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***In vivo* two-photon imaging of T cell motility in joint-draining lymph nodes in a mouse model of rheumatoid arthritis**

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Abstract

Recent imaging studies on intact lymph nodes (LNs)¹ of naïve T-cell receptor (TCR)-transgenic mice have reported that T cells reduce their motility upon contact with relevant antigen-presenting cells (APCs). Using *in vivo* two-photon imaging of T cells in joint-draining (JD) LNs, we examined whether similar changes in T cell motility are observed in wild type mice. Co-transfer of T cells from naïve mice and antigen-experienced T cells from mice with proteoglycan (PG)-induced arthritis into naïve or arthritic recipients resulted in prolonged interactions of antigen-experienced T cells with APCs upon intra-articular antigen (PG) injection, indicating that T cells from arthritic wild type mice recapitulate the motile behavior reported in naïve TCR-transgenic mice. However, naïve T cells also engaged in stable interactions with APCs in the JDLNs of arthritic recipients, suggesting an enhanced ability of APCs in the JDLNs of arthritic hosts to present antigen to either naïve or antigen-experienced T cells.

Keywords

Proteoglycan-induced arthritis; two-photon microscopy; antigen presentation; autoimmunity; dendritic cell

¹ *Non-standard abbreviations used in this paper:* Ag, antigen; APC, antigen-presenting cell; Apc, allophycocyanin; BM, bone marrow; D, displacement; 2D, two-dimensional; DC, dendritic cell; DDA, dimethyldioctadecyl-ammonium bromide; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; JDLN, joint-draining lymph node; L, length; LN, lymph node; OVA, ovalbumin (from chicken egg white); PG, proteoglycan (aggrecan); PGIA, PG-induced arthritis; RA, rheumatoid arthritis; T, time; Tg, transgenic; TPM, two-photon microscopy; V, velocity; WT, wild type.

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Supplementary data

Supplementary material is available at *Cellular Immunology* Online.

Disclosure statement

The authors declare that they do not have any conflict of interest.

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1. Introduction

T-cell recognition of antigens, presented in the context of the major histocompatibility complex (MHC), initiates signaling through the T cell receptor (TCR). The threshold of TCR signaling is usually high, and co-stimulatory signals as well as prolonged physical contact between the antigen-presenting cell (APC) and the T cell are required for full-fledged T cell activation. Visualization of the motility of T cells and APC-T cell interactions *in vivo* by time-lapse two-photon microscopy has greatly advanced our understanding of the dynamics of T cell activation in lymph nodes (LNs) [1–5]. Because the TCR epitope repertoire is diverse, cognate T cells recognizing any single epitope are thought to be rare [6]. Therefore, most imaging studies that focus on the motility of T cells in the lymphoid organs (during priming or secondary exposure to antigen) employ genetically altered mice. These mice are engineered to express in a large population of their T cells a TCR specific for a single epitope of a model antigen, e.g., ovalbumin (OVA), a male-specific antigen (Db_y), or a glycoprotein from lymphocytic choriomeningitis virus (LCMV) [7–13].

Early imaging studies on intact LNs of mice described the motility of naïve wild-type (WT) and TCR-transgenic (TCR-Tg) T cells as a “random walk” [7,8]. However, a few hours after injection of cognate antigen, TCR-Tg, but not WT, T cells reduced their motility and exhibited “swarming” [7], consistent with prolonged APC-T cell interactions and directed (non-random) movement. Studies focusing on the kinetics of T cell motility found that priming of TCR-Tg T cells by antigen-loaded DCs was accompanied by three distinct phases in the motile behavior of T cells [9]. During the first 8 hours after entry into the LN, the T cells made only short contacts with DCs, but the length of these interactions and the number of stationary (immotile) T cells increased progressively during the next 12 hours [9]. On the second day (~48 hours), disengagement of the T cells from DCs was observed, followed by rapid, near-random movement [9], suggesting that at this late phase T cells were either undergoing proliferation or searching for exit routes. Studies using a different TCR-Tg system reported that T cells could establish long-lived interactions with APCs very early after antigen injection [14], and they also repeatedly decelerated due to engagement with APCs during all phases of priming [15]. The differences between the TCR-Tg systems and the experimental strategies employed in these studies make it impossible to time-resolve the exact sequence of motility changes or establish a universal model for the motile behavior of T cells during primary or secondary exposure to antigen. However, the consensus emerging from these studies is that encounter with APCs carrying antigen *in vivo* should lead to the arrest of the cognate T cell in order to ensure its activation *via* long-lived interactions with the relevant APCs, and that T cells resume their high motility when they are ready to divide or leave the LN.

