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1 **Visualising the interaction of CD4 T cells and DCs in the evolution of**
2 **inflammatory arthritis.**

3
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16
17

Abstract

Objectives Successful early intervention in Rheumatoid Arthritis (RA) with the aim of resetting immunological tolerance requires a clearer understanding of how specificity, cellular kinetics and spatial behaviour shape the evolution of articular T cell responses. We aimed to define initial seeding of articular CD4⁺ T cell responses in early experimental arthritis, evaluating their dynamic behaviour and interactions with dendritic cells (DCs) in the inflamed articular environment.

Methods Antigen-induced arthritis was used to model articular inflammation. Flow cytometry and PCR of T cell receptor (TCR) diversity genes allowed phenotypic analysis of infiltrating T cells. The dynamic interactions of T cells with joint residing DCs were visualized using intravital multiphoton microscopy.

Results Initial recruitment of antigen-specific T cells into the joint was paralleled by accumulation of CD4⁺ T cells with diverse antigen-receptor expression and ability to produce TNF α and IFN γ upon mitogenic restimulation. A proportion of this infiltrate demonstrated slower motility speeds and engaged for longer periods with articular DCs *in vivo*. Abatacept treatment did not disrupt these interactions but did reduce T cell expression of inducible costimulatory (ICOS) molecule. We also demonstrated that non-specific CD4⁺ T cells could be recruited during these early articular events.

Conclusions We demonstrate that CD4⁺ T cells engage with articular DCs supporting antigen specific T cell reactivation. This cellular dialogue can be targeted therapeutically to reduce local T cell activation.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the joints¹, characterized by synovial inflammation, degradation of bone and cartilage, progressive loss of function and increasing disability. As RA progresses, inflammatory cells, including T cells and dendritic cells (DCs), infiltrate the synovial compartment². Genome wide association studies (GWAS) support a role for T cells in the pathogenesis of RA; gene polymorphisms associated with CD4⁺ T cell function are associated with susceptibility to RA³. These include HLA-DRB1, PTPN22, CD28 and CTLA-4^{2, 4-6}. Therapeutic targeting of T cells in RA through the use of abatacept provides evidence of their importance in disease pathogenesis. However, there is increasing interest in characterizing the nature of the T cell response in early RA as therapeutics move increasingly in this direction.

Activated CD4⁺ T cells are present in the inflamed joints of RA patients and appear oligoclonal in early disease^{2 7 8}. However, the specificity of these T cells, their site of original activation and how they mediate pathology remain unclear. Lymph node (LN) interactions between T cells and DCs will likely be critical for naïve T cell priming. However, similar interactions could occur within the synovium, whereby joint localized reactivation of T cells would directly contribute to pathology. We employed intravital microscopy to establish the intra-articular dynamics of CD4⁺ T cell interactions with DCs in the context of specificity and clonality in the early stages of experimental 'early arthritis'⁹. Measurement of inducible costimulatory (ICOS) molecule was used as an indicator of T cell engagement in cognate interactions with antigen presenting cells and reception of co-stimulation via CD28 i.e. they had

‘seen’ antigen. In addition, we evaluated the dependence of this dialogue on CD28 co-stimulation.

Methods

Animals

LysMGFP¹⁰, ovalbumin specific OT-II¹¹ T cell receptor (TCR) transgenic (Tg) mice, OT-II DsRed and hCD2-DsRed¹², CD11c-YFP¹³ and TEa TCR RAG2^{-/-} mice¹⁴ were bred in house. C57BL/6J and Ly5.1 mice were purchased from Harlan (Harlan, Bicester, UK). All animals were specified pathogen free and maintained under standard animal house conditions at the University of Glasgow in accordance with UK Home Office Regulations.

Induction of inflammatory arthritis

ArthritoMab antibody cocktail for C57BL/6 (MD Biosciences, Zurich, Switzerland) was used to induce collagen antibody induced arthritis (CAIA). CD4⁺ T cell dependent arthritis was induced as previously reported^{9 15-19} (Supplementary Figure 1A). Abatacept or control IgG was administered at 10 mg/kg (Bristol-Myers Squibb) i.p. 24 hours before induction of arthritis and continuing every 2 days thereafter. For treatments 6 hours prior to imaging, arthritic mice were given 50 µl of abatacept (10 mg/kg) or etanercept (sTNFR-Fc, 0.5 mg/kg, etanercept, Wyeth Pharmaceuticals, Taplow, U.K.) into the inflamed footpad.

Multiphoton laser scanning microscopy (MPLSM)

Mice were anaesthetised using Domitor (50 mg/kg Ketamine and 0.5 mg/kg Medetomidine i.p.) and placed on a heated stage. Articular tissue of the lateral malleolus was surgically exposed

prior to transfer to a warmed saline bath (Supplementary Figure 2A, B). MPLSM was carried out as previously reported¹². Second harmonic generation (SHG) was used to orientate the tissue once under the objective (Supplementary Figure 2C). Naïve joint of LysMGFP showing SHG in blue (Supplementary Figure 2D). CAIA inflamed joint of LysMGFP mouse showing SHG (blue) and LysMGFP expressing cells (green) (Supplementary Figure 2E). Flow cytometric analysis of leukocyte populations in both naïve and CAIA inflamed ankle joints (Supplementary Figure 2F).

