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Keywords:	Early Rheumatoid Arthritis, Synovitis, B cells, Ant-CCP

TITLE PAGE

Mast cells in early rheumatoid arthritis associate with disease severity and support B-cell autoantibody production

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ABSTRACT

Objectives Mast cells (MCs) are involved in the pathogenesis of rheumatoid arthritis (RA). However, their contribution remains controversial. To establish their role in RA, we analysed their presence in the synovium of treatment-naïve early RA patients and their association and functional relationship with histological features of synovitis.

Methods Synovial tissue was obtained by ultrasound-guided biopsy from treatment-naïve patients with early RA (n=99). Immune cells (CD3/CD20/CD138/CD68) and their relationship with $CD117^{+}$ MCs in synovial tissue were analysed by immunohistochemistry (IHC) and immunofluorescence (IF). The functional involvement of MCs in ectopic lymphoid structures (ELS) was investigated *in vitro*, by co-culturing MCs with naïve B cells and anti-citrullinated protein antibodies (ACPA)-producing B cell clones, and *in vivo* in interleukin-27 receptor (IL27*ra*)-deficient and control mice during antigen-induced arthritis (AIA).

Results High synovial MC counts are associated with local and systemic inflammation, autoantibody positivity, and high disease activity. IHC/IF showed that MCs reside at the outer border of lymphoid aggregates. Furthermore, human MCs promote the activation and differentiation of naïve B cells, and induce the production of ACPA, mainly via contact-dependent interactions. In AIA, synovial MC numbers increase in IL27*ra* deficient mice, in association with ELS and worse disease activity.

Conclusions Synovial MCs identify early RA patients with a severe clinical form of synovitis characterised by the presence of ELS.

Key words: Early Rheumatoid Arthritis, Synovitis, B cells, Anti-CCP

INTRODUCTION

Mast cells (MCs) are tissue-resident cells of the innate immunity, involved in several physiological and pathological processes, including infections, cancer and chronic inflammatory diseases[1,2]. They are present in the synovial membrane (SM) and have been implicated in contributing to the inflammatory response in several rheumatic diseases[3], including rheumatoid arthritis (RA)[4]. Notably, MCs are present in healthy synovia[5], but their number significantly increases accompanying the cellular hyperplasia characteristic of RA synovitis[6–8]. Many MC mediators have direct pro-arthritogenic effects[9–12], and MCs can be activated by several stimuli present in the synovium/synovial fluid, such as anti-citrullinated protein antibodies (ACPA) IgG immune complexes[13]. On the other hand, recent evidences suggest that MC contribution to autoimmune diseases can be complex and multifaceted[14]. In the context of RA, for example, human MCs have been shown to exert immunomodulatory functions *in vitro*[15]. In vivo, initial findings were contrasting[16–18], most likely because of the use of animal models in which MC depletion was accompanied by anomalies of other immune cells[19]. In recent years, thanks to the development of new specific models of MC-depletion[20], their contribution has been confirmed to be essential in collagen-induced arthritis (CIA) but redundant in serum-transfer arthritis[21]. Additionally, their depletion in the pre-clinical phases of CIA, rather than in the established phases, was shown to influence the disease outcome[22]. These evidences *in vivo* suggest that MC contribution to RA may be different in various disease stages, i.e. essential during the early phases (assessed by CIA), but somehow dispensable during the late effector phases (serum transfer). However, while these models are self-resolving, in RA there is chronic inflammation with a perpetuation of the aberrant autoimmune response; therefore, the results cannot be easily translated to the clinical setting. Overall, despite the substantial amount of

data, the role of MCs in RA remains to be clarified [4]. As RA is well-recognized as an heterogeneous syndrome in terms of genetic predisposition, pathogenesis, clinical[23,24], and histological[25] features, it could be hypothesised that MC presence and functions in the synovium may be different in various disease subsets. To explore this hypothesis, we systematically analysed the presence of MCs in the synovia of a large cohort of disease-modifying anti-rheumatic drugs (DMARD)-naïve early RA patients. Furthermore, we assessed their interactions with immune cells at synovial level and analysed *in vitro* the crosstalk of MCs with B cells. Finally, we evaluated the relationship between MC synovial infiltration and ectopic lymphoid structures (ELS) in an experimental model of synovial ectopic lymphoid neogenesis.

Methods

Patient samples and ultrasound-guided synovial biopsy

Synovial tissue was obtained by ultrasound-guided synovial biopsy from DMARD-naïve patients with early (<12 months) RA (n=99), enrolled in the Pathobiology of Early Arthritis Cohort (PEAC) cohort of the Centre for Experimental Medicine and Rheumatology of Queen Mary University (London), as previously described[26]. All patients fulfilled the 2010 EULAR criteria for RA[27]. All procedures were performed following written informed consent and were approved by the hospital's ethics committee (REC 05/Q0703/198).

Histological analyses of synovial samples

Synovial sections underwent standard H&E staining and semi-quantitative (SQ) assessment of synovitis according to a previously validated score (Krenn) [28]. Sequentially cut sections underwent Immunohistochemical (IHC) staining and SQ assessment (0-4) for immune cells, as previously reported[29] and automated image analysis and counting for CD117+ve MCs. Patients were classified into high, intermediate and low MC groups (>66th, 33rd-66th, <33rd percentiles, respectively). Supplementary methods.

Peripheral blood-derived MCs

$CD34^+$ hematopoietic stem cells (StemPro® CD34+ kit, ThermoFisher) were differentiated into MCs as previously described[30]. Supplementary methods and Supplementary Figure 1 for MC purity.

Naïve B cell isolation and co-culture with MCs

IgD^+ B cells isolated by immunomagnetic sorting (Miltenyi) from tonsil mononuclear cells were cultured for 7 days alone or together with MCs, in the presence of TLR-9 ligand (CpG ODN-2006, Invitrogen), in contact or separated by a Transwell© membrane. In parallel experiments, B cells were marked with CFSE (Biolegend) to measure proliferation. Where indicated, mouse anti-human CD154 (CD40L) or isotype control (Biolegend) were added at a concentration of 0.1-10 μg/mL. Supplementary methods.

Visualization of MC-B cell interaction

Supplementary methods.

ACPA B cell clone and co-culture with MCs

ACPA-producing immortalized B-cells (2×10^5) were obtained as described (Germar, K et al. Manuscript submitted and[31]) and cultured as indicated in supplementary methods.

Flow cytometry

Flow cytometry staining was performed as previously described[32]. Supplemetary methods

Measurement of immunoglobulins

IgG and IgM were measured using the IgG and IgM ELISA Kits (Bethyl), according to the manufacturer instructions. For the measurement of ACPA, anti-CCP2-IgG was measured by ELISA (Immunoscan-RA Mark 2; Eurodiagnostica).

Antigen induced arthritis(AIA) in wild-type and IL27*ra* KO

AIA was induced in adult (8–12 wk) IL27*ra*-/- mice and age/sex-matched WT as previously described [33]. 5 μm sections of synovia from animals culled at different intervals (days 3, 10 and 35) were processed and analysed as described [33]. To visualize MCs, sequentially-cut sections were stained with acidic Toluidine Blue (Sigma) 0.1% solution (pH 2.0~2.5). Supplementary methods.

Statistical analyses

Measures of central tendency and dispersions and statistical analyses are indicated in each figure legend and in supplementary methods. P values of <0.05 were considered statistically significant.

RESULTS

Mast cells strongly associate with defined histological features of synovitis and markers of disease activity in patients with early RA

To evaluate the association of MCs with different clinical and histological phenotypes of RA in an unbiased setting, we studied their presence in the synovial membranes of patients with early RA, naïve to treatment with DMARDs. Table 1 summarises the demograhic feaures of the patient cohort, which are as expected for a population with early untreated RA i.e. active disease - mean disease activity score (DAS)-28 5.62, high inflammatory markers - mean ESR 38 mm/h, and approximately 70% auto-antibody positive – rheumatoid factor (RF) or ACPA.

Table 1. Summary of patient characteristics (n=99)

Age, years mean (SD), range	52 (16)	19-89
Sex (% Female)	70.7%	
Disease duration (Months) mean (SD), range	6 (3)	1-12
ACPA+ %	75.8%	
RF+ %	73.7%	
ESR mm/h mean (SD), range	38 (30)	2-120
CRP mg/L mean (SD), range	17 (25)	0-162
DAS-28 mean (SD), range	5.62 (1.41)	1.88-8.92
TJC mean (SD), range	11.33 (7.14)	1-28
SJC mean (SD), range	7.33 (5.88)	1-26
VAS (PGA) mean (SD), range	66.25 (24.57)	0-100
HAQ mean (SD), range	1.51 (0.79)	0-4.2

ACPA: anti-citrullinated protein antibodies; RF: rheumatoid factor: ESR: erythrocyte sedimentation rate; CRP: C reactive protein; DAS-28: disease activity score 28 joints; TJC: tender joint count; SJC: swollen joint count; VAS visual analogic scale; PGA Patient Global disease Activity; HAQ: health assessment questionnaire.

First, we assessed the correlation of MC density with markers of both local (i.e. synovial) and systemic inflammation and disease activity. Figure 1A demonstrates that MC counts significantly correlate with inflammatory markers - erythrocyte sedimentation reate (ESR) and c-reactive protein (CRP), disease activity (DAS-28), and synovial inflammation (Krenn Score). Interestingly, MCs correlated with other immune cells in synovial membranes, with particularly high correlation indexes for B cell) and T cells scores (Spearman r 0.617 and 0.519, respectively, $p<0.001$). Since MC infiltration in synovial specimens was heterogenous, we stratified patients according to the number of MCs into three groups (low, medium and high MC counts), as shown in figure 1B. Consistent with the strong correlation shown in figure 1A, high T and B cell scores were predominant in patients with high MC counts (figure 1C-D). As these data indicate that MCs correlate with synovial inflammation and the degree of lymphocyte infiltration, we evaluated the presence of MCs in three classified forms of synovitis (pathotypes): Lymphoid, Myeloid and Pauci-immune/Fibroid [25]. Interestingly, more than 80% of patients with high MC synovial counts displayed a lymphoid-rich pathotype, characterised by synovial T- and B-cell aggregates; *viceversa*, as expected, the number of MCs was significantly higher in patients with a lymphoid pathotype (figure 1E and 1F). These data indicate that MCs are strongly associated with lymphoid aggregates in the synovia of patients with early RA. Next, we investigated the heterogeneity of synovial MCs. Two types of MCs have been described in humans, expressing tryptase alone (MC_T) or tryptase and chymase (MC_TC), with the following distribution in synovium: predominance of MC_TC in normal synovium[5], expansion of both in RA[7], with relative increase of MC_T described in early [8] and late RA [34]. By performing double immunofluorescence in a subgroup of patients from our early RA cohort (n=15), we found both types of MCs expressed in the synovia, with significantly higher levels of MC_TC (Supplementary Figure S2A), and an average ratio MC_TC:MC_T of 1:3. When patients were stratified according to

pathotypes, we observed a significant increase of both types of MCs in the lymphoid pathotype (Supplementary Figure S2B), with the ratio MC_TC:MT_C changing from 1:6 (fibroid) to 1:2 (lymphoid). Additionally, MC_T, and not MC_TC, showed a significant correlation with synovial inflammation (Supplementary Figure S2C-D). Supplementary Figure S2E shows a representative image with a predominance of MC_T in a patient with lymphoid pathotype. These data suggest an enrichment of tryptase expressing synovial MCs (MC_T) in the lymphoid pathotype in association with the degree of inflammation.