Our primary goal in the present study was to determine whether antigen-experienced T cells from immunized WT mice could mimic the motile behavior of T cells isolated from naïve TCR-transgenic mice (reported in other systems) upon exposure to cognate antigen *in vivo*. An additional goal was to examine the kinetics of T cell motility in the context of an autoimmune inflammatory disease, since data resulting from studies on a disease model may provide clinically useful information. We used proteoglycan (PG)-induced arthritis (PGIA), a mouse model of rheumatoid arthritis (RA). PGIA can be induced in WT BALB/c mice by repeated intraperitoneal immunization with human articular cartilage PG (also termed aggrecan) [16–18]. Populations of human PG-specific (antigen-experienced) T cells gradually expand in the PG-immunized mice, and arthritis development depends on the loss of self-tolerance, i.e., emergence of T- and B-cell responses to mouse cartilage PG [17–19]. The autoimmune character and relevance of the PGIA model to the human disease are further underscored by the presence of “RA-specific” antibodies such as those against immunoglobulins (rheumatoid factor) and citrullinated proteins in the serum of arthritic

mice [18]. Since PGIA can be adoptively transferred to immunocompromised BALB/c mice by co-injection of T and B cells from arthritic donors and soluble antigen (human PG) [19–21], the transferred T cells represent a “pathogenic” population that can trigger arthritis upon re-activation with PG *in vivo*. Unlike naïve TCR-Tg T cells whose response to cognate antigen is an isolated immunological event, the response of antigen-experienced T cells from arthritic animals to *in vivo* antigen challenge is linked to the initiation of autoimmune arthritis. Thus, the antigen (PG)-induced changes in the motile behavior of these antigen-experienced T cells in the LNs may provide some information about the *in vivo* conditions of T cell activation, ultimately leading to destructive autoimmunity.

In the present study, we co-transferred T cells from naïve and arthritic donor mice into syngeneic naïve or arthritic recipients, and then challenged the donor cells with antigen (PG) injected into the ankle joints of the recipient mice. Using time-lapse two-photon microscopy [19], we then monitored the motility of transferred T cells in the ankle joint-draining (popliteal) LNs *in vivo* at different time points between 2 and 72 hours after the intra-articular injection of antigen. We asked the following questions: (i) Are differences in motility between naïve and antigen-experienced WT T cells detectable in the LNs of naïve recipients? If so, (ii) are these differences antigen-specific, and (iii) also observed in the LNs of arthritic hosts? (iv) Is the motile behavior of antigen-experienced WT T cells reminiscent of the reported motility changes of naïve TCR-Tg T cells after *in vivo* exposure to antigen? (v) Do naïve and antigen-experienced T cells compete with each other for access to APCs carrying the relevant antigen? Finally, we also sought to determine if (vi) the presence or absence of local inflammation (arthritis in the joint being drained by the LN) had any influence on the motile behavior of T cells in the LN.

2. Materials and methods

2.1. Mice, antigens, immunization for PGIA, and intra-articular antigen administration

Adult female WT BALB/c mice were purchased from the National Cancer Institute (Frederick, MD, USA). Transgenic BALB/c mice expressing a TCR specific for human cartilage proteoglycan (PG-TCR-Tg mice) have been described previously [22,23]. The majority of T cells in homozygous PG-TCR-Tg mice recognize the “5/4E8” immunodominant epitope (⁸⁹ATEGRVRVNSAYQDK¹⁰³) located in the first globular (G1) domain of human PG [23,24].

For immunization, PG was extracted from human osteoarthritic knee cartilage as described elsewhere [17,25]. Femoral condyles or tibial plateaus were obtained from consenting patients who had undergone total knee joint replacement surgery. Human cartilage collection was approved by the Institutional Review Board of Rush University Medical Center (Chicago, IL, USA). The antigen-adjuvant emulsion used for immunization contained 100 µg sterile PG protein dissolved in 100 µl phosphate buffered saline (PBS) and 1 mg dimethyldioctadecyl-ammonium bromide (DDA) (Sigma-Aldrich, St. Louis, MO, USA) adjuvant dissolved in 100 µl PBS per injection. This emulsion was injected intraperitoneally into WT female BALB/c mice three times at three-week intervals [16,17,25]. The mice were inspected for the symptoms of arthritis (swelling and redness of distal limbs) twice a week after the third immunization. As reported before [25,26], over 95% of the PG/DDA-injected animals developed arthritis within 8–14 days after the last immunization. Mice with PGIA were used as either cell donors or recipients within one week of disease onset. Naïve cell donors and recipients were non-immunized age-matched female BALB/c mice. Sterile PG or chicken ovalbumin (OVA; Sigma-Aldrich), 20 µg each, was dissolved in 10 µl PBS (without adjuvant) and injected into the ankle joint to activate T cells in the joint-draining popliteal lymph node (LN) prior to *in vivo* imaging. As a protein-free control, the same volume of PBS was injected into the ankle joints of a separate group

of mice. All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Rush University Medical Center.