Recovery of cells from tissues

Ankle joints were teased apart and the tissue digested for 25 minutes at 37 °C in 2.5 mg/mL collagenase D (Roche/Sigma Aldrich, Dorset, UK) in HBSS. Samples were then homogenized using Miltenyi Tissue Dissociators and strained to obtain single cell suspensions. Popliteal lymph nodes (pLN) were forced through Nitex mesh (Cadisch Precision Meshes) to obtain single cell suspensions. Cell suspensions were then washed in FACS buffer (PBS containing 2% FCS) and subsequently stained for flow cytometry.

Generation of bone marrow derived dendritic cells (BMDCs)

Bone marrow from C57BL/6 mice was cultured in GM-CSF conditioned medium as previously described²⁰. After 6 days, BMDC were cultured +/- OVA (100 µg/ml) for 2 hours before maturation overnight with the addition of *Escherichia coli* 055:B5 LPS (100 ng/ml) (Sigma-Aldrich). BMDC were then washed in fresh medium and co-cultured with T cells isolated from joint draining LN.

107 *Flow cytometry*

108 Cell viability was determined by Fixable Viability Dye eFluor 506 (eBioscience). Cells were
109 subsequently washed (400 x g for 5 minutes) in FACS buffer and incubated with Fc Block for 15
110 minutes at 4 °C prior to addition of fluorochrome-conjugated antibodies (30 minutes at 4 °C).
111 Antibodies used (eBioscience): anti-CD3 (clone 17A2); anti-CD4 (clone GK1.5); anti-CD45
112 (clone 30-F11); anti-CD45.1 (clone A20); anti-CD69 (clone H1.2F3); anti-CD44 (clone IM7);
113 anti-ICOS (clone 7E.17G9); anti-CD62L (clone MEL-14); anti-CD19 (clone eBio1D3); anti-
114 MHC-II (I-A/I-E) (clone M5/114.15.2); anti-CD11c (clone N418). For fluorescence-activated
115 cell sorting (FACS), joint draining popliteal LNs were pooled on day 4 post-HAO challenge and
116 stained for viability, anti-CD45, anti-CD3 and anti-CD4. Live CD45⁺ CD3⁺ CD4⁺ T cells were
117 isolated using a FACS Aria IIU (BD Biosciences, Oxford, UK) and co-cultured with BMDC at a
118 1:10 ratio for 72 hours at 37 °C and 5% CO₂. Intracellular cytokine staining was performed on
119 cells isolated from joints and draining LNs using anti-IFN γ (XMG1.2) and anti-TNF α (MP6-
120 XT22) following stimulation with either OVA₃₂₃₋₃₃₉ (0.5 μ g/mL) or PMA (10 ng/ml) and
121 ionomycin (500 ng/ml) for 4 hours in the presence of brefeldin A. Cells were acquired on an
122 LSRII (BD Biosciences) and data analysed using FlowJo software (TreeStar version 7.6.5,
123 Oregon, USA).

124

125 *TCR V β repertoire clonality*

126 V β complementarity determining region 3 (CDR3) clonality was assessed using
127 SuperTCRExpressTM (BioMed Immunotech, Florida, USA) on endogenous CD4⁺ T cells sorted
128 from inflamed joints and popliteal LNs using the BD FACs ARIAIII. Sorted cells were lysed and

the RNA extractions, cDNA synthesis and PCR performed as per the manufacturer's instructions. PCR products were run on a 4% agarose gel and visualized on a GelDoc system (BioRad, Hertfordshire, UK).

Statistics

Data is shown as mean $\pm$ SD. Groups were compared using one-way ANOVA or unpaired Student's *t* test. Specific tests and significance levels are stated in the appropriate figure legends. Using data from previous studies^{12 17} we estimated that a minimum of three animals per time point/group would provide 80% power to detect significant differences ($p < 0.05$) in numbers of T cells induced following antigen challenge versus carrier alone. Statistical analysis of results was performed using Prism version 6 (GraphPad, Inc, CA, USA).