Finally, we assessed the clinical phenotype of patients stratified according to MCs. As shown in table 2, patients with medium and high MC counts have significantly raised ESR and disease activity (DAS28) compared to low MC counts, and patients with high MCs have a significantly higher prevalence of auto-antibody positivity (ACPA and RF) compared to low and medium MCs.. To exclude that the association of MCs with disease severity was exclusively driven by their association with lymphoid cells, we performed additional analyses excluding lymphoid patients, and found that MCs were significantly correlated with ESR (Spearman r=0.272 p=0.007), CRP (r=0.217 p=0.033), and DAS-28 (r=0.308 p=0.002). Overall, this suggests that the stratification of patients according to synovial MCs identifies patients with a severe clinical phenotype.

Table 2. Clinical phenotype of patients stratified according to MC numbers.

		MAST CELLS			
		Low (n=32)	**Medium (n=33)**	**High (n=34)**	
Age mean (SD)	52 (16)	50 (15)	54 (17)	52 (15)	0.300
Female %	70.7%	68.8%	71.9%	72.7%	0.704
ESR mean (SD)	38 (30)	27 (28)	42 (30)	45 (30)	0.033
CRP mean (SD)	17 (25)	10 (29)	21 (23)	18 (24)	0.174
RF+ %	73.7%	68.8%	60.6%	91.2%	0.031
ACPA+ %	75.8%	78.1%	60.6%	88.2%	0.009
DAS28 mean (SD)	5.65(1.41)	4.97 (1.54)	6.05 (1.22)	5.91 (1.24)	0.003

Fisher's exact test or ANOVA, as appropriate

Mast cells interact with T and B cells in follicular structures in RA synovium and tonsil tissue

Having established the presence of MCs in the synovial biopsies from early RA patients with lymphoid-rich sysnovitis, we next investigated the distribution of MCs in the synovia of patients with a lymphoid pathotype. A representative example of the synovial histology of these patients is shown in Figure 2A. Immunohistochemical staining of sequential sections confirmed the presence of CD117+ synovial MCs (figure 2B). By immunofluorescence, we identified MCs bordering lymphoid aggregates, in close contact with B and T cells (figure 2C). A similar distributon was observed in the highly organised secondary lymphoid organs (SLO) from tonsil tissue, used as controls (figure 2D).

Thus, MCs reside on the outer boundary of B and T cell aggregates, and are a histological feature of both synovial ELS, and SLOs.

Mast cells enhance B cells survival, proliferation and differentiation and production of class-switched Ig and ACPA via CD40L

As MCs were found in the proximity of B and T cell aggregates in synovial membranes, and because the activation of B cells toward the production of autoantibodies locally contributes to the pathogenic process in RA[29], we hypothesised that human MCs could influence the activation of B cells. To test this hypothesis, we cultured naïve B cells isolated from tonsils with *in vitro* differentiated human MCs, using the TLR9 ligand CpG to boost B cell activation[35]. MCs enhanced the survival of naïve B cells (figure 3A) with a significant increase in IgG secretion but only minor changes in IgM production (figure 3B). Since CpG *per se* lacks the ability to induce a full differentiation of naïve B cells[36,37], the production of IgG upon co-culture of MCs with naïve B cells suggest that MCs can provide additional signals allowing B cell differentation and the isotype switch toward IgG (figure 3B).

Interestingly, cell contact was not necessary to induce the MC-mediated effect on B cell survival, indicating that soluble factors were sufficient (figure 3C). Additionally, MCs were enhancing the proliferation of CFSE-labelled naïve B cells, and this effect was again not dependent on cell contact (figure 3D). On the contrary, the production of IgG was significantly dependent on cell contact, suggesting that membrane-bound factors were responsible (figure 3E). Similarly, MCs were able to ehnahce the production of RA specific autoantibodies (ACPA) by B cells (figure 3F), an effect again dependent on cell contact. Collectively, these data indicate that MCs can induce the survival, proliferation and differentiation of naïve B cells toward IgG-secreting B cells via indirect and direct cell-cell contact.

Next, we investigated the mechanisms by which MCs promote IgG production by B cells and demonstrated that this is CD40L-dependent (figure 3G), confirming previous reports indicating that murine MCs mediate B cell activation through this cell surface costimulatory molecule[38,39].

To further confirm the ability of MCs to induce the differentiation of naïve B cells toward antibody-producing memory B cells, we analysed B cells by flow cytometry after 7 days of co-culture with MCs. Figure 3H shows that co-culture of naïve B cell with MCs increased the number of antibody-producing memory B cells (CD27+CD38+). Interestingly, this effect could be inhibited by treatment with anti-CD40L in a dose-dependent manner, further confirming the ability of MCs to induce of B cell differentiation *via* CD40L-CD40 interaction (figure 3H).

As cell contact was crucial for the MC-induced differentiation of B cells, we performed phalloidin staining on MCs and B cells after 24h of co-culture, which showed actin re-organisation in the region of contact between MCs and B cells (figure 3I). This suggests an active cellular interaction between MCs and B cells.

Synovial mast cell infiltration occurs early and is associated with ELS and disease severity in antigen-induced arthritis in IL27*ra* deficient mice

Having demonstrated that human MCs enhance B cell activation and differentiation *in vitro* and are associated with synovial ELS in patients with early RA, we wished to investigate *in vivo* the relevance of the interaction between MCs and B cells within ELS in the pathogenesis of arthritis. To this end, we examined MCs synovial infiltration in AIA, a model in which acute inflammatory arthritis is induced by intra-articular injection of methylated bovine serum albumin (mBSA) following systemic immunization with the same antigen. We utilised IL27*ra*-deficient mice, which develop exacerbated synovitis comprising ELS[33].

Figure 4A and B show that MCs were present already in the early phases of AIA (three days after intra-articular injection), with their numbers further increasing at day 10 (d10) and day 35 (d35) post-arthritis induction. Importantly, in IL27*ra*-deficient mice, synovial MC infiltration was significantly higher compared to wild-type littermates at d10 and d35 (figure 4C-D). Moreover, at the peak of inflammation (day 10) synovial MCs showed a positive correlation with arthritis index, synovial infiltrate and the presence of erosions (figure 4E). Finally, in IL27*ra* deficient animals at day 10, there was a trend towards a correlation between MCs and the area of lymphoid aggregates (figure 4F) and MCs were found at the borders of lymphoid aggregates (figure 4G), reflecting the observations in RA patients with a lymphoid pathotype.

Overall, these observations confirm the association of MCs with ectopic lymphoid neogenesis and disease severity, during arthritis induction *in vivo*.

Discussion

In this manuscript, we present the first systematic analysis of MCs in the synovial membrane (SM) of early RA patients obtained by US-guided synovial biopsy prior to therapeutic intervention. Our data demonstrate that synovial MCs are strongly associated with specific pathobiological and clinical phenotypes potentially linked to their ability to induce the activation/differentiation of B cells and the production of ACPA autoantibodies.

Although MCs have long known to be part of the inflammatory infiltrate in RA, their presence in the SM has only been analysed in a few studies, which described increased MC numbers and mediators in the SM and synovial fluid of RA patients, possibly in correlation with disease activity[6–8]. More recently, in a study describing the immunopathologic characteristics of ultrasound-defined synovitis in RA patients in remission, the presence of synovial MCs and B cells at baseline was associated with disease reactivation at follow-up[40]. At the same time, studies *in vitro* and *in vivo* have yielded contradictory results, so that their exact contribution is still unclear[4].

To obtain further insight into the relevance of MCs in RA, we analysed the SM of a large cohort of patients with early (<12 months) RA (n=99), unbiased by treatment and disease duration.

Our data demonstrate high synovial MC counts in patients with a severe clinical phenotype at baseline. Furthermore, MCs correlate with synovial inflammation and, in particular, with ectopic lymphoid structures (ELS), which are found in approximately 40% of RA patients and have been associated with disease severity, T cell priming and autoantibody production, including the local on-going production of class-switched autoantibodies, such as ACPA[29,41]. Interestingly, we found a higher prevalence of tryptase-expressing MCs (MC_T) in the synovia of patients with ELS. Although the concept of MC heterogeneity is

still ill-defined[42], the prevelence of MC_T in association with the lymphoid pathotype would be in line with the pro-inflammatory role of this subpopulation described in asthma[43]. The strong association of MCs with ELS led us to hypothesise that MCs could modulate the local adaptive immune response. The ability of human MCs to influence T cells interaction has already been shown[44,45]. Murine MCs have been shown to activate B cells and promote their differentiation toward effector cells[38,46]. Interestingly, murine MCs were also shown to control, via CD40/CD40L interaction, the expansion and differntiation of IL-10-competent B cells, which is in line with their immunomodulatory functions[39]. The interaction of human MCs and B cells has been poorly studied: in the context of allergic responses, MCs have been shown to express CD40L and induce IgE production by B cells[47,48], while in the context of cancer they have been shown to activate lymphoplasmacytic cells via CD40L[49]. Nonetheless, the interaction between human MCs and B cells has never been studied in the context of autoimmune diseases characterised by local B cell responses.

Here, we show that MCs are at the border of B and T cell aggregates in the synovia of RA patients, similarly to the distribution observed in secondary lymphoid organs. Furthermore, we show that human MCs support the survival, activation, proliferation and differentiation of naive B cells into IgG-producing B cells, in line with recent results in mice [46]. This effect is dependent on cell contact and, specifically, on CD40L-CD40 interaction. Importantly, we demonstrate that MCs induce ACPA production by B cells, also in a contact-dependent manner. Furthermore, we provide the first evidence of an active interaction between human MCs and B cells, as previously shown for MC-T cells[50] and MC-DCs[51]. *In vivo*, we used antigen-induced arthritis (AIA) to assess the timecourse and magnitude of synovial MC infiltration, and IL27*ra* deficient mice as a model of exacerbated synovitis accompanied by ELS formation[33], thus resembling RA patients with a lymphoid pathotype. In both wild-

type and IL27*ra* deficient animals, we observed an early synovial MC infiltration, before the formation of ELS, further increasing at later stages. Whether the early synovial infiltration of MCs means that they are essential to lymphoid neogenesis remains to be established. In fact, in addition to the direct modulation of lymphocyte activation, MCs are also known to produce several mediators[52], including angiogenic and lymphangiogenic factors[53][54], that could support the organization of ELS [55]. Interestingly, MC numbers were significantly higher in IL27*ra* deficient animals, in association with synovial ELS and a worse disease outcome. As IL27*ra* deficiency has been shown to enhance the activation of MCs in the context of Th2 responses [56,57], it will be of interest to further assess the specific role of IL27-mediated MC inhibition in autoimmune diseases.

In conclusion, our study points to the relevance of MCs in RA and their role as novel markers of synovial inflammation. In fact, their presence contributes to the definition of a MC-rich highly inflamed synovial pathotype and helps identifying patients with a severe clinical phenotype.

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Competing interests: None declared

Contributorship: FR: study design, experiments, data acquisition, data analysis, manuscript preparation and revision; DM: study design, experiments, data acquisition, data analysis; LFJ, GJ: experiments, data acquisition, data analysis (animal data); SP, TM: experiments, data acquisition; AN, FH data acquisition, data analysis (clinical data); AR, SR, FK, RT, GS, SJ , FWR, AD, GM: interpretation of experimental results, manuscript revision; CP: study design, interpretation of experimental results, manuscript preparation. FR wrote the manuscript and all authors critically revised its final preparation and approved its submission.