2.2. Separation and labeling of T cells

These procedures have been described earlier [19]. In brief, spleens and joint draining (brachial, axillary, popliteal, and inguinal) LNs and spleens were harvested from naïve (non-immunized) or arthritic BALB/c mice. The spleen/LN cell suspensions were enriched in T cells by immunomagnetic removal of all non-T cells using a mouse T-cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) [19]. The purity of separated T cells was typically greater than 95%, as assessed by flow cytometry after cell surface immunostaining with anti-CD3 antibody (eBioscience, San Diego, CA, USA) using a FACS Canto II flow cytometer and FACS Diva software (BD Flow Cytometry Systems, San Jose, CA, USA) [19]. T cells separated from arthritic mice were usually labeled with a red fluorescent CellTracker dye (CMTPX, 5 μ M), and those separated from naïve mice with a green fluorescent CellTracker dye (CMFDA, 2.5 μ M) (both obtained from Molecular Probes, Invitrogen, Carlsbad, CA, USA). According to the manufacturer, intracellular conversion of these non-fluorescent compounds to fluorescent dyes requires esterase activity, thus the dyes stain only viable cells. After staining, the labeled cells were washed and incubated for 30 min at 37°C in culture medium containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Cell counts and viability were determined by trypan blue exclusion prior to transfer.

2.3 T cell transfer

Three major series of T cell transfer experiments were performed. First, we sought to visualize and analyze the motile behavior of naïve versus antigen-experienced T cells in the PG-loaded popliteal LNs of naïve hosts. Therefore, in the first series of experiments, we co-transferred 5×10^6 green fluorescence-labeled T cells isolated from naïve mice and 5×10^6 red-labeled T cells isolated from mice with PGIA intravenously into naïve recipients. Approximately 3 hours after cell transfer, PG was delivered to the popliteal LN *via* injection into the ankle joint cavity of the recipient mice. Control mice were transferred with similar mixtures of naïve and antigen (PG)-experienced T cells, but they were injected intra-articularly with either an irrelevant protein (OVA) instead of PG, or with PBS, as described above. *In vivo* imaging of T cells in the popliteal LN by two-photon microscopy (TPM) was performed at defined time points between 2 and 48 hours after antigen injection. To prevent the entry of circulating (new) lymphocytes to the LN during the imaging sessions [9], 250 μ g of the L-selectin blocking monoclonal antibody (mAb) MEL-14 [27] was injected intravenously at the time of antigen injection (to mice imaged between 2 and 8 hours), or at 22, 46 and 70 hours, respectively, to mice imaged at 24, 48, and 72 hours after antigen administration.

To study the motility of T cells in the popliteal LN of arthritic hosts (experimental series 2), green fluorescent T cells from naïve and red fluorescent T cells from arthritic donor mice were co-transferred into arthritic recipients that also received intra-articular PG injections after cell transfer. The popliteal LNs of the T cell-recipient mice were subjected to *in vivo* TPM imaging at 4, 24, 48 and 72 hours after PG injection. As described above, recipients received MEL-14 mAb 2 hours before the scheduled imaging sessions.

Finally, to determine if competition occurred between naïve and antigen-experienced T cells [13] (experimental series 3), we transferred 1×10^7 T cells, isolated from naïve donors only, into arthritic recipient mice. PG was injected into either inflamed or non-inflamed ankle joints of otherwise arthritic recipients (i.e., recipient mice having inflammation in other

joints). The animals received MEL-14 mAb at the same time points as in experimental series 2, and TPM imaging was performed at 4, 24, 48, and 72 hours after T cell transfer.

In a limited set of experiments, we injected bone marrow (BM)-derived fluorescence-labeled dendritic cells (DCs) together with PG into the arthritic joints before T cell transfer and visualized the interaction of DCs with naïve or antigen-experienced T cells in the popliteal LN. DCs were generated *in vitro* by culturing BM cells in the presence of murine granulocyte-macrophage colony stimulating factor (GM-CSF) for 10 days [28,29].

2.4. In vivo two-photon microscopy (TPM)

In vivo TPM imaging of cells in the popliteal LN has been described in detail in a previous paper [19]. In brief, imaging was conducted on the popliteal LN of live anesthetized mice. We used a Prairie Ultima TPM system (Prairie Technologies, Middleton, WI, USA) composed of a Ti:Sapphire femtosecond-pulse laser driven by a Chameleon Ultra pumping laser (Coherent, Santa Clara, CA, USA) tuned to 810 nm, and an Olympus BX51WI microscope (Olympus, Center Valley, PA, USA) with a 40X (0.8 numerical aperture) water-dipping lens wrapped in an objective heater (Bioscience Tools, San Diego, CA, USA). The position of the microscope stage, laser scanning, and image acquisition were controlled *via* Prairie View software (Prairie Technologies). To image the fluorescent cells in the tissue, images were acquired at different emission wavelengths: red CMTX labeled cells were visualized using a fluorescence filter with a cut-off range of 570–626 nm, and green CMFDA labeled cells with a 500–550 nm filter. Emitted fluorescent light was detected by photo-multiplier tubes (Hamamatsu, Hamamatsu City, Japan). During image acquisition, laser power and detector gain and offset levels were set to minimize both photodamage to the tissue and background noise. To monitor cell motility, two-dimensional (2D) time-lapse series were created by acquiring images every 30 second at the same z plane within the T cell zone, usually at a depth of 70 μm or greater below the surface of the LN [19]. 3D image stacks were occasionally acquired by scanning 2 to 5 x-y planes within a 2–10 μm -thick tissue segment along the z axis.