Results

Characterising T cell recruitment to the joint

Employing a previously described model of articular inflammation displaying histological evidence of bone and cartilage remodeling and autoreactive T and B cell responses^{9 15-19}, we determined appropriate timings to visualize articular T cell and DC behaviour by detailing the kinetics of cellular recruitment *ex vivo* (Supplementary figure 1A). Induction of arthritis using heat aggregated ovalbumin (HAO) resulted in an influx of neutrophils, dendritic cells, and CD4⁺ T cells to the joint (Supplementary figure 1B-F). Maximal accumulation of DCs and CD4⁺ T cells occurred at day 4, with distinguishable populations observed for donor transferred OT-II cells and endogenous CD45.1⁻CD4⁺ T cells (Supplementary figure 1E-F). OT-II T cells were the minor T cell population relative to endogenous CD4⁺ T cells (Figure 1A, B). Both transferred OT-II cells and endogenous CD4⁺ T cells infiltrating the joint displayed an activated proinflammatory phenotype, being CD44^{hi}CD62L^{lo/neg} and produced TNF α and IFN γ following *ex vivo* stimulation with PMA/ionomycin (Figure 1C, D). Notably, endogenous CD4⁺ T cells from popliteal lymph nodes (pLN) only produced TNF α upon restimulation, suggesting that subsequent reactivation in the target tissue contributed to further differentiation allowing production of IFN γ (Figure 1E). We tested the possibility that a proportion of these endogenous T cells may be specific for OVA, having been primed in response to immunization with OVA/CFA used to maintain the transferred OT-II cells. T cells were FACS sorted from inflamed joints and popliteal LNs for co-culture with OVA-pulsed bone-marrow derived DCs. The very low numbers of T cells recovered from the inflamed joint proved prohibitive to performing a robust analysis. However, T cells sorted from the popliteal LN showed endogenous T cells did not respond to OVA-pulsed bone-marrow derived DCs whereas OT-II T cells significantly

increased expression of ICOS and CD44 (Supplementary Figure 2A). In addition, *ex vivo* stimulation of unfractionated cells with OVA₃₂₃₋₃₃₉ revealed that OT-II T cells from inflamed joints and pLNs were able to respond, whereas the endogenous T cells were not (Supplementary Figure S2B-E). Thus, while the specificity of these cells remains unknown, the majority are unlikely to be specific for the immunodominant epitope of the inciting antigen.

T cells and DC undergo long-lived interactions in the joint

Having established the recruitment kinetics of CD4⁺ T cells into the joint, we next visualized their behaviour using intravital multiphoton laser scanning microscopy (MPLSM) and lineage specific fluorescent reporter mice (Supplementary Figure S3, Supplementary Movie S1). Following periarticular injection of HAO, a rare population of slow moving and stationary OT-II DsRed articular T cells were consistently observed (Figure 2A, Movie 1). Their typical behavioural parameters namely low speed, displacement rate and meandering index (Figure 2C-E) were consistent with recognition of cognate antigen and consequent local activation²¹⁻²³. This was reflected in the proportion of OT-II cells interacting with fluorescent DCs for longer than 10 minutes (Figure 2F). Clustering of CD11c-YFP cells was also observed around tendons and the joint capsule.

To investigate the behaviour of infiltrating endogenous T cells, we crossed CD11c-YFP mice to intact hCD2-DsRed mice (CD11c-YFPxDsRed), allowing analysis of endogenous T cell interactions. Induction of articular inflammation resulted in recruitment of endogenous T cells to the joint (Figure 2B, Movie 2) – these T cells exhibited a broader range of speeds, displacement

rate and meandering index than the monoclonal antigen-specific OT-II population (Figure 2C-E). Notably, some endogenous T cells displayed behaviour consistent with recognition of antigen and activation, moving slowly and remaining in contact with CD11c⁺ cells for longer than 10 minutes (Figure 2F). In parallel, many cells exhibited scanning behaviour, moving quickly from DC to DC, only contacting for short periods of less than 5 minutes (Figure 2F). Thus, OT-II and a sub-population of endogenous T cells interact with CD11c⁺ cells within the inflamed articular environment in a manner consistent with antigen recognition and activation.

Heterogeneity in motility of endogenous CD4⁺ T cells likely arises from diversity of antigen specificity. Given the evidence of broader autoreactive responses in this model^{9 15-17 19}, and in RA patients¹, it seemed unlikely that a monoclonal T cell clone would dominate the inflammatory infiltrate. Therefore, in the absence of information regarding the target antigens of the endogenous T cells, we probed T cell receptor (TCR) use as an indicator of diversity among articular endogenous CD4⁺ T cells. By performing V β complementarity determining region 3 (CDR3) clonality analyses we were able to assess whether there was selective accumulation of few or multiple T cell clones. CD4⁺ T cells from naive LN displayed diverse V β usage consistent with multiple (poly)clonality (Supplementary Figure 4A). Diversity was also broad in LNs draining the inflamed site - all V β were expressed with the exception of V β 13 (Supplementary Figure 4B). By contrast, when articular T cells were examined, although the majority of V β chains were represented, only one or two CDR3 clones were identified per V β used (Supplementary Figure 4C). While the reduced clonality of V β use by the joint infiltrating CD4⁺ T cell population implies a role for antigen, the number of V β chains present may also explain the heterogeneity in behaviour seen in our imaging studies.