Ethical approval: All procedures were performed following written informed consent and were approved by the hospital's ethics committee (REC 05/Q0703/198).

Data sharing statement: n.a. **FIGURE LEGENDS**

Figure 1 Association of high mast cell counts with synovial inflammation, disease severity and lymphoid aggregates. (A) Correlations of MC numbers with inflammatory markers, disease activity and histological scores. Line at p=0.05. (B) Patients stratified according to mast cell numbers into low (<33rd percentile) medium (33rd-66th percentile) and high (>66th percentile) groups (C-D) Distribution of CD3 (C) and CD20 (D) scores in patients stratified according to MC numbers as in B. (E) Distribution of pathotypes in patients stratified according to MC numbers. (F) MC density in patients stratified according to pathotypes. n=99 *p=0.05, Spearman correlation in A, Chi-Square in C-E, One Way ANOVA with Bonferroni post-hoc test in F.

Figure 2 Mast cells border synovial lymphoid aggregates (A) IHC staining for CD3, CD20, CD138 and CD68 in an US-guided biopsy of a patient with early RA classified as lymphoid pathotype. Semi-quantitative scores for each marker are indicated. (B) IHC staining for CD117 (c-kit) showing synovial MCs. High magnification, on the right, shows synovial MCs (arrows) close to cellular aggregates (C) Immunofluorescence staining of the RA synovia, showing the interactions between CD20 B cells (in green) and CD3+ T cells (in red), forming an ectopic lymphoid aggregate, and CD117+ MCs (in clear blue). The high magnification shows MCs surrounded by B and T cells at the edge of one aggregate. (D) Immunofluorescence of a human tonsil, showing MCs (blue) at the edge of a germinal centre formed by aggregates of B (green) and T cells (red).

Figure 3 Mast cell support survival, proliferation and differentiation of naïve B cells into antibody secreting cells and induce the production of ACPA autoantibodies**.** (A) Proportion of live B cells, measured by FACS staining, after 7 days of culture without (o) or with (■) MCs, at a ratio of 1:6, together with CPG at 1 µg/mL, n=4 (B) IgG and IgM measured by ELISA in the supernatants of naïve B cells harvested after 7 days of culture, n=13 for IgG and 7 for IgM. (C) Proportion of live cells, when naïve B cells were cultured alone or co-cultured with MCs in contact or in transwell, n=3. (D) Proliferation measured by CFSE staining and FACS analysis after 7 days of culture. Representative histograms on the left and cumulative data with division index on the right, n=3. (E) IgG production, naïve B cells cultured alone or co-cultured with mast cells in contact or in transwell, n=3. (F) ACPA measured by CCP2 ELISA upon co-culture ACPA producing B cell clone with MCs in contact or transwell, n=3. (G) IgG production upon inhibition of CD40L in the co-culture of MCs and B cells. n=3 (H) Proportion of antibody-producing cells (CD27+CD38+), after gating on live/$CD117^-$/$CD19^+$.Representative histograms from 3 independent experiments. (I) IF of MCs (light blue) and B cells (red) after 24h of co-culture and CPG triggering. Actin re-organisation is shown in green (phalloidin). Nuclei in blue (DAPI). Measure bar 5µm. Representative image of 3 independent experiments. *=$p<0.05$, Mann-Whitney (A and B), one way ANOVA with Bonferroni post-hoc (C,D, E, H). n= number of independent experiments with n MC donors and n B cell donors)

Figure 4 Mast cells associate with ELS and disease severity in antigen induced arthritis in IL27*ra* deficient mice (A) Representative images of toluidine blue staining showing metachromatic mast cells (arrows) at different time-points during antigen-induced arthritis, with results summarized on in (B); n=>6 for each time-point. (C) Comparison of MC infiltration in wild type (WT) and WSX1 (IL27*ra* knock out) mice, and (D) representative images of toluidine blue staining with metachromatic MCs (red arrows) at d35 time-point. N= >6 /time-point/group. (E) Correlation of synovial MC numbers with arthritis index, synovial infiltrate and erosions at day 10. (F) Correlation of MC number with ELS area in IL27R KO mice at day 10 and (G) representative image. Size bars 100 μm, unless specified. *p<0.05, one way ANOVA with Bonferroni in B, Mann-Whitney comparing WT and WSX1 at each timepoint in C, Spearman correlation in E and F.

Supplementary Figure S1. Panel A shows an example of FACS staining of in vitro cultured MCs, with purity, expressed as percentage of CD117 positive cells, of 99.7% (range from 95 to 99.7%)

Supplementary Figure S2. Immunofluorescence for tryptase and chymase

A Density of MCs expressing tryptase (MC_T) or tryptase and chymase (MC_TC) in synovia. B Density of MC_T and MC_TC in different pathotypes C Correlation between the density of MC_TC and Krenn synovitis score D Correlation between MC_T and Kreen Synovitis Score E Representative example of a patients with a lymphoid pathotype. Blue=DAPI (nuclei); red=tryptase; green=chymase; n= 15, representative image out of 5 patients with lymphoid pathotype in E. Mann-Whitney in A, One-way ANOVA with Bonferroni post-test in B. Spearman in C-D.

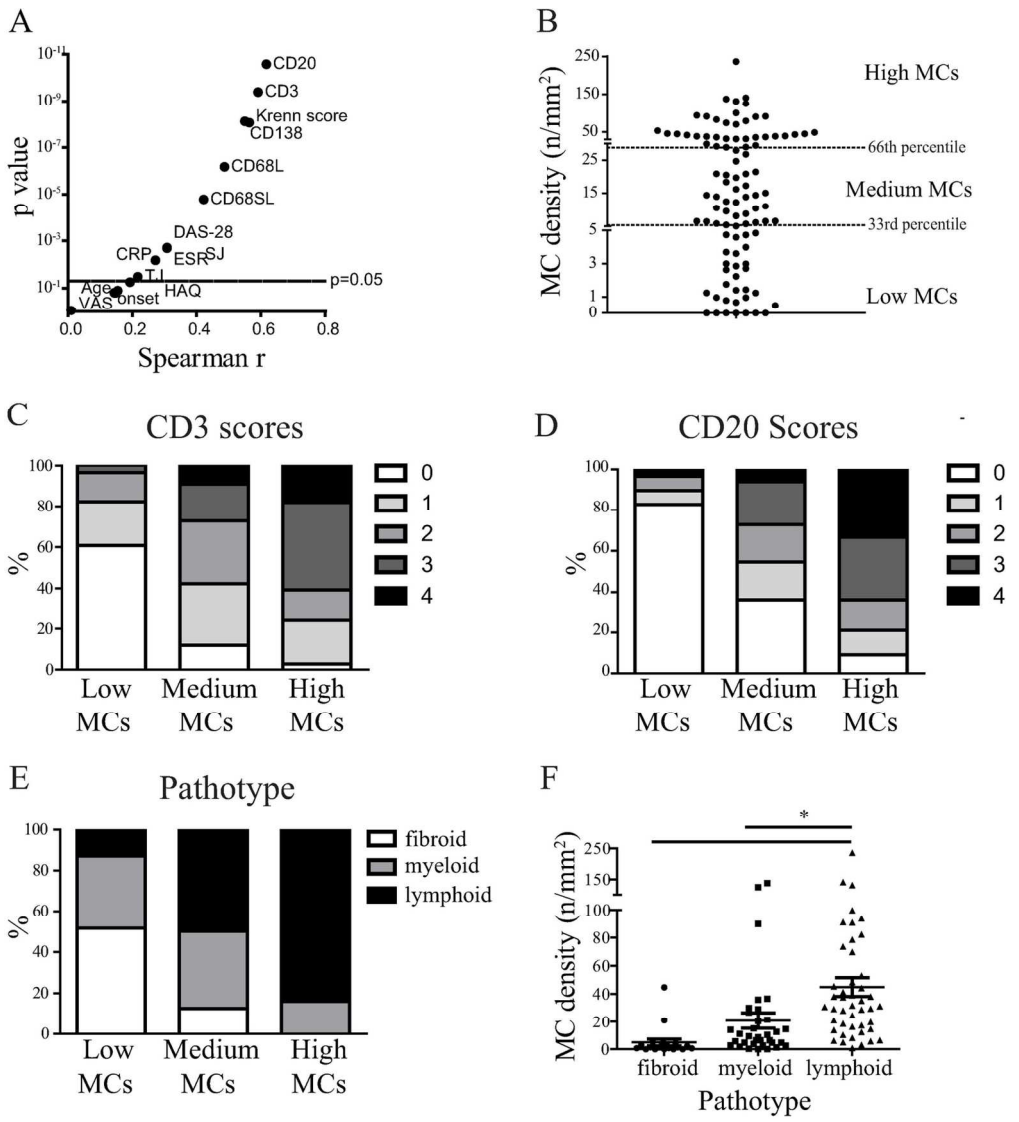

Figure 1 Association of high mast cell counts with synovial inflammation, disease severity and lymphoid aggregates.

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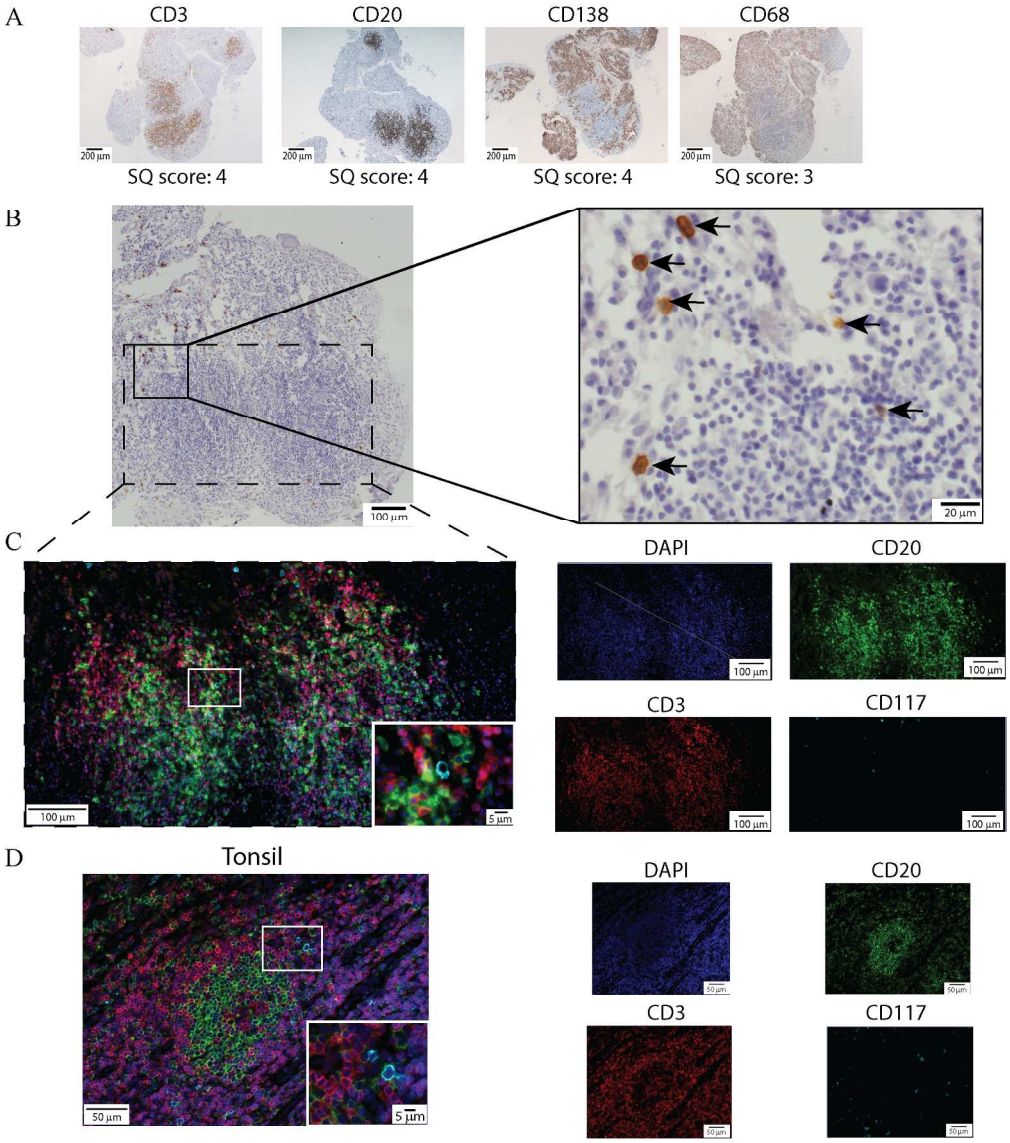