2.5 Image processing and analysis

TPM images were processed using Imaris software (v. 6.1.3 or 7.2.3; Bitplane, South Windsor, CT, USA). Post acquisition, serial 2D images were used to build time-lapse series, and 3D images were reconstructed from z series stacks. The images were adjusted for optimal brightness, contrast, background, and color intensity. Cell movement in 2D time-lapse series was analyzed using Imaris Track. Drift correction was performed using tissue structure elements that moved together (due to the micro-movement of the breathing animal) and employing a displacement filter built in Imaris Track. The computer-rendered cell tracks were then reviewed and edited manually until they matched the real cell paths. The cell motility parameters analyzed were as follows: (i) displacement ([D], the distance between the start and end points of a cell track; in μm), (ii) average velocity ([V] path length [L] divided by time [T] [L/T; in $\mu\text{m}/\text{min}$), (iii) 2D motility coefficient ($D^2/4T$; in $\mu\text{m}^2/\text{min}$), which was calculated as the slope of the mean displacement plotted against the square root of time ($D/T^{1/2}$; in $\mu\text{m}/\text{min}^{1/2}$) [5,7], and (iv) the proportion of immotile cells as the percent (%) of all cells tracked. T cells were considered immotile if they fulfilled the following criteria: (i) They were visible for at least 10 min (did not leave the imaging plane), (ii) their paths were confined to a 20 $\mu\text{m} \times 20 \mu\text{m}$ area within the x-y plane [5], and (iii) their velocity did not exceed 4.5 μm (approximately half of a cell's diameter) per min at any time during the imaging session. The data was exported to Microsoft Excel spreadsheets for analysis. In all T cell transfer experiments, the total number of T cells (either naïve or antigen-experienced) analyzed in the JDLNs ranged from 111 to 216, using 3–5 mice at each time point post antigen injection.

2.6 MHC II-peptide tetramer binding assay

We used an allophycocyanin (Apc)-labeled human PG (aggrecan)-specific tetramer (PG tetramer) composed of the human PG “5/4E8” peptide (⁸⁹ATEGRVVRVNSAYQDK [22–24]) linked to I-A^d (BALB/c haplotype-matched MHC class II molecule), and an irrelevant control Apc-labeled tetramer composed of a human class II-associated invariant chain peptide (CLIP; ¹⁰³PVSKMRMATPLLMQA) linked to I-A^d (CLIP tetramer). Both tetramers were provided by the NIH Tetramer Core Facility at Emory University (Atlanta, GA, USA). T cells were purified from the spleens and LNs of naïve and PG-immunized (arthritic) WT BALB/c mice as described in section 2.2. T cells purified from naïve heterozygous PG-TCR-Tg BALB/c mice (in which up to 30% of T cells express the vβ4 chain of the PG-specific TCR [30]) served as a positive control for PG tetramer binding. Following purification, the three populations of T cells (naïve WT, antigen [PG]-experienced WT, and naïve PG-TCR-Tg) were cultured separately for 24 hours with 5 ng/ml murine interleukin (IL)-2, as we found that the robustness of tetramer binding could be greatly increased by IL-2 pretreatment. Before addition of tetramers, the antigen-experienced WT T cell population was labeled with red fluorescence as described in section 2.2, while the other two populations (naïve WT and naïve PG-TCR-Tg cells) were left unlabeled. The T cell samples for the tetramer binding/competition assay were set up as follows: (i) naïve T cells alone, (ii) antigen-experienced WT T cells alone, (iii) PG-TCR-Tg T cells alone, or antigen-experienced (red fluorescent) WT T cells mixed with unlabeled T cells from either (iv) naïve WT or (v) naïve PG-TCR-Tg mice. The single cell populations contained 1.2×10^6 cells, and the mixtures contained 0.6×10^6 cells of each of the two cell types. The cells samples were incubated for 16 hours in the presence of 0, 0.3, 0.6, 1.2, or 2.4 μg Apc-labeled PG tetramer or Apc-labeled CLIP tetramer in 100 μl DMEM medium containing 10% FBS under standard tissue culture conditions. The cells were then washed and surface-labeled with Fitc-conjugated anti-mouse CD4 antibody or Fitc-rat IgG2b (isotype control) antibody (both from eBioscience). The percent of tetramer-binding (Apc⁺) CD4⁺ T cells in each sample was determined by flow cytometry (as described in section 2.2) after gating separately on red fluorescent (antigen-experienced WT) and unlabeled (naïve WT or naïve PG-TCR-Tg) cells within the Fitc-CD4⁺ population. Following preliminary dose optimization experiments, two replicate assays were performed as described above.

2.7 Statistical analysis

Statistical analysis was performed using Microsoft Excel's Analysis ToolPak and SPSS software (v. 15, SPSS, Chicago, IL, USA). Two groups were compared using Student's t test, or the Mann-Whitney u test (for non-parametric data). P values of less than 0.05 were accepted as statistically significant.