210

211 **CD4⁺ T cells of irrelevant specificity can be recruited to the inflamed joint.**

212 To assess recruitment of effector/memory CD4⁺ T cells of unrelated/irrelevant antigen
213 specificity, we adoptively transferred Th1 polarised TEa TCR transgenic CD4⁺ T cells (specific
214 for E α ₅₂₋₆₈ I-A^b) into congenic recipients and induced arthritis in the absence or presence of their
215 cognate antigen (HAO or HAO + E α respectively). Induction of arthritis recruited Th1 OT-II into
216 the joint as expected, however a significant population of TEa Th1 cells were also detected
217 (Figure 3A, B). Induction of arthritis with HAO and E α ₅₂₋₆₈ (HAO + E α) did not significantly
218 increase numbers of TEa in the inflamed tissue (Figure 3A, B) but was required for their up-
219 regulation of inducible costimulatory (ICOS) molecule expression (Figure 3C, D) and expansion
220 of TEa in the pLN (Figure 3E, F). Thus, the presence of a diverse TCR repertoire and the
221 observation that a proportion of T cells continually exhibit scanning behaviour at the inflamed
222 site could therefore be explained by recruitment of antigen experienced cells of an irrelevant
223 antigen specificity.

224

225 **Abatacept has no immediate impact on T cell/DC interactions in the inflamed joint.**

226 As well as its clinical success²⁴, abatacept is also effective in murine models of disease²⁵⁻²⁷.
227 Abatacept, a CTLA4-Ig fusion protein, targets one of the most important co-stimulators of T cell
228 activation, blocking binding of T cell CD28 to CD80/CD86 on antigen presenting cells (APCs)
229²⁸⁻³⁰. Previously we reported abatacept inhibition of pathology in our arthritis model¹⁸ and the
230 priming of LN T cells³¹. We extended these studies to determine the effect of abatacept upon the
231 intra-articular T cell response. Mice were treated with abatacept one day prior to HAO challenge

and then on day 1 and day 3 post-challenge. To assess whether abatacept could have a more immediate impact on T cell/DC communication, a single administration of drug was given 6 hours prior to tissue harvesting. Abatacept treatment, either throughout or 6 hours prior to harvest, resulted in reduced accumulation of endogenous T cells in the joint (Figure 4A, B), but did not impact on OT-II articular accumulation (Figure 4A, C). A failure to prime endogenous T cells in the pLN was found to underpin their failed accumulation in the joint (Supplementary Figure S5). Although abatacept treatment did not significantly alter OT-II proportions in the joint draining LNs, it did prevent the upregulation of ICOS (Supplementary Figure S6). Abatacept treatment also impacted upon reactivation of endogenous and donor T cells in the joint evidenced as lower ICOS expression, even when given 6 hours prior to harvesting of cells (Figure 4D, E respectively).

Consistent with the flow cytometry data, MPLSM of PBS challenged joints revealed few T cells or DCs, whereas a large population of endogenous T cells was observed after HAO challenge (Figure 5A, Movie 3). Abatacept treatment throughout reduced endogenous T cell infiltration of inflamed joints (Figure 5A, Movie 3), consistent with our flow data. T cells that were observed exhibited reduced speed (Figure 5B). The displacement rate (Figure 5C), directionality of their movement (meandering index, Figure 5D) and interaction time with DCs (Figure 5E) appeared to be unaffected. Previous studies show that motility is influenced by numerous extrinsic factors (reviewed in ³²). Published data suggests that the availability of such factors is altered/limited in the reduced inflammatory environment post abatacept treatment ¹⁸. Abatacept treatment 6 hours prior to imaging did not impact on T cell speed, displacement rate, meandering index or interaction times with articular DCs (Figure 5A-E, Movie 3). Anti-TNF (etanercept), given as an

additional control 6 hours prior to imaging, similarly had no impact on the behaviour of T cells in the joint (Figure 5A-E, Movie 3). Although abatacept did not alter endogenous T cell/DC articular interactions, even 6 hours of localized exposure was sufficient to reduce expression of ICOS, indicative of continued dialogue between APC and T cell in the inflamed joint. Abatacept is therefore able to affect the re-activation of T cells within the inflamed joint itself.

Discussion

We present the first examination of the dynamics of T cell recruitment and interactions in the articular environment. Using a model of early articular inflammation, we find that recruitment of a small population of antigen-specific CD4⁺ T cells is paralleled by influx of a larger oligoclonal CD4⁺ T cell population, a significant proportion of which engages with DCs in a manner consistent with recognition of cognate antigen. Signaling through CD28 was not required for ongoing articular interactions, but was critical for full activation of CD4⁺ T cells in the joint. These findings not only enhance our fundamental understanding of the location and dynamics of the cellular interactions that control the development of articular inflammation, but will also be informative for future studies in clinical RA examining the evolution of articular T cell responses.