Figure 2 Mast cells border synovial lymphoid aggregates

193x218mm (300 x 300 DPI)

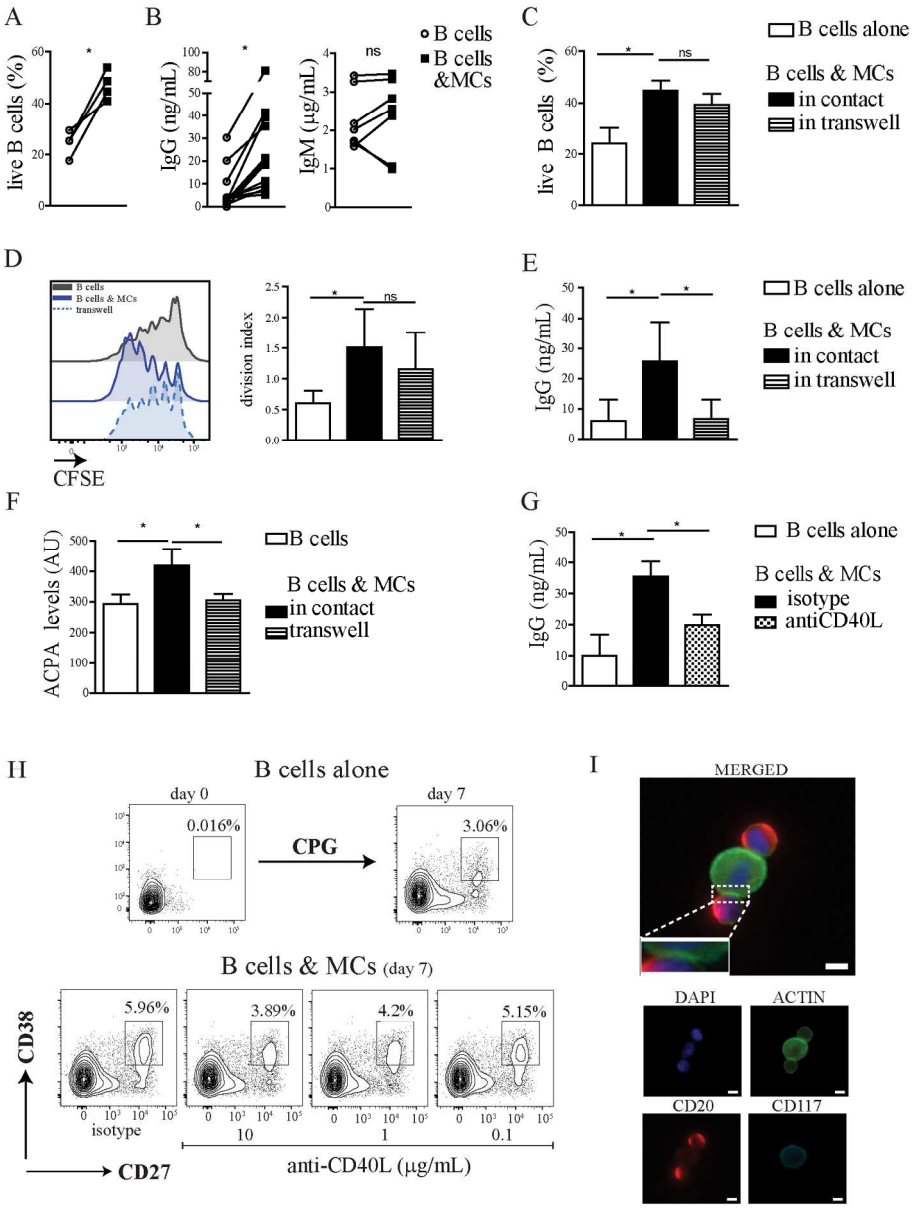


Figure 3 Mast cell support survival, proliferation and differentiation of naïve B cells into antibody secreting cells and induce the production of ACPA autoantibodies.

146x194mm (300 x 300 DPI)

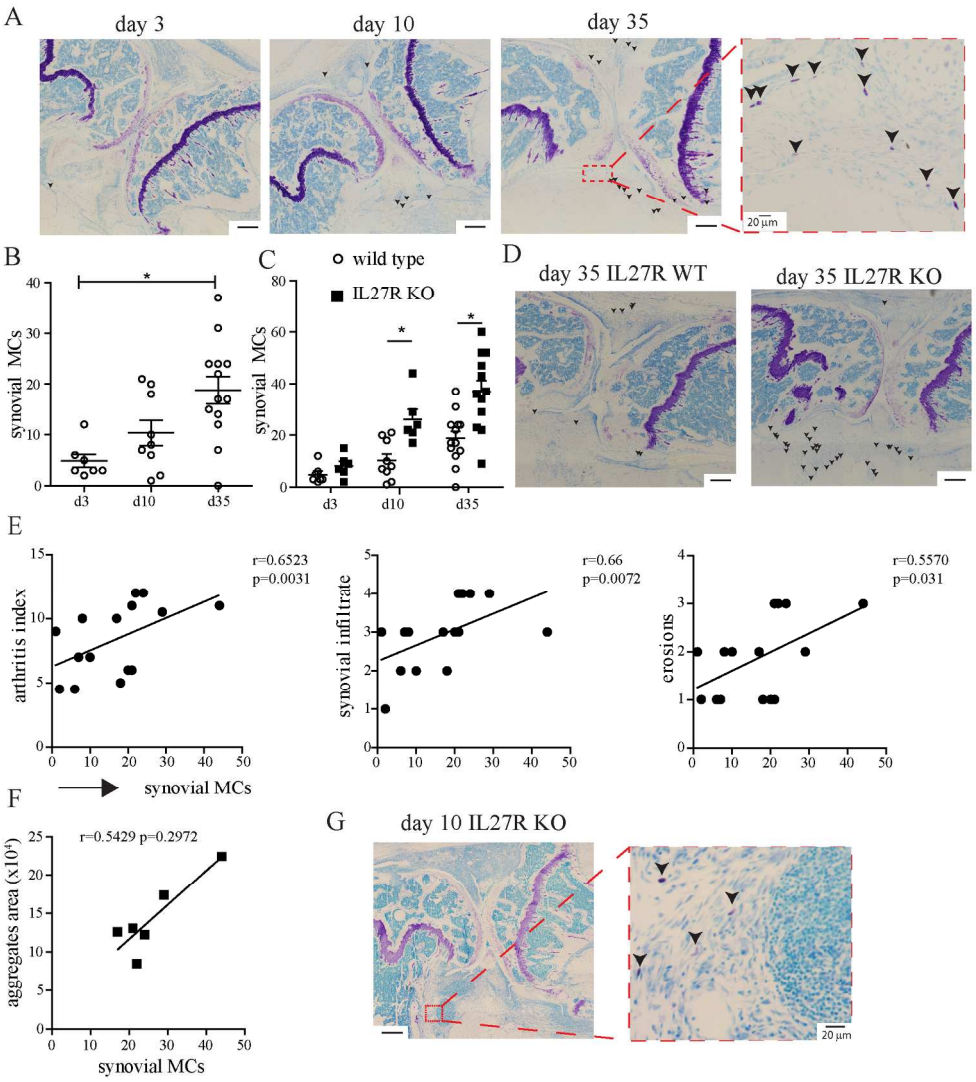
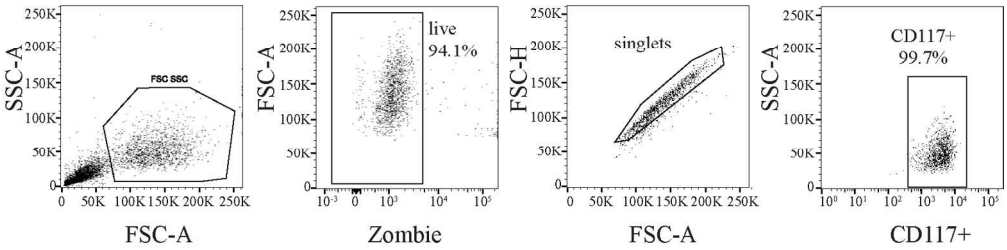


Figure 4 Mast cells associate with ELS and disease severity in antigen induced arthritis in IL27ra deficient mice

184x198mm (300 x 300 DPI)

A



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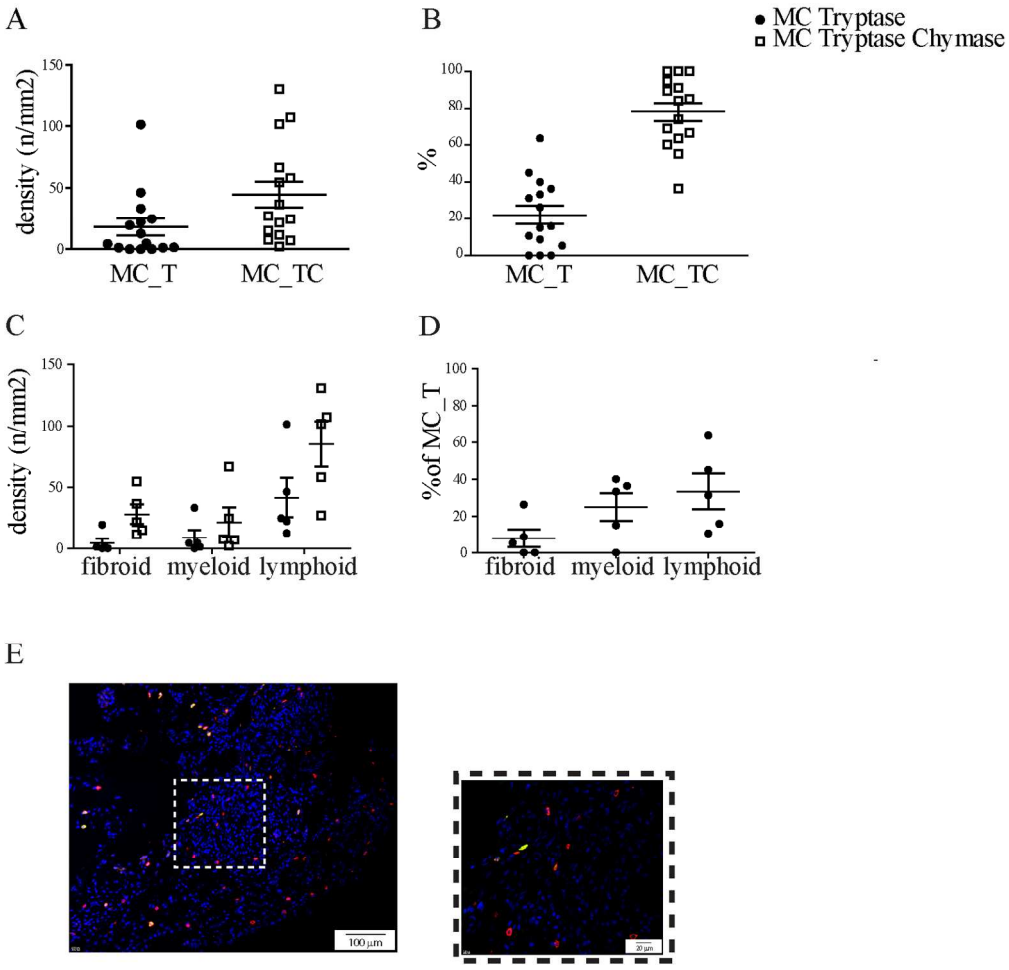