3. Results and discussion

3.1 Naïve and antigen (PG)-experienced T cells show differences in motility in the joint-draining popliteal LN of the naïve host upon encountering intra-articularly injected PG

First, we sought to determine if cognate T cells from PG-immunized (arthritic) BALB/c mice and cognate T cells from naïve (non-immunized) BALB/c mice display differences in motile behavior in the LN of the naïve host in response to *in vivo* antigen (PG) challenge. In this setting, the rare naïve cognate T cells would be primed, and the more numerous cognate T cells in the antigen-experienced population would be re-stimulated, by APCs found within the LN. The TCRs of antigen-experienced cognate T cells would also possess higher affinity to the MHC-peptide complex than the TCRs of naïve cognate cells [12]. We hypothesized that the combination of these quantitative (cognate cell frequency) and qualitative (affinity of the TCR of individual cells) differences between the two T cell populations could be sufficient to result in different motile behavior in the LN upon transfer to naïve mice. To test

this, we co-transferred naïve and antigen (PG)-experienced T cells to naïve recipient mice, injected PG into the ankle joint, and then monitored the motility of the T cells by *in vivo* TPM. Consistent with an initial “transient interactions” phase, observed between antigen-pulsed dendritic cells (DCs) and T cells in some other model systems [4,9,12], both naïve and antigen-experienced T cells exhibited high motility in the LNs at an early time point (2 hours) after antigen administration (Fig. 1A and B). T cell motility decreased by 4 hours, and slightly increased afterwards, but remained slightly below the initial level at 6 hours after antigen injection. Importantly, over this 4-hour period, the antigen-experienced T cells exhibited significantly reduced motility as compared with naïve T cells (Fig. 1A and B). At 4 hours, the percent of immotile (stationary) T cells in the antigen-experienced population (Fig. 1B, hatched bars) was also significantly higher than the percent of immotile naïve T cells (Fig. 1B, open bars). The low level of cell motility at this time point suggested that the presentation of antigen became the most efficient in the joint-draining LN 4 hours after intra-articular administration, leading to the arrest of T cells, preferentially of those from the antigen-experienced population. Studies using other model systems [9,15] have reported that cognate T cells may exhibit low motility for several hours upon engagement with antigen-loaded APCs (such as DCs). To investigate this, we took the 4-hour time point as a start, and monitored the motile behavior of naïve and antigen-experienced T cells in the joint-draining LN for up to 48 hours after intra-articular PG injection. Again, at 4 hours, antigen-experienced T cells showed significantly reduced motility as compared with the naïve counterparts (Fig. 1C and D). Naïve T cells exhibited nearly “random walk” [5,7,31], whereas the movement of antigen-experienced T cells was much more constrained, as indicated by the differences between them with regard to their motility coefficients (Fig. 1D) and displacement plots (Fig. 1E, left-side panel). The reduced motility of antigen (PG)-experienced T cells versus naïve T cells was clearly associated with the preferential recognition of PG by the former, as injection of an irrelevant protein (OVA) or PBS did not lead to different motile behavior between these populations at 4 hours after intra-articular injection (Fig. 1E, middle and right-side panels) or later (data not shown). However, at 8 hours after PG administration and beyond, both naïve and antigen-experienced T cells became highly motile again (Fig. 1C and D). The proportions of immotile cells in both populations also dropped dramatically after 4 hours (Fig. 1D and Fig. 2A and B). Together, these results suggest that a single injection of PG into the naïve host leads to short-term presentation of this antigen and transient interaction of T cells with APCs. Although APCs were not visualized in this study, it is conceivable that the more pronounced deceleration of antigen-experienced versus naïve T cells was due to the presence of a larger number of cognate cells and their higher affinity to MHC-PG peptide complexes in the antigen-experienced than in the naïve T cell population.

3.2 Antigen-experienced, but not naïve, T cells engage in long-lived interactions with APCs in the joint-draining LN when co-transferred into arthritic hosts

APCs isolated from mice with PGIA present PG to antigen-experienced T cells much more efficiently than APCs from naïve mice *in vitro* [17]. Therefore, we asked whether the arrest of antigen-experienced T cells would be more profound and would last longer in the LN of the arthritic than in the naïve host. We also sought to determine if the high efficiency of antigen presentation in this milieu would affect the motility of naïve T cells compared to their behavior in the “naïve” environment. To address these questions, we co-transferred naïve and antigen-experienced T cells into arthritic mice, and monitored the motility of the two cell populations after intra-articular administration of PG. As seen in Fig. 3A and B, the velocity and overall motility of antigen-experienced T cells were dramatically reduced for up to 48 hours, which prompted us to add an even later time point (72 hours). By 72 hours after PG injection, the motility of these cells increased considerably. The arrest of naïve T cells was also more pronounced in the arthritic than in the naïve LN at 4 hours (compare

Fig. 1 with Fig. 3). In fact, both naïve and antigen-experienced T cells were arrested in nearly equal proportions in the LN of the arthritic host at this early time point (Fig. 3B and Movie 1). However, naïve cells showed signs of “disengagement” by 24 hours, and resumed high motility by 72 hours after PG administration (Fig. 3 and Movie 2). This behavior of naïve T cells suggested that they were strongly, but still only transiently, engaged with APCs in the arthritic LN, or that their access to APCs was limited because they were outcompeted by the antigen-experienced cells in this environment [13].