Genetic studies have implicated CD4⁺ T cells in the pathogenesis of RA²⁻⁶. Consistent with this, our model displayed recruitment of immune system cells, including neutrophils, macrophages, B cells, DCs, and crucially, CD4⁺ T cells. These T cells displayed an antigen-experienced phenotype with the potential to produce pro-inflammatory TNF α and IFN γ . As expected, an

increased proportion of CD4⁺ T cells capable of producing TNF α were found in the LN draining the inflamed joint. It has long been known that T cells in somatic tissues have a memory phenotype and T cells in the inflamed joint also have an antigen experienced phenotype. However, the role of antigen in the recruitment, retention or activation of memory T cells remains unclear. To address this, we imaged T-DC interactions in the joint.

The dynamics of T cell interactions with DC have been characterised using *in vivo* imaging and revealed particular behaviours indicative of certain outcomes. For example, activation of T cells in LN occurs with three phases: the first (typically the initial 8 hours of antigen exposure) is characterised by transient interactions (<5 minutes); the second, 8–20 hours after exposure to antigen, is characterized by long lived T cell/DC interactions (>10 minutes) reflecting recognition of cognate antigen and costimulation, underpinning priming of the T cell^{21 22 33}; after ~48 hours, T cells recover motility and subsequent interactions with DCs are short. Equally, these interactions can also impact on T cell differentiation¹² and even tolerance^{23 34}. Our studies directly demonstrate stable interactions between endogenous CD4⁺ T cells and DCs in the inflamed joint consistent with recognition of specific antigen. These interactions likely underpin our observation that TNF α producing T cells in the joint co-expressed IFN γ unlike their counterparts in the joint draining lymph node. A similar “two hit” theory has already been proposed for IL-4 production^{35 36}.

Although the specificity of T cells in RA joints remains unclear, diversity in TCR expression by synovial T cells is reduced, indicating their selective accumulation³⁷⁻⁴⁰. Notably, comparable clonal responses are detected in healthy siblings of RA sufferers⁴¹. Thus, changes in clonality of

the response are likely to occur early in disease and reflect disease status. Indeed, selected V β clonal expansion seen in peripheral blood of RA patients⁴² is reduced in the face of successful anti-TNF α therapy⁴³, indicating alterations in repertoire can inform as to responsiveness to therapeutic intervention. Endogenous T cells infiltrating joints in our model exhibited reduced V β CDR3 repertoire diversity, consistent with the oligoclonal nature of human RA synovial T cells^{39 44-46}. The similarity in clonal responses in the joint and draining LN also supports the hypothesis that T cell activation and clonal expansion occurs in secondary lymphoid organs prior to migration and accumulation in the joint. Given the propensity for autoreactivity arising in the endogenous CD4⁺ T cell compartment in our model⁹ and the observation of sustained interactions with DCs in the inflamed joint, our data supports a model where autoreactive T cells may constitute a small but significant population of articular infiltrating T cells and that cognate interactions with DCs would support their activation/proliferation in response to local antigen^{47 48}.

As some endogenous T cells did not undergo interactions with DC in the inflamed joint and the TCR usage analysis demonstrated an oligoclonal response we tested whether activated/memory T cells of irrelevant antigen-specificity could be recruited to the inflamed joint. Indeed, a range of viral and bacterial T cell specificities have been identified in RA synovial tissue⁴⁹, and consistent with this, we directly demonstrated that irrelevant-specificity CD4⁺ T cells can be recruited to the inflamed joint independently of cognate antigen. Such memory T cells may be activated by the inflammatory environment as reported by Brennan^{50 51} and others⁵²⁻⁵⁴ in the instance of cytokine-activated T cells (Tck). While recruitment of irrelevant specificity T cells was not dependent upon cognate antigen, the latter was required for up-regulation of ICOS,

323 stressing the importance of cognate T/DC interaction in the inflamed joint for full T cell
324 activation.

325
326 As we found antigen-specific, oligoclonal and unrelated T cells in the joint we examined whether
327 a current therapeutic targeting T-DC interactions affected these cells in the joint or draining LN.
328 While abatacept did not directly inhibit the ability of T cells and DCs to interact in the inflamed
329 joint, the diminished ability of these T cells to up-regulate ICOS suggested a failure in their
330 activation. Thus, in addition to inhibiting priming of potentially autoreactive T cells in the joint
331 draining LN, abatacept influences the quality of DC-T cell interactions directly in the inflamed
332 joint.

333
334 In conclusion, our findings demonstrate that T cells make cognate interactions with DCs
335 in the inflamed joint. Recruitment of a small number of inciting-antigen-specific T cells into the
336 joint environment is paralleled by a larger oligoclonal population of pro-inflammatory CD4⁺ T
337 cells, a proportion of which interact with DCs in a manner consistent with recognition of cognate
338 antigen. We also demonstrate that T cells of irrelevant specificity can enter the joint. However,
339 recognition of cognate antigen with costimulation is necessary for full activation in the articular
340 environment.