137x132mm (300 x 300 DPI)

SUPPLEMENTARY METHODS

Patient samples and ultrasound-guided synovial biopsy

Synovial tissue was obtained by ultrasound-guided synovial biopsy from DMARD-naïve patients with early (<12 months) RA (n=99), enrolled in the Pathobiology of Early Arthritis Cohort (PEAC) cohort (http://www.peac-mrc.mds.qmul.ac.uk) of the Centre for Experimental Medicine and Rheumatology of Queen Mary University (London), as previously described[26]. All patients fulfilled the 2010 EULAR criteria for RA[27] . Patients had clinically defined synovitis but duration of symptoms of less than 12 months and were all naïve to DMARD and steroid therapy. Upon enrollment and acquisition of demographic and clinical disease parameters, patients underwent ultrasound-guided synovial biopsy of a clinically active joint [26]. All procedures were performed following written informed consent and were approved by the hospital's ethics committee (REC 05/Q0703/198).

Histological analyses of synovial samples

3 μm formalin-fixed and paraffin-embedded (FFPE) synovial sections were deparaffinised in xylene (2x) and rehydrated in 100% ethanol (2x) (Sigma) and underwent standard H&E staining and semi-quantitative (SQ) assessment (0-9) of synovitis according to a previously validated score (Krenn)[28]. Sequentially cut sections, deparaffinized and rehydrated as above, underwent antigen retrieval in citrate buffer (pH 6, DAKO) at 95 °C for 30 min. After washing, cells were stained with the following antibodies (clone) for 1h at room temperature: CD20cy (L26), CD3 (F7.2.38), CD68 (KP1), CD138 (MI15), CD117, c-kit (rabbit polyclonal), all from DAKO. Sections were then incubated with anti-rabbit/mouse secondary antibody (DAKO Envision HRP) for 30 minutes at room temperature and visualised using

DAB (DAKO). Slides were dehydrated with 2x washes each in Xylene and 100% ethanol and mounted. Each slide underwent SQ scoring (0-4), as previously reported[29].

Synovial biopsies were categorized into synovial pathotypes according to the following criteria:

i) Lymphoid (L) CD20≥2 and/or CD138>2

ii) Myeloid (M) CD68SL≥2, CD20≤1 and/or CD3≥1, CD138≤2 and

iii) Fibroid (F) CD68SL<2 and CD3, CD20, CD138<1

To assess the presence of MCs, we performed IHC staining for CD117 (c-kit) (DAKO) and used automated image analysis and counting (Olympus CellSens) to calculate the density of MCs. Patients were classified into high, intermediate and low MC groups (>66th, 33rd-66th, <33rd percentile, respectively).

Immunofluorescence

Sections of FFPE synovial tissue from RA patients with lymphoid pathotype (n=5) and tonsils from routine tonsillectomy (n=3), after deparaffinization, dehydration and antigen retrieval as above, underwent Immunofluorescence (IF) staining for CD3, CD20, CD117 (DAKO) and corresponding secondary antibodies (Invitrogen) Additional sections of FFPE synovial tissue from early RA patients with lymphoid (n=5), myeloid (n=5) and fibroid (n=5) pathotype, after deparaffinization, dehydration and antigen retrieval as above, underwent Immunofluorescence (IF) staining for tryptase and chymase and corresponding secondary antibodies (Invitrogen). Images were visualised using an Olympus microscope and CellSens software (Olympus).

Peripheral blood-derived mast cells

CD34^{+} hematopoietic stem cells (StemPro® CD34+ kit, ThermoFisher) were differentiated into MCs as previously described[30]. Briefly, stem cells were cultured in StemPro medium and supplement (ThermoFischer) with 100 ng/mL of recombinant human IL-6 and SCF (Peprotech) and, for the first week, 5 ng/mL of IL-3 (Peprotech). Emi-depletion was performed weekly. After 6-8 weeks, the purity of mast cells was determined by flow cytometry analyses of CD117 (c-kit) and FcεRI and ranged from 90 to 99% (Supplementary Figure 1).

Naïve B cell isolation and co-culture with MCs

Tonsils were obtained from Barts Health NHS Trust Human Tissue Resource Centre during routine tonsillectomy, following written informed consent (Biobank LREC 07/Q0605/29). Tonsil single cell suspension were obtained by mechanical distructon through a cell strainer and naïve B cells (IgD+) were from isolated by immunomagnetic sorting following the the manufacturer's instructions (Miltenyi). Tonsil IgD+ B cells (purity > 95%) were cultured in IMDM 10%FCS at a density of 1x106/mL, alone, or together with mast cells at a ratio of 1:6 (MC:B cells), in the presence of TLR-9 ligand (1 μg/mL of CpG ODN-2009, Invitrogen) for 7 days. MCs were in direct contact with B cells or separated by a Transwell© membrane. In parallel experiments, B cells were marked with CFSE (Biolegend) to measure proliferation. Where indicated, mouse anti-human CD154 (CD40L) or corresponding isotype control (Biolegend) were added to the co-culture at a concentration of 0.1-10 μg/mL.

Visualization of MC-B cell interacton

Human B cells (CD19+) were immunomagnetically selected from Buffy coat of healthy blood donors (NHSBT, National Blood Service, UK) using CD19+ magnetic beads, according to the manufacturer's instructions (Miltenyi). B cells were cultured with human

MCs at a ratio of 1:1. After 24 h, cells were harvested, washed in PBS and stained with anti-CD117 APC (Miltenyi), anti-CD19 PE (R&D) for 20min at 4°C. Cells were washed, fixed in PFH 4% for 10 minutes at room temperatures, then incubated with CytoPainter Phalloidin-iFluor 488 Reagent (Abcam) diluted 1:1000 in PBS/1%BSA for 30 min at RT. After washing, cells were transfererd on slide by cytospin and visualized using an Olympus microscope and CellSens Software.

ACPA B cell clone and co-culture with MCs

ACPA-producing immortalized B-cells (2 x 105) were obtained as described (Germar, K et al. Manuscript submitted and[31]) and cultured on irradiated mouse L cell fibroblast stably expressing CD40L ($5x10^4$) in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo) supplemented with P/S (Thermo), 8% FCS (Gibco) and IL-21 (25 ng/ml). B-cells were passaged twice a week until used for the coculture experiment. For coculture experiments, B-cells were cultured with mast cells at a ration of 1:6 for 7 days.

Flow cytometry

Flow cytometry staining was performed as previously described[32]. Zombie NIR (Biolegend) was used to exclude dead cells and assess the proportion of live B cells, and the antibodies are listed in the supplementary materials. Cells were acquired on LSR Fortessa (BD) and the results were analysed using FlowJo software (v X.0.7, Tree Star Inc.). The following antibodies were used: FcεRI FITC (MAR-1), CD27 APC (0323), CD20 PerCPCy5.5 (2H7) from eBioscience; CD19 BV510 (HIB19), CD38 BV711 (HIT2), and IgD PE (IA6-2) from Biolegend, CD117 APC (A3C6E2) from Miltenyi.

Measurement of immunoglobulins

IgG and IgM were measured using the IgG and IgM ELISA Kits (Bethyl), according to the manufacturer instructions. For the measurement of ACPA, anti-CCP2-IgG was measured by ELISA (Immunoscan-RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands).

Antigen induced arthritis in wild-type and IL-27R KO

AIA was induced in adult (8–12 wk) Il-27ra-/- mice and age/sex-matched WT as previously described[33]. FFPE 5 μm sections of synovia from animals culled at different intervals (days 3, 10 and 35) were processed, stained and analysed as described in [29]. Sequentially-cut sections were deparaffinized and stained in acidic Toluidine Blue (Sigma) 0.1% solution (pH 2.0~2.5) for 2 min, then washed, dehydrated 2x with 100% ethanol, cleared 2x with xylene and mounted. Slides were acquired using an Olympus microscope with CellSens Software (Olympus) and metachromatic MCs in synovia were counted by two blind independent observers (FR, DM) and expressed as the mean of two observations for each sample. Interobserver agreement was evaluated using the intraclass correlation coefficient (with a cutoff value of .0.7 to indicate acceptable agreement).

Statistical analyses

Measures of central tendency and dispersions and statistical analyses are indicated in each figure legend. The following statistical tests were used: Mann-Whitney for comparison between two groups; one-way ANOVA with Bonferroni's post-hoc for comparison between multiple groups; Chi-Square for proportions; Spearman for correlations. Analyses were performed using IBM SPSS v23 (IBM) for clinical datasets and Graphpad Prism v5 for experimental datasets. P values of <0.05 were considered statistically significant.

Reviewer: 2

Comments to the Author

The authors have addressed most comments from the reviewers and included new data on mast cell subsets that have increased the value of the current mansucript. However, the potential causality between mast cells and ELS formation is not addressed properly. The authors state that the background of the mice that could be used for studying development of ELS (chronic model of antigen-induced arthritis) is incompatible with the genetic background of mast cell-deficient mice. This may be true, but there are other ways to deplete mast cells in vivo, for instance by administration of depleting antibodies (i.e. anti–c-kit mAb) or via CRISPR/Cas.

Therefore, I would strongly urge the authors to perform these studies to enhance their data.

We would like to thank the Reviewer for his additional comments. We do agree the manuscript has improved significantly with the addition of data on MC subsets, an analysis performed following a relevant suggestion from the Reviewer, for which we are extremely thankful.

As for the potential causality between mast cells and ELS formation, we do agree this is an important point. To overcome the issues mentioned in our previous reply the Reviewer suggests trying alternative approaches. Although we appreciate this feedback, we feel that the use of anti-C-kit antibodies would lead to inconclusive results, as it would deplete all hematopoietic progenitors expressing C-kit and NOT exclusively MC. In fact, c-kit-depletion has been explored as a bone marrow pre-transplant conditioning strategy (Blood 2004 104:4963). As for the use of CRISPR/Car technology, we are sure the Reviewer appreciates the complexity and time required to create new animal strains de novo, with delaying of publication of the human observations by a year or more.

In any case, even if we were able to get a different MC-deficient strain from collaborators, the results of such experiments would not be necessarily transferable to the human disease (the focus of our manuscript). In addition, while we agree that confirmation in animal models would be important to show causality in that system, our manuscript does not claim we demonstrate **causality** but only that MCs are **associated** with ELS, as without spontaneous gene mutations in humans is almost impossible to demonstrate causality.