3.3 Competition for antigen can be demonstrated *in vitro* and is proportional with the frequency of antigen-specific T cells among the competing populations

T cells keep re-circulating among lymphoid organs until they gain access to both cognate antigen and co-stimulatory signals sufficient to initiate an activation program, or they die in a few days if activating stimuli are absent. Because the amount of antigen (presented by APCs in the contexts of MHC and co-stimulatory molecules) is usually low within individual LNs [12], T cells need to compete with each other. Antigen-experienced T cells from an immunized animal should have a clear advantage in this competition, due to both their higher precursor frequency and higher affinity to the relevant antigen in comparison with naïve T cells.

As described above (section 3.2), we co-transferred large numbers of both naïve and antigen-experienced T cells to the host mice, but the amount of intra-articularly injected antigen (PG) that could reach the hosts' JDLNs was likely very low, together establishing the basis for T cell competition [32]. Therefore, we sought to determine if antigen (PG epitope)-specific competition between naïve and PG-experienced T cells could be demonstrated *in vitro*. To measure antigen binding to T cells by flow cytometry, we used two fluorochrome (Apc)-labeled MHC class II-peptide tetramers: an antigen-specific tetramer composed of I-A^d and a PG peptide representing an immunodominant epitope (“5/4E8” epitope [24]) within the PG molecule, and a non-specific I-A^d-CLIP tetramer as a control. The binding of the PG tetramer to each T cell population was clearly detectable even at a suboptimal (non-saturating) dose of the tetramer (Fig. 3C and D, and Supplementary Table 1). However, fewer T cells of the naïve WT population bound the PG tetramer in the presence than in the absence of PG-experienced WT T cells (Fig. 3C, middle left and bottom panels, respectively), indicating that naïve T cells were outcompeted by the antigen-experienced T cells for tetramer binding. The specificity of PG tetramer binding could be confirmed by demonstration of further competition between antigen-experienced WT T cells and naïve PG-TCR-Tg T cells (Fig. 3D), the latter of which contains a significant proportion of cells expressing a TCR specific for the exact epitope represented by the PG peptide in the tetramer [22,23]. Competition was not observed at higher (saturating) doses of the PG tetramer, neither was it observed for T cells incubated with the control CLIP tetramer at any dose (Supplementary Table 1).

Collectively, the results of the *in vitro* antigen (PG tetramer) binding assay indicate that T cells compete for binding of an antigenic peptide when the availability of this particular epitope is limited, and that the binding capacity of T cells is proportional with the relative number (and perhaps TCR affinity) of cognate T cells in the competing populations. The *in vitro* data also suggest that the differences in the motility of co-transferred naïve and antigen-experienced T cells *in vivo* are likely due to competition between these populations for access to APCs presenting PG epitopes.

3.4 Naïve T cells engage in long-term interactions with APCs in the LN of arthritic host when exogenous antigen-experienced T cells are absent

To examine the effect of the lack of competition on the *in vivo* motility of naïve T cells, we next asked whether removal of antigen-experienced competitors from the transferred (exogenous) population would increase the length or frequency of interactions between APCs and naïve T cells in the LN of the arthritic host. Therefore, we transferred only naïve T cells (total cell number was as the same as in the mixture) into arthritic mice, and monitored their motility for up to 72 hours after intra-articular PG injection. As shown in Fig. 4A and B, naïve T cells were found arrested at 4 hours, and remained in a nearly immotile state at 24 hours. Interestingly, their motility coefficient increased and the percent of stationary cells dropped in an almost linear fashion between 24 and 72 hours (Fig. 4B). Overall, the motility dynamics of naïve T cells in this setting suggested that they, indeed, had better access to antigen presented by APCs in the absence than in the presence of transferred antigen-experienced competitors [13], although they probably still needed to compete with endogenous antigen-experienced T cells in the LN of the arthritic host.

3.5 Joint inflammation modulates the motile behavior of naïve T cells in the draining LN

Since antigen was delivered to the ankle joint-draining (JD)LNs after injection into inflamed ankles of arthritic hosts, we sought to determine whether the motility of naïve T cells was the same if antigen was injected into a non-arthritic joint. To test this, after the i.v. transfer of naïve T cells, we injected PG into non-inflamed (not visibly swollen) ankle joints of recipients that had arthritis in other joints. Although the velocity of T cells in the non-arthritic JDLNs was slightly higher than the velocity of those in the arthritic JDLNs at 4 and 24 hours, T cells in the former resumed their high motility much later than those in the latter (compare Fig. 4A and B with Fig. 4C and D). Whether the delayed disengagement of naïve T cells in non-inflamed JDLNs is a result of less efficient priming as compared to priming in inflamed JDLNs at 4 and 24 hours, or a result of the absence of the effects of inflammatory mediators on the process of antigen presentation, cannot be determined solely on the basis of T cell motility. However, this observation does suggest that inflammation (in this case, arthritis) has a modulating effect on the dynamics of antigen presentation in LNs that drain inflammatory sites.