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Figure 1: CD4⁺ T cells recruited to the joint have an activated, proinflammatory phenotype. (A) Representative flow cytometry plots, gated on viable CD45⁺ cells isolated from the joints on day 4 post PBS or HAO periarticular challenge, showing the expression of CD4 and CD45.1 representing the endogenous T cells (CD4⁺CD45.1⁻) and donor OT-II T cells (CD4⁺CD45.1⁺). (B) Absolute number of OT-II donor T cells and endogenous T cells isolated from the joints after PBS or HAO periarticular challenge. (C) Representative flow cytometry plots of CD44 and CD62L expression by OT-II donor T cells and endogenous CD4⁺ T cells from inflamed joints. (D) IFN γ and TNF α production by cells was assessed following PMA/Ionomycin *ex vivo* re-stimulation. Representative flow cytometry plots showing IFN γ and TNF α responses of OT-II donor and endogenous CD4⁺ T cells isolated from joints. (E) Percentage of TNF α expressing endogenous and OT-II donor that co-express IFN γ at both draining popliteal LN and joint in HAO challenged mice. Data shows the mean with points representing individual experimental animals. Data is representative of three individual experiments, * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$.

Figure 2. Imaging T cell interactions with DCs within the joint. Adoptive transfer of DsRed OT-II Th1 cells into CD11cYFP recipients allowed visualization of antigen specific T cell/DC interactions in the inflamed articular microenvironment (A), while crossing CD11cYFP animals with CD2DsRed transgenic mice allowed imaging of endogenous T cell interactions with DCs (B) in our T cell dependent model of arthritis. Arthritis was induced by periarticular injection of heat aggregated ovalbumin (HAO) as before and MPLSM of inciting-antigen specific OT-II T

cells or endogenous T cells interacting with DCs in the inflamed articular environment performed four days later. T cells are seen in red, DCs in green and SHG from collagen structure in blue. T cell speed (C), displacement rate (D), meandering index (E) and duration of interaction with DCs (F) were determined using Volocity image analysis software for three fields of view and three animals. Data shows the mean with points representing individual cells. Data is representative of two independent experiments. Scale bars represent 50 μ m.

Figure 3: Antigen experienced Th1 cells of irrelevant specificity can be recruited to the inflamed joint. OT-II Th1 cells (DSRed) and TEa Th1 cells (CD45.2⁺) were transferred into CD45.1 B6 hosts and mice subsequently challenged in the footpad with PBS, HAO or HAO + E α ₅₂₋₆₈. (A) Each transferred T cell population could be tracked in the CD45.1 B6 hosts based on their expression of either DSRed (OT-II T cells) or CD45.2 (TEa T cells). Representative flow cytometry plots, previously gated on the CD45⁺ CD4⁺ T cells in the joint showing expression of CD45.2 and DSRed. (B) Absolute number of CD45.2⁺ TEa T cells in the joint after PBS, HAO only or HAO + E α challenge. (C) Representative flow cytometry plots showing CD44 and ICOS expression on CD45.2⁺ TEa T cells in the joint after PBS, HAO only or HAO + E α challenge. (D) Absolute number of ICOS⁺ CD45.2⁺ TEa T cells in the joint after PBS, HAO only or HAO + E α challenge. (E) Percentage of CD45.2⁺ TEa T cells in the draining lymph node (popliteal LN) as a percentage of total CD4⁺ T cells and (F) absolute number of CD45.2⁺ TEa T cells in the draining lymph node after PBS, HAO only or HAO + E α challenge. Data representative of two independent experiments; n=5 mice per group. ** represents p<0.01; *** represents p<0.001.

Figure 4. The therapeutic impact of abatacept on CD4⁺ T cell accumulation in inflamed joints. C57BL/6J recipient mice were adoptively transferred with OT-II Th1 cells, boosted with OVA/CFA and periarticularly challenged with PBS or HAO (as in Figure S1A). In addition, mice were either treated throughout with abatacept (Aba) (given 24 hours prior to footpad challenge and every 2 days thereafter) or 6 hours prior to harvest of the tissues. Mice were taken 4 days after footpad challenge. Single cell suspension of inflamed joints were analysed by flow cytometry to determine the proportions of endogenous and inciting-antigen-specific Th1 OT-II cells accumulating after challenge. (A) Representative flow cytometry plots showing both endogenous (CD4⁺CD45.1⁻) and OT-II (CD4⁺CD45.1⁺) T cells on day 4 post-challenge with PBS or HAO. Proportions of viable leukocytes isolated from joints found to be (B) endogenous CD4⁺CD45.1⁻ T cells and (C) CD4⁺CD45.1⁺ OT-II cells. Proportion of ICOS expressing (D) endogenous CD4⁺ T cells and (E) OT-II donor T cells isolated from PBS, HAO with IgG isotype control, HAO with abatacept throughout or HAO with abatacept 6 hours prior to harvest. Data is representative of 3 experiments; n=5 mice. * represents p<0.05; ** represents p<0.01; *** represents p<0.001; **** represents p<0.0001.