Nonetheless, we provide data at different levels to indicate that MCs are not simple bystanders in the pathogenesis of RA, including the presence of MC in the disease tissue of a large cohort of patients with early rheumatoid arthritis prior to therapeutic intervention, their functional ability to activate / differentiate B cells leading to auto-antibody production and their association with disease severity.

Accordingly, following the helpful Reviewer's comments, we have modified the title omitting the association with *"lymphoid aggregates"* and inserting the association with *"disease severity"* (see also below) as follows: "Mast cells in early rheumatoid arthritis associate with disease severity and support B-cell autoantibody production".

We hope that Reviewer will agree that by deleting the association with lymphoid aggregates in the title we avoid possible misinterpretation on causality between MC and ELS formation.

In their response 1.1. the authors conclude that synovial mast cells associate with disease severity, independently of their association with lymphoid aggregates. Therefore, the title of the manuscript should be changed (i.e. incorporate disease activity) and this information should also be included in the abstract and discussion.

We thank the Reviewer for this comment. We do agree that mentioning *"disease severity"* in the title (see above) provides a more balanced reflection of the clinical and immunological data presented in the manuscript. Following the Reviewer' suggestion, we have also specifically mentioned this new analysis in the results (page 12 "To exclude that the association of MCs with disease severity was exclusively driven by their association with lymphoid cells, we performed additional analyses excluding lymphoid patients, and found that MCs were significantly correlated with ESR (Spearman r=0.272 p=0.007), CRP (r=0.217 p=0.033), and DAS-28 (r=0.308 p=0.002). Overall, this suggests that the stratification of patients according to synovial MCs identifies patients with a severe clinical phenotype"). The association of MCs with disease activity has been already highlighted in the abstract results ("High synovial MC counts are associated with local and systemic inflammation, autoantibody positivity, and high disease activity"), and manuscript conclusions ("… their presence contributes to the definition of a MC-rich highly inflamed synovial pathotype and helps identifying patients with a severe clinical phenotype"). We have now stressed the correlation of MCs with disease severity also in the abstract conclusions: "Synovial MCs identify early RA patients with a severe clinical form of synovitis characterised by the presence of ELS".

In their response 2, the authors state that they have included a sentence on mast cells and IL-10 competent B cells (p.18). However, they should also include the essential information that the interaction between these cells is also mediated via CD40/CD40L interaction which consequently may also have a regulatory function.

We have now specified this: "Interestingly, MCs were also shown to control, via CD40/CD40L interaction, the expansion and differentiation of IL-10-competent B cells, which is in line with their immunomodulatory functions".

In their response 3, the authors do not reference the Mion paper that they mentioned in their response 2.

We mention the previous work by Merluzzi et al 2010, from the same group. Both papers were already cited in our manuscript. Furthermore, it is important to emphasise that in both papers the functional experiments were performed in animals, NOT humans.

In addition, these authorsy overlook quite a few papers that either demonstrate human B cell activation by mast cells (1: Ryzhov S, Goldstein AE, Matafonov A, Zeng D, Biaggioni I, Feoktistov I. Adenosine-activated mast cells induce IgE synthesis by B lymphocytes: an A2B-mediated process involving Th2 cytokines IL-4 and IL-13 with implications for asthma. J Immunol. 2004 Jun 15;172(12):7726-33. PubMed PMID: 15187156.
2: Pawankar R, Okuda M, Yssel H, Okumura K, Ra C. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. J Clin Invest. 1997 Apr 1;99(7):1492-9. PubMed PMID: 9119992; PubMed Central PMCID: PMC507968.) or even highlight the importance of CD40/CD40L in this process (1: Tournilhac O, Santos DD, Xu L, Kutok J, Tai YT, Le Gouill S, Catley L, Hunter
Z, Branagan AR, Boyce JA, Munshi N, Anderson KC, Treon SP. Mast cells in Waldenstrom's macroglobulinemia support lymphoplasmacytic cell growth through CD154/CD40 signaling. Ann Oncol. 2006 Aug;17(8):1275-82. Epub 2006 Jun 20. PubMed
PMID: 16788002.
2: Gauchat JF, Henchoz S, Mazzei G, Aubry JP, Brunner T, Blasey H, Life P, Talabot D, Flores-Romo L, Thompson J, et al. Induction of human IgE synthesis in B cells by mast cells and basophils. Nature. 1993 Sep 23;365(6444):340-3. PubMed PMID: 7690905.). These papers should definitely be referenced.

We do agree with the Reviewer that there is additional literature on this specific topic that would have been worth citing. However, we had to consider the space limitations for ARD both in terms of total word counts (3000 words) and total citations (suggested limit of 50). Therefore, we had to select the most recent and relevant literature. For example, we had to leave out many significant manuscripts in the field of allergy, since we are already citing several recent and comprehensive reviews on mast cells.
As for the specific articles mentioned, the articles by Ryzhov et al used mast cell lines (e.g. HMC-1), which have been shown to be poorly representative of primary mast cells.
We agree with the reviewer that it is appropriate to cite the other articles mentioned, as they deal with the functional role of CD40L. We have now cited these manuscripts (page 18).

In their response 5, the authors do not respond satisfactorily to the question of the reviewer. Blocking the CD40/CD40L axis with blocking antibodies in the in vitro experiments with mast cells and ACPA+ B cell clones is quite straightforward and should not take a lot of time. Therefore, I persist in my request to perform these studies as in my opinion they will substantially increase the value of the manuscript.

While we agree with the Reviewer that these experiments would be technically straightforward, they would be logistically challenging, as performed by external collaborators (the Leiden group), who currently do not have dedicated resources to carry out these time consuming experiments (MC differentiation takes up to 8 weeks).

Importantly, we feel that these experiments would not change dramatically the impact of the paper, as this already shows, for the first time, that human MC can induce the differentiation of **naïve B cells** into IgG producing B cells via CD40L and the production of ACPA in a contact-dependent manner. Nonetheless, to be on the caution side, we have now removed the mention on the possible role of CD40L expressed by MCs in the induction of ACPA by B cells (page 18, line 21).

TITLE PAGE

Mast cells in early rheumatoid arthritis associate with ~~synovial lymphoid aggregates~~disease severity and support B-cell autoantibody production

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ABSTRACT

Objectives Mast cells (MCs) are involved in the pathogenesis of rheumatoid arthritis (RA). However, their contribution remains controversial. To establish their role in RA, we analysed their presence in the synovium of treatment-naïve early RA patients and their association and functional relationship with histological features of synovitis.

Methods Synovial tissue was obtained by ultrasound-guided biopsy from treatment-naïve patients with early RA (n=99). Immune cells (CD3/CD20/CD138/CD68) and their relationship with $CD117^{+}$ MCs in synovial tissue were analysed by immunohistochemistry (IHC) and immunofluorescence (IF). The functional involvement of MCs in ectopic lymphoid structures (ELS) was investigated *in vitro*, by co-culturing MCs with naïve B cells and anti-citrullinated protein antibodies (ACPA)-producing B cell clones, and *in vivo* in interleukin-27 receptor (IL27*ra*)-deficient and control mice during antigen-induced arthritis (AIA).

Results High synovial MC counts are associated with local and systemic inflammation, autoantibody positivity, and high disease activity. IHC/IF showed that MCs reside at the outer border of lymphoid aggregates. Furthermore, human MCs promote the activation and differentiation of naïve B cells, and induce the production of ACPA, mainly via contact-dependent interactions. In AIA, synovial MC numbers increase in IL27*ra* deficient mice, in association with ELS and worse disease activity.

Conclusions Synovial MCs identify ~~are closely associated with the presence of synovial ELS in early RA patients where they contribute to B cell activation and the development of local inflammatory and autoimmune responses. Thus, high numbers of synovial MCs in~~ early RA

patients with ~~identify~~ a severe clinical form of synovitis ~~defined~~ characterised by the presence of ELS.

Key words: Early Rheumatoid Arthritis, Synovitis, B cells, Anti-CCP

INTRODUCTION

Mast cells (MCs) are tissue-resident cells of the innate immunity, involved in several physiological and pathological processes, including infections, cancer and chronic inflammatory diseases[1,2]. They are present in the synovial membrane (SM) and have been implicated in contributing to the inflammatory response in several rheumatic diseases[3], including rheumatoid arthritis (RA)[4]. Notably, MCs are present in healthy synovia[5], but their number significantly increases accompanying the cellular hyperplasia characteristic of RA synovitis[6–8]. Many MC mediators have direct pro-arthritogenic effects[9–12], and MCs can be activated by several stimuli present in the synovium/synovial fluid, such as anti-citrullinated protein antibodies (ACPA) IgG immune complexes[13]. On the other hand, recent evidences suggest that MC contribution to autoimmune diseases can be complex and multifaceted[14]. In the context of RA, for example, human MCs have been shown to exert immunomodulatory functions *in vitro*[15]. In vivo, initial findings were contrasting[16–18], most likely because of the use of animal models in which MC depletion was accompanied by anomalies of other immune cells[19]. In recent years, thanks to the development of new specific models of MC-depletion[20], their contribution has been confirmed to be essential in collagen-induced arthritis (CIA) but redundant in serum-transfer arthritis[21]. Additionally, their depletion in the pre-clinical phases of CIA, rather than in the established phases, was shown to influence the disease outcome[22]. These evidences *in vivo* suggest that MC contribution to RA may be different in various disease stages, i.e. essential during the early phases (assessed by CIA), but somehow dispensable during the late effector phases (serum transfer). However, while these models are self-resolving, in RA there is chronic inflammation with a perpetuation of the aberrant autoimmune response; therefore, the results cannot be easily translated to the clinical setting. Overall, despite the substantial amount of

data, the role of MCs in RA remains to be clarified [4]. As RA is well-recognized as an heterogeneous syndrome in terms of genetic predisposition, pathogenesis, clinical[23,24], and histological[25] features, it could be hypothesised that MC presence and functions in the synovium may be different in various disease subsets. To explore this hypothesis, we systematically analysed the presence of MCs in the synovia of a large cohort of disease-modifying anti-rheumatic drugs (DMARD)-naïve early RA patients. Furthermore, we assessed their interactions with immune cells at synovial level and analysed *in vitro* the crosstalk of MCs with B cells. Finally, we evaluated the relationship between MC synovial infiltration and ectopic lymphoid structures (ELS) in an experimental model of synovial ectopic lymphoid neogenesis.

Methods

Patient samples and ultrasound-guided synovial biopsy

Synovial tissue was obtained by ultrasound-guided synovial biopsy from DMARD-naïve patients with early (<12 months) RA (n=99), enrolled in the Pathobiology of Early Arthritis Cohort (PEAC) cohort of the Centre for Experimental Medicine and Rheumatology of Queen Mary University (London), as previously described[26]. All patients fulfilled the 2010 EULAR criteria for RA[27]. All procedures were performed following written informed consent and were approved by the hospital's ethics committee (REC 05/Q0703/198).

Histological analyses of synovial samples

Synovial sections underwent standard H&E staining and semi-quantitative (SQ) assessment of synovitis according to a previously validated score (Krenn) [28]. Sequentially cut sections underwent Immunohistochemical (IHC) staining and SQ assessment (0-4) for immune cells, as previously reported[29] and automated image analysis and counting for CD117+ve MCs. Patients were classified into high, intermediate and low MC groups (>66th, 33rd-66th, <33rd percentiles, respectively). Supplementary methods.