3.6 Antigen-experienced T cells make contacts with recently migrated DCs but preferentially engage in long-lived interactions with LN-resident APCs

In order to visualize APC-T cell interactions [4], in a limited set of experiments we co-injected fluorescence-labeled DCs and antigen into the joints of arthritic mice following transfer of either naïve or antigen-experienced T cells. By 24 hours after the intra-articular injection, a number of DCs could be found in the T cell zones of the JDLNs (Supplementary Fig. S1). Both naïve T cells (Fig. S1A) and antigen-experienced T cells (Fig. S1B) made contacts with these DCs, but the interactions were mainly transient (lasting for less than 10 min). However, close inspection of TPM movies revealed that both naïve (not shown) and antigen-experienced T cells (Fig. S1C) preferentially sought long-term engagement with autofluorescent “spots” that presumably represented the endocytic vesicles of resident APCs in the JDLNs of arthritic hosts (Supplementary Fig. S1C, and Movie 3). From this observation, we conclude that visualization of endogenous APCs, e.g., by imaging host mice expressing fluorescent proteins in DCs [10,33] and/or in other APCs, would be required for the correct interpretation of interactions between APCs and T cells in the lymphoid organs of PG-immunized/arthritic mice after antigen injection.

In summary, using *in vivo* TPM, here we show that antigen (PG)-experienced T cells from PG-immunized, but genetically unmanipulated, mice recapitulate the reported motile behavior of naïve TCR-Tg cells, as the motility of antigen-experienced WT T cells appears

to change in a similar fashion as naïve TCR-Tg cells upon encountering APCs that carry cognate antigen in the LNs. In our system, the differences between co-transferred naïve and antigen-experienced WT T cells in motility dynamics were clearly detectable regardless whether the cells were co-transferred into naïve or PG-immunized (arthritic) recipient mice. This indicates that repeated immunization of WT mice with an antigen (such as PG) in adjuvant can considerably increase the proportion of antigen-experienced (memory) T cells that express PG-specific TCRs. Therefore, at least in the PGIA model, the frequency of PG-specific T cells in the transferred population is sufficient for meaningful analyses of T cell motility in the context of the presentation of this antigen in the LNs of either naïve or arthritic recipient mice. It is likely that the TCRs of PG-experienced memory cells have higher affinity to MHC-PG peptide complexes (as suggested by our PG tetramer binding experiment) and higher expression of co-stimulatory and adhesion molecules than naïve T cells, which together would facilitate the establishment of long-lived contacts between antigen-experienced T cells and APCs. The prolonged engagement of antigen-experienced T cells with APCs may limit the access of the co-transferred naïve counterparts to the same APCs, as demonstrated in the *in vivo* and *in vitro* competition experiments of our study. Comparison of the motile behavior of naïve T cells in naïve versus arthritic recipients also reveals that the arrest of naïve cells, at least at the early phase of antigen presentation, is much more robust in the arthritic than in the naïve LN environment. The increased frequency and high antigen-presenting potency of APCs in the arthritic host partly explains this difference, but additional factors may contribute. For example, besides PG epitopes, new peptide epitopes (including autoepitopes), generated in the host mice upon repeated immunization and/or inflammatory joint destruction [17,25], might be presented to the transferred naïve T cells. It has been shown that self-tolerance is broken in BALB/c mice during the induction of PGIA, as the arthritic mice exhibit reactivity with self PG [17], as well as with PG-unrelated other antigens such as citrullinated proteins and self IgG [18]. Such reactivities are thought to arise as a result of intra- and intermolecular epitope spreading in either BALB/c mice with PGIA or patients with RA [18,34–37]. It is likely that APCs in the LNs of mice with PGIA are able to present a large repertoire of antigenic peptides with diverse specificities, for many of which cognate T cells may exist in the transferred naïve, as well as in the antigen-experienced populations. Consistent with this, even antigen-experienced T cells seem to preferentially engage in long-lasting interactions with endogenous APCs, and they only transiently contact recently emigrated exogenous DCs in the LNs after the transfer of these DCs into arthritic recipient mice. Finally, we have found that naïve T cells disengage from APCs in the LNs of arthritic hosts more rapidly the presence than in the absence of inflammation (arthritis) at the site of antigen injection. The biological significance of this latter phenomenon, and whether it is observed only in arthritis (PGIA) or also occurs in other models of autoimmunity and inflammation, remain to be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We monitor activation-related T cell motility in vivo in genetically unaltered mice.

We visualize T cells in joint-draining lymph nodes in an arthritis model.

Antigen-experienced T cells show motility similar to that in TCR transgenic mice.

T cells are preferentially arrested in joint-draining lymph nodes of arthritic mice.

The motility of naïve T cells is influenced by cellular competition and inflammation.

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Fig. 1.