Figure 5. Imaging T cell/DC interactions in response to abatacept. CD11cYFPxDsRed recipient mice were adoptively transferred with non-fluorescent OT-II Th1 cells, boosted with OVA/CFA and periarticularly challenged with PBS or HAO (as in Figure S1A). In addition, mice were either treated throughout with Abatacept (Aba) (given 24 hours prior to challenge and every 2 days thereafter) or 6 hours prior to imaging. MPLSM of ankle joints was performed on day 4 following challenge. In addition, one group received Etanercept (Etan) given 6 hours prior to imaging. (A) Representative MPLSM images showing CD11c⁺ cells (yellow), endogenous T

586 cells (red) and SHG from joint capsule and tendon (blue). Cell tracking was performed to
587 determine T cell (B) speed, (C) displacement rate, (D) meandering index and (E) duration of
588 contact with CD11c⁺ cells using Volocity image analysis software for three fields of view and
589 three animals. Data shows the mean with points representing individual cells. Data is
590 representative of two independent experiments. Scale bars represent 50 μ m.

591

Supplementary Figure S1. CD4⁺ T cell recruitment to inflamed joints. Flow cytometry was used to establish the kinetics of cellular infiltrate of joints. (A) A CD4⁺ T cell dependent model of articular inflammation using trackable antigen specific T cells was used to allow tracking of inciting CD4⁺ T cell responses. Infiltration of CD11b⁺Ly6G⁺ neutrophils (B), CD11b⁺CD11c⁺ dendritic cells (C), CD19⁺ B cells (D), endogenous CD4⁺CD45.1⁻ T cells (E) and CD4⁺CD45.1⁺ donor OT-II T cells (F) to inflamed joints was determined by flow cytometry on days 1, 4, 7 and 21 post PBS or HAO challenge. Data shows percentage (%) of total cells recovered from the joint. Data representative of two independent experiments; n=3-4 mice per group. * represents p<0.05.

Supplementary Figure S2. Endogenous T cells isolated from joints and draining LN do not make significant responses to OVA. CD4⁺ T cells were FACsorted from pooled pLNs on day 4 post-HAO challenge and co-cultured with OVA-pulsed BMDC. (A) Percentage of OT-II donor T cells and endogenous T cells from pLN expressing CD44 and ICOS after co-culture with medium or OVA-pulsed BMDC. T cells were FACsorted from a pool of 20 mice; data shows 4 technical replicates. IFN γ and TNF α production by cells was assessed following medium or OVA₃₂₃₋₃₃₉ (OVAp) *ex vivo* re-stimulation. (B) Representative flow cytometry plots showing IFN γ and TNF α responses of OT-II donor (CD4⁺CD45.1⁺) and endogenous CD4⁺ T cells (CD4⁺CD45.1⁻) isolated from joints. (C) Percentage of OT-II donor T cells and endogenous T cells from joints that produced cytokine (either IFN γ or TNF α or both) in response to stimulation. (D) Representative flow cytometry plots showing IFN γ and TNF α responses of OT-II donor (CD4⁺CD45.1⁺) and endogenous CD4⁺ T cells (CD4⁺CD45.1⁻) from joint draining pLNs. (E) Percentage of OT-II donor T cells and endogenous T cells from joint draining pLNs

that produced cytokine in response to stimulation (either IFN γ or TNF α or both). Data shows five individual mice, *** represents p<0.0001; *** represents p<0.001.

Supplementary Figure S3. MPLSM was used to image the ankle in real time from a medial aspect (A), with anaesthetized mice being transferred to a warmed saline bath prior to imaging (B). Second harmonic generation (SHG) localized tendons to allow anatomical localization and orientation (C). The validity of our methodology was confirmed by imaging responses in LysM-GFP reporter mice during collagen-antibody-induced-arthritis (CAIA). Neutrophils were not observed in the control joint (D) however they were abundant in the inflamed joint (E), consistent with ex vivo confirmatory flow cytometry analysis (F). Neutrophils were highly motile, consistent with other studies of neutrophil behaviour in inflamed joints. Mean cell proportions of 5 mice per group are shown. MPLSM images are representative of 7 mice.

Supplementary Figure S4: TCR diversity in endogenous CD4⁺ T cells recruited to the inflamed joint. To determine TCR diversity of endogenous CD4⁺ T cells infiltrating the draining lymph nodes and the joints, endogenous CD4⁺CD44^{hi} T cells were sorted by FACS from naïve C57BL/6J popliteal lymph nodes (A), joint-draining lymph nodes (popliteal) (B) and inflamed joints (C) 4 days post HAO challenge. PCR was performed to determine the clonality of these cells for each V β using the SuperTCR*Express* kit (BioMed Immunotech). Endogenous T cells were sorted from pooled samples (naïve n=3; HAO popliteal LN n=20; HAO joint n=20 mice). pLN denotes popliteal lymph node. Numbers above represent each V β ; ‘L’ denotes DNA ladder; ‘-’ denotes negative control; ‘+’ denotes positive control. Data is representative of three independent experiments.

