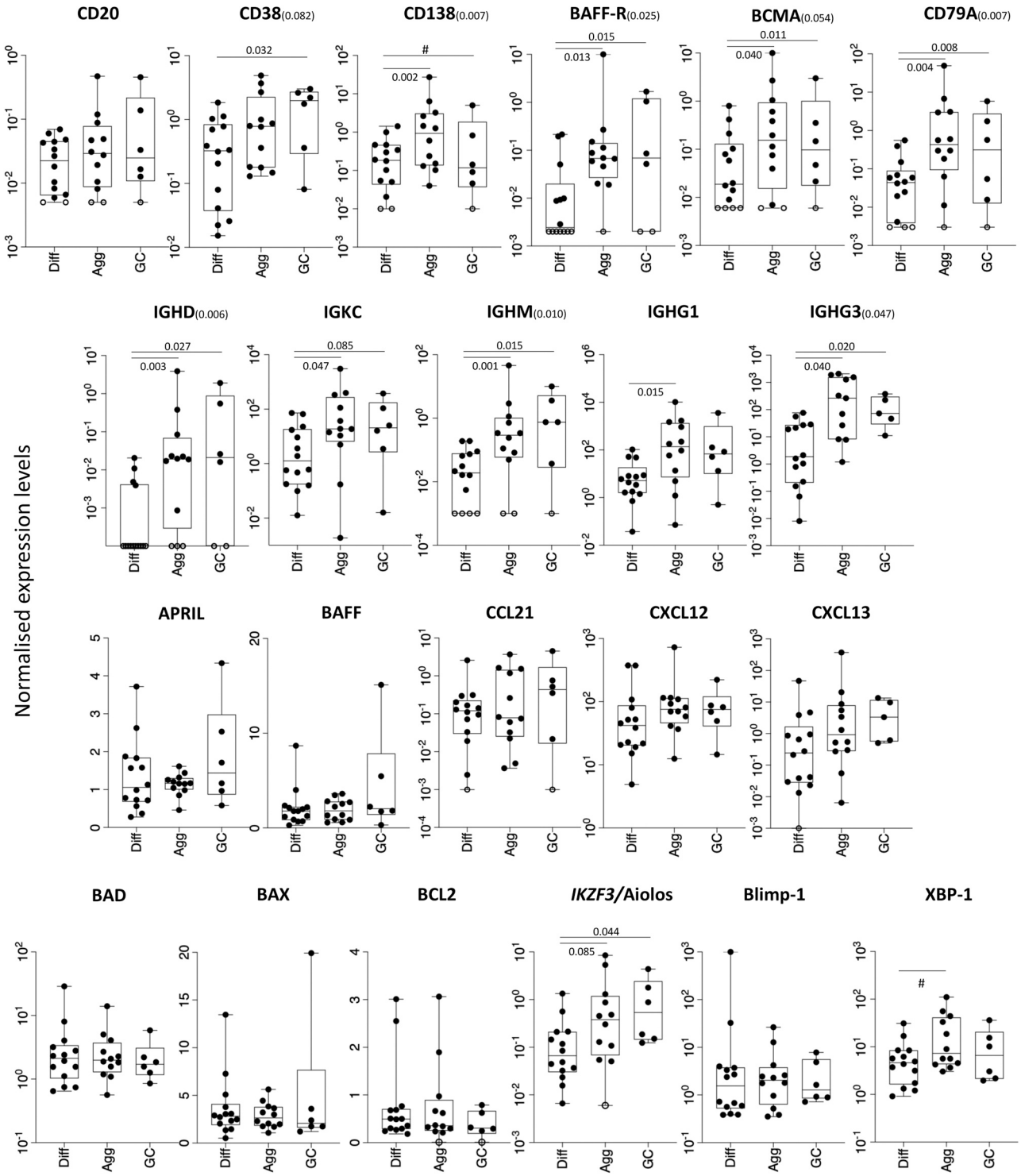
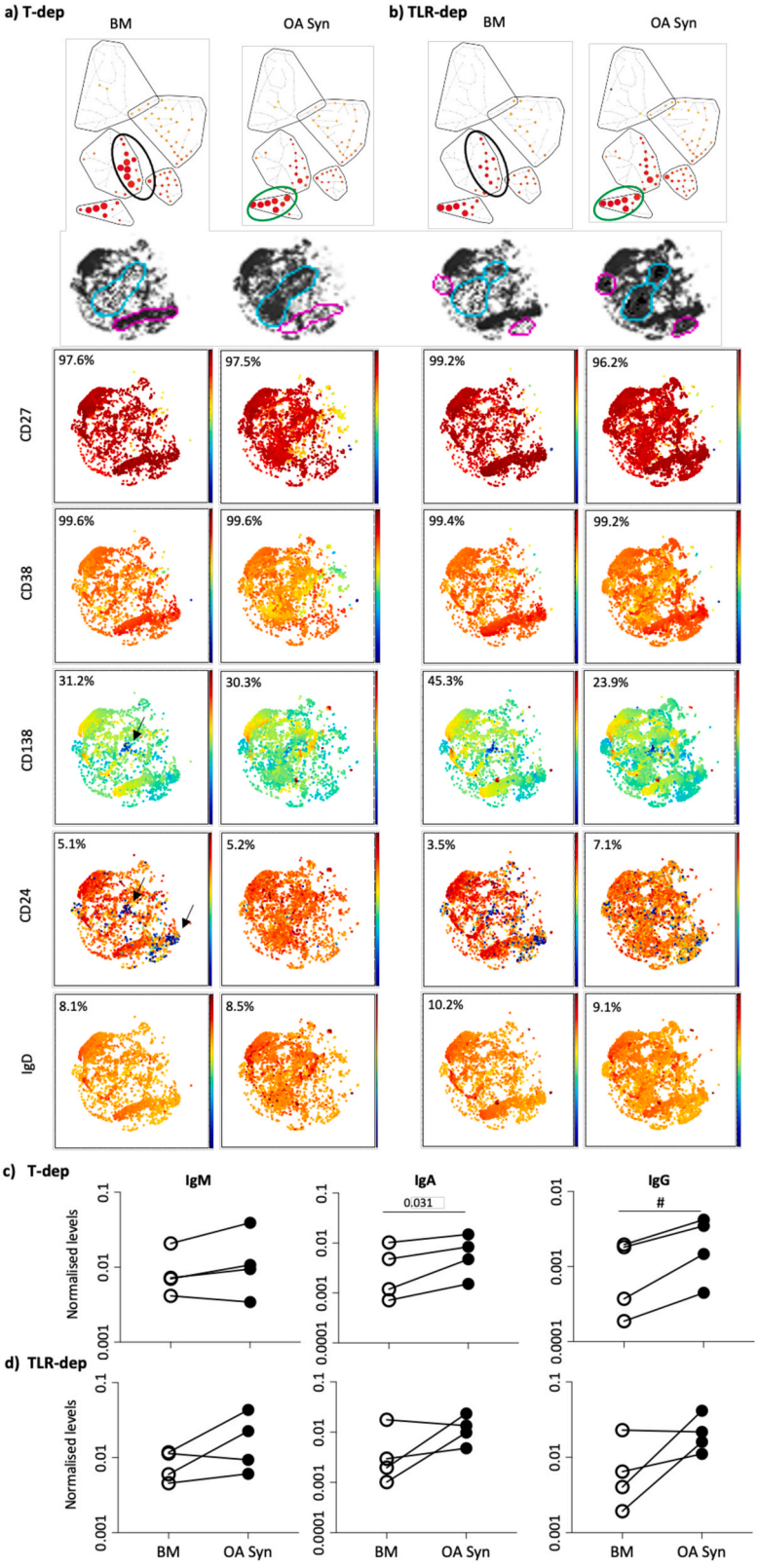