Changes in the motile behavior of co-transferred wild type (WT) naïve and antigen (Ag)-experienced T cells over time in the popliteal lymph node (LN) of naïve hosts in response to intra-articular antigen administration. (A and B) Naïve WT BALB/c mice received a 1:1 mixture of fluorescence-labeled T cells isolated from naïve donors and from mice with proteoglycan (PG)-induced arthritis (PGIA). Differentially labeled naïve and PG antigen (Ag)-experienced T cells in the popliteal LNs were subjected to two-photon microscopy (TPM) hourly between 2 and 6 hours after intra-articular injection of PG into the hosts. (A) Average velocities of naïve (open circles) and Ag-experienced (closed circles) T cells are shown at the indicated time points of this short-term experiment. (B) The percent of immobile naïve (open bars) and Ag-experienced (cross-hatched bars), and their motility coefficients are shown at each time point. The results represent the means $\pm$ SEM. Asterisks denote statistically significant differences between the naïve and Ag-experienced T cells ($p < 0.05$). (C and D) A similar but longer-term imaging experiment with a time frame of 4 to 48 hours after intra-articular PG injection. (C) Velocity and (D) the percent of immobile naïve and Ag-experienced T cells and the motility coefficients are shown at the indicated

four time points. The results are expressed and significant differences are indicated as in (A and B). (E) The motility profiles of naïve (open circles) and Ag(PG)-experienced (closed circles) T cells were compared in the LNs of naïve mice 4 hours after intra-articular injection of PG (left-side panel), ovalbumin (OVA; middle panel), or phosphate buffered saline (PBS; right-side panel). In each panel, the mean displacement is plotted against the square root of time. The data demonstrate that the movement of PG-experienced T cells is constrained after PG injection, but not after OVA or PBS injection.

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Fig. 2.

Representative TPM images and illustration of the motility of co-transferred naïve and antigen (Ag)-experienced T cells in the popliteal LN of the naïve host between 4 and 48 hours after intra-articular antigen injection. (A) Representative images of naïve (green fluorescence-labeled) and antigen-experienced (red fluorescence-labeled) T cells in the LN at 4, 24, and 48 hours after intra-articular injection of PG into naïve recipient mice. The images (taken from the long-term experiment shown in Fig. 1C and D) show a large proportion of immotile (round) T cells in each population at 4 hours, whereas the T cells display elongated shapes (consistent with higher motility) at later time points. (B) Illustration of T cell motility in the same experiment by color-coded cell tracks. Blue and magenta spheres (both with short tracks) indicate immotile naïve and Ag-experienced T cells, respectively. Motile naïve and motile Ag-experienced T cells are depicted with long green and long red tracks, respectively. The number of immotile (blue and magenta) T cells decreases, and the relative proportion of motile Ag-experienced T cells (red spheres with long red tracks) noticeably increases over time.

Fig. 3.

Motility of co-transferred naïve and antigen (Ag)-experienced T cells in the popliteal LN of the arthritic host *in vivo*, and competition between naïve and Ag-experienced T cells for MHC-PG peptide tetramer binding *in vitro*. The (A) velocity and (B) the motility coefficients with the percentage of immotile cells are shown for the co-transferred naïve (open circles/open bars) and Ag-experienced (closed circles/hatched bars) T cells in the LNs of antigen-injected arthritic recipient mice between 4 and 72 hours after PG injection. The results are expressed and significant differences are indicated as in Fig. 1. (C and D) Flow cytometry scatter plots demonstrate competition between T cell populations for MHC-PG peptide tetramer binding *in vitro* at a suboptimal dose (0.6 μg for 1.2 million cells) of the tetramer. (C) The percent of naïve T cells that bind the I-A^d-PG peptide tetramer (middle

left panel) is lower when they are mixed with Ag (PG)-experienced T cells (middle right panel) than if incubated alone (bottom panel) with the tetramer. (D) The Ag (PG) specificity of tetramer binding is confirmed by the observation that fewer Ag-experienced T cells bind the tetramer in the presence (middle right panel) than in the absence (bottom panel) of competing T cells from naïve PG-TCR-Tg mice (middle left panel). Red fluorescence was used to distinguish Ag-experienced T cells from unlabeled naïve WT or PG-TCR-Tg T cells within the CD4⁺ cell gate (top panels). The percentages of cells that bound the allophycocyanin (Apc)-labeled PG tetramer (PG tetramer-Apc) are indicated in boldface within the middle and bottom scatter plots. This type of competition was not observed at higher (saturating) doses of the tetramer (Supplementary Table 1). The data shown are from one of two replicate experiments with similar results.

Fig. 4.

Motility of transferred naïve T cells in the popliteal LNs of arthritic hosts over time in response to antigen injection into inflamed (A and B) or non-inflamed (C and D) ankle joints. (A) Velocity and (B) percent of immotile cells (open bars) and their motility indices (open circles) are shown for the naïve T cells (transferred without antigen-experienced T cells) in the LNs of arthritic recipient mice between 4 and 72 hours after PG injection into inflamed joints. (C and D) A similar set of experiments was carried out, except that the motility parameters of transferred naïve T cells in the popliteal LN were determined between 4 and 72 hours after injection of PG into non-inflamed ankle joints of otherwise arthritic recipients. The data shown are the (A) velocity, and (B) percent of immotile cells and their motility coefficients. The results are expressed as in Fig. 1. Asterisks indicate statistically significant changes over time relative to the starting time point (4 hours).