638

639 **Supplementary Figure S5. Abatacept treatment inhibits CD4⁺ T cell activation in the joint**
640 **draining lymph node.** C57BL/6J recipient mice were adoptively transferred with OT-II Th1
641 cells, boosted with OVA/CFA and periarticularly challenged with PBS or HAO (as in Figure
642 S1A). In addition, mice were either treated throughout with IgG isotype control or abatacept
643 (given 24 hours prior to footpad challenge and every 2 days thereafter). Mice were taken 4 days
644 after footpad challenge. Single cell suspension of draining lymph nodes (popliteal LNs) were
645 analysed by flow cytometry. Absolute number of (A) total cells and (B) endogenous CD4 T cells
646 in the popliteal LNs after PBS or HAO challenge and treatment with IgG or abatacept. (C)
647 Representative flow plots showing expression of CD44 and CD62L on endogenous CD4⁺ T
648 cells. (D) Percentage of endogenous CD4⁺ T cells that are CD44^{hi}CD62L^{lo} in the popliteal LN
649 after PBS or HAO challenge. (E) Representative flow plots of ICOS expression on endogenous
650 CD4⁺ T cells. (F) Percentage of endogenous CD4⁺ T cells that are ICOS⁺ in the popliteal LN
651 after PBS or HAO challenge and treatment with IgG or abatacept. Data is representative of 3
652 experiments; n=5 mice per group. **** represents p<0.0001.

653

654 **Supplementary Figure S6. Abatacept inhibits ICOS expression by antigen experienced**
655 **CD4⁺ T cell donor cells in the joint draining lymph node.** C57BL/6J recipient mice were
656 adoptively transferred with OT-II Th1 cells, boosted with OVA/CFA and periarticularly
657 challenged with PBS or HAO (as in Figure S1A). In addition, mice were either treated
658 throughout with IgG isotype control or abatacept (given 24 hours prior to footpad challenge and
659 every 2 days thereafter). Mice were taken 4 days after footpad challenge. Single cell suspension
660 of draining lymph nodes (popliteal LNs) were analysed by flow cytometry. Proportion (A) and

absolute number (B) of donor OT-II cells in the popliteal LNs after PBS or HAO challenge and treatment with IgG or abatacept are shown. (C) Representative flow plots showing expression of CD44 and CD62L on donor OT-II CD4⁺ T cells. (D) Percentage of donor OT-II CD4⁺ T cells that are CD44^{hi}CD62L^{lo} in the popliteal LN after PBS or HAO challenge with isotype control or abatacept. (E) Representative flow plots of ICOS expression by OT-II CD4⁺ T cells. (F) Percentage of endogenous CD4⁺ T cells that are ICOS⁺ in the popliteal LN after PBS or HAO challenge and treatment with IgG or abatacept. Data is representative of 3 experiments; n=5 mice per group. **** represents p<0.0001.

Movies

- 1. Movie 1:** Dynamics of OT-II T cell interactions with DCs in the inflamed joint.
- 2. Movie 2:** Dynamics of endogenous T cell interactions with DCs in the inflamed joint.
- 3. Movie 3:** Endogenous T cell/DC interactions in PBS, HAO challenged joints, with abatacept throughout, abatacept 6hrs or etanercept 6hrs prior to imaging.

Supplementary Movies

- 1. Supplementary movie S1:** Intravital MPLSM imaging of non-arthritis joints and CAIA arthritic joints in LysMGFP mice.

Figure 1: CD4⁺ T cells recruited to the joint have an activated phenotype and proinflammatory profile.

Figure 2: Imaging and analysis of T cell – DC interactions within the joint.

Figure 3: Antigen experienced Th1 cells of irrelevant specificity can be recruited to inflamed joints.

Figure 4: The impact of Abatacept on CD4⁺ T cell accumulation in joints in a model of RA.

Figure 5: Impact of therapeutic intervention on T cell – DC interactions in the joint

Supplemental Figure S1: CD4+ T cell recruitment to inflamed joints

Supplemental Figure S2: Endogenous T cells isolated from joints and draining LN do not make significant responses to OVA.

Supplemental Figure S3: Imaging inflammatory responses in the articular environment using the CAIA model.

Supplemental Figure S4 : TcR diversity in endogenous CD4⁺ T cells recruited to the inflamed joint.

Supplemental Figure S5: Abatacept inhibits CD4⁺ T cell activation in the joint draining lymph node

Supplemental Figure S6: Abatacept inhibits ICOS expression by antigen experienced CD4⁺ T cell donor cells in the joint draining lymph node