Fig. 6

Osteoarthritis and Cartilage

Tissue architectures in 32 synovial biopsies shows PC in tissues with aggregate of B-cells and Germinal centre like structure. Gene expression levels (by qPCR, displayed as boxplot, median line, IQR-box and extremes whiskers) in 32 synovial biopsies grouped based on tissue architectures (TA). Genes relate to B-cell lineage cell surface markers; Ig-isotypes; B-cell survival factor receptors; cytokines/chemokines, apoptosis and transcription factor genes. Biopsies were classified for TA by histology into tissues containing: diffuse B-cell infiltration (Diff, n = 14), aggregates of B-cells (Agg, n = 12) and GC-like structures (GC, n = 6). Open dots represent levels below detection by qPCR, set 10-fold lower than the lowest detectible level. ANCOVA analysis confirmed that age and gender were not affecting results. *p*-Value for the overall assays between the three groups were calculated by ANCOVA accounting for age/gender (indicated next to the gene name) and for post hoc tests for individual differences between two TAs by lines/*p*-values. $^{\#}$ $p < 0.100$.



(caption on next page)

Fig. 7 Osteoarthritis and Cartilage

Differentiation of OA B-cells with BM and OA Synovium stromal cell support. The capacity of stromal cells derived from OA knee synovitis to support late B-cell differentiation/survival was compared at Day 13, to that of BM cells in paired B-cell cultures from Day 6 in both T-dep and TLR-dep assays. (a) T-dep and (b) TLR-dep assays: SPADE trees and viSNE maps. Dense subpopulations are highlighted in the grey-shaded map. Coloured maps for five markers at Day 13 following differentiation, in four paired assays. Positivity (mean % of total B-cells) is indicated in the left-top corner (significant for CD138 by *t*-test, detailed in Suppl-Fig. 8). (c) Level of Ig secreted at Day 13 (normalised to number of cells in the culture) in the same four pairs. Changes were tested by paired *T*-test. Open: BM and Black OA Syn stromal cell support. $^{\#}p < 0.100$.

CRediT authorship contribution statement

XX, FP: Conception and design, collection and assembly of data, analysis and interpretation of data, statistical analysis. FS: statistical analysis. PGC: provision of study materials, funding supporting the clinical studies providing the samples used. XX, FP, FS, PGC, GMD: Drafting of the article. XX, FP: obtained funding related to this work. All authors critically contributed and approved the Final version of the article.

Acknowledgements

XX was supported via a joint PhD scholarship between the University of Leeds and the China Research Council (CSC, No. 201708330243). PGC and FP are supported in part by the National Institute for Health Research (NIHR) Leeds Biomedical Research Centre. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Conflict of interest

All authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2023.03.017.

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