Peripheral blood-derived MCs

$CD34^+$ hematopoietic stem cells (StemPro® CD34+ kit, ThermoFisher) were differentiated into MCs as previously described[30]. Supplementary methods and Supplementary Figure 1 for MC purity.

Naïve B cell isolation and co-culture with MCs

IgD^+ B cells isolated by immunomagnetic sorting (Miltenyi) from tonsil mononuclear cells were cultured for 7 days alone or together with MCs, in the presence of TLR-9 ligand (CpG ODN-2006, Invitrogen), in contact or separated by a Transwell© membrane. In parallel experiments, B cells were marked with CFSE (Biolegend) to measure proliferation. Where indicated, mouse anti-human CD154 (CD40L) or isotype control (Biolegend) were added at a concentration of 0.1-10 μg/mL. Supplementary methods.

Visualization of MC-B cell interaction

Supplementary methods.

ACPA B cell clone and co-culture with MCs

ACPA-producing immortalized B-cells (2×10^5) were obtained as described (Germar, K et al. Manuscript submitted and[31]) and cultured as indicated in supplementary methods.

Flow cytometry

Flow cytometry staining was performed as previously described[32]. Supplemetary methods

Measurement of immunoglobulins

IgG and IgM were measured using the IgG and IgM ELISA Kits (Bethyl), according to the manufacturer instructions. For the measurement of ACPA, anti-CCP2-IgG was measured by ELISA (Immunoscan-RA Mark 2; Eurodiagnostica).

Antigen induced arthritis(AIA) in wild-type and IL27*ra* KO

AIA was induced in adult (8–12 wk) IL27*ra*-/- mice and age/sex-matched WT as previously described [33]. 5 μm sections of synovia from animals culled at different intervals (days 3, 10 and 35) were processed and analysed as described [33]. To visualize MCs, sequentially-cut sections were stained with acidic Toluidine Blue (Sigma) 0.1% solution (pH 2.0~2.5). Supplementary methods.

Statistical analyses

Measures of central tendency and dispersions and statistical analyses are indicated in each figure legend and in supplementary methods. P values of <0.05 were considered statistically significant.

RESULTS

Mast cells strongly associate with defined histological features of synovitis and markers of disease activity in patients with early RA

To evaluate the association of MCs with different clinical and histological phenotypes of RA in an unbiased setting, we studied their presence in the synovial membranes of patients with early RA, naïve to treatment with DMARDs. Table 1 summarises the demograhic feaures of the patient cohort, which are as expected for a population with early untreated RA i.e. active disease - mean disease activity score (DAS)-28 5.62, high inflammatory markers - mean ESR 38 mm/h, and approximately 70% auto-antibody positive – rheumatoid factor (RF) or ACPA.

Table 1. Summary of patient characteristics (n=99)

Age, years mean (SD), range	52 (16)	19-89
Sex (% Female)	70.7%	
Disease duration (Months) mean (SD), range	6 (3)	1-12
ACPA+ %	75.8%	
RF+ %	73.7%	
ESR mm/h mean (SD), range	38 (30)	2-120
CRP mg/L mean (SD), range	17 (25)	0-162
DAS-28 mean (SD), range	5.62 (1.41)	1.88-8.92
TJC mean (SD), range	11.33 (7.14)	1-28
SJC mean (SD), range	7.33 (5.88)	1-26
VAS (PGA) mean (SD), range	66.25 (24.57)	0-100
HAQ mean (SD), range	1.51 (0.79)	0-4.2

ACPA: anti-citrullinated protein antibodies; RF: rheumatoid factor: ESR: erythrocyte sedimentation rate; CRP: C reactive protein; DAS-28: disease activity score 28 joints; TJC: tender joint count; SJC: swollen joint count; VAS visual analogic scale; PGA Patient Global disease Activity; HAQ: health assessment questionnaire.

First, we assessed the correlation of MC density with markers of both local (i.e. synovial) and systemic inflammation and disease activity. Figure 1A demonstrates that MC counts significantly correlate with inflammatory markers - erythrocyte sedimentation reate (ESR) and c-reactive protein (CRP), disease activity (DAS-28), and synovial inflammation (Krenn Score). Interestingly, MCs correlated with other immune cells in synovial membranes, with particularly high correlation indexes for B cell) and T cells scores (Spearman r 0.617 and 0.519, respectively, $p<0.001$). Since MC infiltration in synovial specimens was heterogenous, we stratified patients according to the number of MCs into three groups (low, medium and high MC counts), as shown in figure 1B. Consistent with the strong correlation shown in figure 1A, high T and B cell scores were predominant in patients with high MC counts (figure 1C-D). As these data indicate that MCs correlate with synovial inflammation and the degree of lymphocyte infiltration, we evaluated the presence of MCs in three classified forms of synovitis (pathotypes): Lymphoid, Myeloid and Pauci-immune/Fibroid [25]. Interestingly, more than 80% of patients with high MC synovial counts displayed a lymphoid-rich pathotype, characterised by synovial T- and B-cell aggregates; *viceversa*, as expected, the number of MCs was significantly higher in patients with a lymphoid pathotype (figure 1E and 1F). These data indicate that MCs are strongly associated with lymphoid aggregates in the synovia of patients with early RA. Next, we investigated the heterogeneity of synovial MCs. Two types of MCs have been described in humans, expressing tryptase alone (MC_T) or tryptase and chymase (MC_TC), with the following distribution in synovium: predominance of MC_TC in normal synovium[5], expansion of both in RA[7], with relative incresase of MC_T described in early [8] and late RA [34]. By performing double immunofluorescence in a subgroup of patients from our early RA cohort (n=15), we found both types of MCs expressed in the synovia, with significantly higher levels of MC_TC (Supplementary Figure S2A), and an average ratio MC_TC:MC_T of 1:3. When patients were stratified according to

pathotypes, we observed a significant increase of both types of MCs in the lymphoid pathotype (Supplementary Figure S2B), with the ratio MC_TC:MT_C changing from 1:6 (fibroid) to 1:2 (lymphoid). Additionally, MC_T, and not MC_TC, showed a significant correlation with synovial inflammation (Supplementary Figure S2C-D). Supplementary Figure S2E shows a representative image with a predominance of MC_T in a patient with lymphoid pathotype. These data suggest an enrichment of tryptase expressing synovial MCs (MC_T) in the lymphoid pathotype in association with the degree of inflammation.

Finally, we assessed the clinical phenotype of patients stratified according to MCs. As shown in table 2, patients with medium and high MC counts have significantly raised ESR and disease activity (DAS28) compared to low MC counts, and patients with high MCs have a significantly higher prevalence of auto-antibody positivity (ACPA and RF) compared to low and medium MCs. ~~Overall, this suggests that the stratification of patients according to synovial MCs identifies patients with a severe clinical phenotype~~. To exclude that the association of MCs with disease severity was exclusively driven by their association with lymphoid cells, we performed additional analyses excluding lymphoid patients, and found that MCs were significantly correlated with ESR (Spearman r=0.272 p=0.007), CRP (r=0.217 p=0.033), and DAS-28 (r=0.308 p=0.002). Overall, this suggests that the stratification of patients according to synovial MCs identifies patients with a severe clinical phenotype.

Table 2. Clinical phenotype of patients stratified according to MC numbers.

		MAST CELLS			
		Low (n=32)	**Medium (n=33)**	**High (n=34)**	
Age mean (SD)	52 (16)	50 (15)	54 (17)	52 (15)	0.300
Female %	70.7%	68.8%	71.9%	72.7%	0.704
ESR mean (SD)	38 (30)	27 (28)	42 (30)	45 (30)	0.033
CRP mean (SD)	17 (25)	10 (29)	21 (23)	18 (24)	0.174
RF+ %	73.7%	68.8%	60.6%	91.2%	0.031
ACPA+ %	75.8%	78.1%	60.6%	88.2%	0.009
DAS28 mean (SD)	5.65(1.41)	4.97 (1.54)	6.05 (1.22)	5.91 (1.24)	0.003

Fisher's exact test or ANOVA, as appropriate

Mast cells interact with T and B cells in follicular structures in RA synovium and tonsil tissue

Having established the presence of MCs in the synovial biopsies from early RA patients with lymphoid-rich sysnovitis, we next investigated the distribution of MCs in the synovia of patients with a lymphoid pathotype. A representative example of the synovial histology of these patients is shown in Figure 2A. Immunohistochemical staining of sequential sections confirmed the presence of CD117+ synovial MCs (figure 2B). By immunofluorescence, we identified MCs bordering lymphoid aggregates, in close contact with B and T cells (figure 2C). A similar distributon was observed in the highly organised secondary lymphoid organs (SLO) from tonsil tissue, used as controls (figure 2D).

Thus, MCs reside on the outer boundary of B and T cell aggregates, and are a histological feature of both synovial ELS, and SLOs.

Mast cells enhance B cells survival, proliferation and differentiation and production of class-switched Ig and ACPA via CD40L

As MCs were found in the proximity of B and T cell aggregates in synovial membranes, and because the activation of B cells toward the production of autoantibodies locally contributes to the pathogenic process in RA[29], we hypothesised that human MCs could influence the activation of B cells. To test this hypothesis, we cultured naïve B cells isolated from tonsils with *in vitro* differentiated human MCs, using the TLR9 ligand CpG to boost B cell activation[35]. MCs enhanced the survival of naïve B cells (figure 3A) with a significant increase in IgG secretion but only minor changes in IgM production (figure 3B). Since CpG *per se* lacks the ability to induce a full differentiation of naïve B cells[36,37], the production of IgG upon co-culture of MCs with naïve B cells suggest that MCs can provide additional signals allowing B cell differentation and the isotype switch toward IgG (figure 3B).

Interestingly, cell contact was not necessary to induce the MC-mediated effect on B cell survival, indicating that soluble factors were sufficient (figure 3C). Additionally, MCs were enhancing the proliferation of CFSE-labelled naïve B cells, and this effect was again not dependent on cell contact (figure 3D). On the contrary, the production of IgG was significantly dependent on cell contact, suggesting that membrane-bound factors were responsible (figure 3E). Similarly, MCs were able to ehnahce the production of RA specific autoantibodies (ACPA) by B cells (figure 3F), an effect again dependent on cell contact. Collectively, these data indicate that MCs can induce the survival, proliferation and differentiation of naïve B cells toward IgG-secreting B cells via indirect and direct cell-cell contact.

Next, we investigated the mechanisms by which MCs promote IgG production by B cells and demonstrated that this is CD40L-dependent (figure 3G), confirming previous reports indicating that murine MCs mediate B cell activation through this cell surface costimulatory molecule[38,39].

To further confirm the ability of MCs to induce the differentiation of naïve B cells toward antibody-producing memory B cells, we analysed B cells by flow cytometry after 7 days of co-culture with MCs. Figure 3H shows that co-culture of naïve B cell with MCs increased the number of antibody-producing memory B cells (CD27+CD38+). Interestingly, this effect could be inhibited by treatment with anti-CD40L in a dose-dependent manner, further confirming the ability of MCs to induce of B cell differentiation *via* CD40L-CD40 interaction (figure 3H).

As cell contact was crucial for the MC-induced differentiation of B cells, we performed phalloidin staining on MCs and B cells after 24h of co-culture, which showed actin re-organisation in the region of contact between MCs and B cells (figure 3I). This suggests an active cellular interaction between MCs and B cells.

Synovial mast cell infiltration occurs early and is associated with ELS and disease severity in antigen-induced arthritis in IL27*ra* deficient mice

Having demonstrated that human MCs enhance B cell activation and differentiation *in vitro* and are associated with synovial ELS in patients with early RA, we wished to investigate *in vivo* the relevance of the interaction between MCs and B cells within ELS in the pathogenesis of arthritis. To this end, we examined MCs synovial infiltration in AIA, a model in which acute inflammatory arthritis is induced by intra-articular injection of methylated bovine serum albumin (mBSA) following systemic immunization with the same antigen. We utilised IL27*ra*-deficient mice, which develop exacerbated synovitis comprising ELS[33].

Figure 4A and B show that MCs were present already in the early phases of AIA (three days after intra-articular injection), with their numbers further increasing at day 10 (d10) and day 35 (d35) post-arthritis induction. Importantly, in IL27*ra*-deficient mice, synovial MC infiltration was significantly higher compared to wild-type littermates at d10 and d35 (figure 4C-D). Moreover, at the peak of inflammation (day 10) synovial MCs showed a positive correlation with arthritis index, synovial infiltrate and the presence of erosions (figure 4E). Finally, in IL27*ra* deficient animals at day 10, there was a trend towards a correlation between MCs and the area of lymphoid aggregates (figure 4F) and MCs were found at the borders of lymphoid aggregates (figure 4G), reflecting the observations in RA patients with a lymphoid pathotype.

Overall, these observations confirm the association of MCs with ectopic lymphoid neogenesis and disease severity, during arthritis induction *in vivo*.

Discussion

In this manuscript, we present the first systematic analysis of MCs in the synovial membrane (SM) of early RA patients obtained by US-guided synovial biopsy prior to therapeutic intervention. Our data demonstrate that synovial MCs are strongly associated with specific pathobiological and clinical phenotypes potentially linked to their ability to induce the activation/differentiation of B cells and the production of ACPA autoantibodies.

Although MCs have long known to be part of the inflammatory infiltrate in RA, their presence in the SM has only been analysed in a few studies, which described increased MC numbers and mediators in the SM and synovial fluid of RA patients, possibly in correlation with disease activity[6–8]. More recently, in a study describing the immunopathologic characteristics of ultrasound-defined synovitis in RA patients in remission, the presence of synovial MCs and B cells at baseline was associated with disease reactivation at follow-up[40]. At the same time, studies *in vitro* and *in vivo* have yielded contradictory results, so that their exact contribution is still unclear[4].

To obtain further insight into the relevance of MCs in RA, we analysed the SM of a large cohort of patients with early (<12 months) RA (n=99), unbiased by treatment and disease duration.

Our data demonstrate high synovial MC counts in patients with a severe clinical phenotype at baseline. Furthermore, MCs correlate with synovial inflammation and, in particular, with ectopic lymphoid structures (ELS), which are found in approximately 40% of RA patients and have been associated with disease severity, T cell priming and autoantibody production, including the local on-going production of class-switched autoantibodies, such as ACPA[29,41]. Interestingly, we found a higher prevalence of tryptase-expressing MCs (MC_T) in the synovia of patients with ELS. Although the concept of MC heterogeneity is

still ill-defined[42], the prevelence of MC_T in association with the lymphoid pathotype would be in line with the pro-inflammatory role of this subpopulation described in asthma[43]. The strong association of MCs with ELS led us to hypothesise that MCs could modulate the local adaptive immune response. The ability of human MCs to influence T cells interaction has already been shown[44,45]. Murine MCs have been shown to activate B cells and promote their differentiation toward effector cells[38,46]. Interestingly, murine MCs were also shown to control, via CD40/CD40L interaction, the expansion and differntiation of IL-10-competent B cells, which is in line with their immunomodulatory functions[39]. ~~Nonetheless, t~~The interaction of human MCs and B cells has ~~never~~ been poorly studied~~;~~ ~~particularly~~ in the context of allergic responses, MCs have been shown to express CD40L and induce IgE production by B cells[47,48], while in the context of cancer they have been shown to activate lymphoplasmacytic cells via CD40L[49]. Nonetheless, the interaction between human MCs and B cells has n~~ot~~ever been studied in the context of autoimmune diseases characterised by local B cell responses.

Here, we show that MCs are at the border of B and T cell aggregates in the synovia of RA patients, similarly to the distribution observed in secondary lymphoid organs. Furthermore, we show that human MCs support the survival, activation, proliferation and differentiation of naive B cells into IgG-producing B cells, in line with recent results in mice [46]. This effect is dependent on cell contact and, specifically, on CD40L-CD40 interaction. Importantly, we demonstrate that MCs induce ACPA production by B cells, also in a contact-dependent manner~~, and thus most likely because of CD40L~~. Furthermore, we provide the first evidence of an active interaction between human MCs and B cells, as previously shown for MC-T cells[50] and MC-DCs[51]. *In vivo*, we used antigen-induced arthritis (AIA) to assess the timecourse and magnitude of synovial MC infiltration, and IL27*ra* deficient mice as a model of exacerbated synovitis accompanied by ELS formation[33], thus resembling RA patients

with a lymphoid pathotype. In both wild-type and IL27*ra* deficient animals, we observed an early synovial MC infiltration, before the formation of ELS, further increasing at later stages. Whether the early synovial infiltration of MCs means that they are essential to lymphoid neogenesis remains to be established. In fact, in addition to the direct modulation of lymphocyte activation, MCs are also known to produce several mediators[52], including angiogenic and lymphangiogenic factors[53][54], that could support the organization of ELS [55]. Interestingly, MC numbers were significantly higher in IL27*ra* deficient animals, in association with synovial ELS and a worse disease outcome. As IL27*ra* deficiency has been shown to enhance the activation of MCs in the context of Th2 responses [56,57], it will be of interest to further assess the specific role of IL27-mediated MC inhibition in autoimmune diseases.

In conclusion, our study points to the relevance of MCs in RA and their role as novel markers of synovial inflammation. In fact, their presence contributes to the definition of a MC-rich highly inflamed synovial pathotype and helps identifying patients with a severe clinical phenotype.

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Contributorship: FR: study design, experiments, data acquisition, data analysis, manuscript preparation and revision; DM: study design, experiments, data acquisition, data analysis; LFJ, GJ: experiments, data acquisition, data analysis (animal data); SP, TM: experiments, data acquisition; AN, FH data acquisition, data analysis (clinical data); AR, SR, FK, RT, GS, SJ , FWR, AD, GM: interpretation of experimental results, manuscript revision; CP: study design, interpretation of experimental results, manuscript preparation. FR wrote the manuscript and all authors critically revised its final preparation and approved its submission.

Ethical approval: All procedures were performed following written informed consent and were approved by the hospital's ethics committee (REC 05/Q0703/198).

Data sharing statement: n.a. **FIGURE LEGENDS**

Figure 1 Association of high mast cell counts with synovial inflammation, disease severity and lymphoid aggregates. (A) Correlations of MC numbers with inflammatory markers, disease activity and histological scores. Line at p=0.05. (B) Patients stratified according to mast cell numbers into low (<33rd percentile) medium (33rd-66th percentile) and high (>66th percentile) groups (C-D) Distribution of CD3 (C) and CD20 (D) scores in patients stratified according to MC numbers as in B. (E) Distribution of pathotypes in patients stratified according to MC numbers. (F) MC density in patients stratified according to pathotypes. n=99 *p=0.05, Spearman correlation in A, Chi-Square in C-E, One Way ANOVA with Bonferroni post-hoc test in F.

Figure 2 Mast cells border synovial lymphoid aggregates **(**A) IHC staining for CD3, CD20, CD138 and CD68 in an US-guided biopsy of a patient with early RA classified as lymphoid pathotype. Semi-quantitative scores for each marker are indicated. (B) IHC staining for CD117 (c-kit) showing synovial MCs. High magnification, on the right, shows synovial MCs (arrows) close to cellular aggregates (C) Immunofluorescence staining of the RA synovia, showing the interactions between CD20 B cells (in green) and CD3+ T cells (in red), forming an ectopic lymphoid aggregate, and CD117+ MCs (in clear blue). The high magnification shows MCs surrounded by B and T cells at the edge of one aggregate. (D) Immunofluorescence of a human tonsil, showing MCs (blue) at the edge of a germinal centre formed by aggregates of B (green) and T cells (red).

Figure 3 Mast cell support survival, proliferation and differentiation of naïve B cells into antibody secreting cells and induce the production of ACPA autoantibodies**. (**A) Proportion of live B cells, measured by FACS staining, after 7 days of culture without (o) or with (■) MCs, at a ratio of 1:6, together with CPG at 1 µg/mL, n=4 (B) IgG and IgM measured by ELISA in the supernatants of naïve B cells harvested after 7 days of culture, n=13 for IgG and 7 for IgM. **(**C) Proportion of live cells, when naïve B cells were cultured alone or co-cultured with MCs in contact or in transwell, n=3. (D) Proliferation measured by CFSE staining and FACS analysis after 7 days of culture. Representative histograms on the left and cumulative data with division index on the right, n=3. (E) IgG production, naïve B cells cultured alone or co-cultured with mast cells in contact or in transwell, n=3. (F) ACPA measured by CCP2 ELISA upon co-culture ACPA producing B cell clone with MCs in contact or transwell, n=3. (G) IgG production upon inhibition of CD40L in the co-culture of MCs and B cells. n=3 (H) Proportion of antibody-producing cells (CD27+CD38+), after gating on live/$CD117^{-}$/$CD19^{+}$.Representative histograms from 3 independent experiments. (I) IF of MCs (light blue) and B cells (red) after 24h of co-culture and CPG triggering. Actin re-organisation is shown in green (phalloidin). Nuclei in blue (DAPI). Measure bar 5µm. Representative image of 3 independent experiments. *=$p<0.05$, Mann-Whitney (A and B), one way ANOVA with Bonferroni post-hoc (C,D, E, H). n= number of independent experiments with n MC donors and n B cell donors)

Figure 4 Mast cells associate with ELS and disease severity in antigen induced arthritis in IL27*ra* deficient mice (A) Representative images of toluidine blue staining showing metachromatic mast cells (arrows) at different time-points during antigen-induced arthritis, with results summarized on in (B); n=>6 for each time-point. (C) Comparison of MC infiltration in wild type (WT) and WSX1 (IL27*ra* knock out) mice, and (D) representative images of toluidine blue staining with metachromatic MCs (red arrows) at d35 time-point. N= >6 /time-point/group. (E) Correlation of synovial MC numbers with arthritis index, synovial infiltrate and erosions at day 10. (F) Correlation of MC number with ELS area in IL27R KO mice at day 10 and (G) representative image. Size bars 100 μm, unless specified. *p<0.05, one way ANOVA with Bonferroni in B, Mann-Whitney comparing WT and WSX1 at each timepoint in C, Spearman correlation in E and F.

Supplementary Figure S1. Panel A shows an example of FACS staining of in vitro cultured MCs, with purity, expressed as percentage of CD117 positive cells, of 99.7% (range from 95 to 99.7%)

Supplementary Figure S2. Immunofluorescence for tryptase and chymase

A Density of MCs expressing tryptase (MC_T) or tryptase and chymase (MC_TC) in synovia. B Density of MC_T and MC_TC in different pathotypes C Correlation between the density of MC_TC and Krenn synovitis score D Correlation between MC_T and Kreen Synovitis Score E Representative example of a patients with a lymphoid pathotype. Blue=DAPI (nuclei); red=tryptase; green=chymase; n= 15, representative image out of 5 patients with lymphoid pathotype in E. Mann-Whitney in A, One-way ANOVA with Bonferroni post-test in B. Spearman in C-D.