Characterization of the trafficking and motility of T cells in the joints and joint-draining lymph nodes in an autoimmune murine model of rheumatoid arthritis

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Contents

Keywords and List of Abbreviations

Keywords

Proteoglycan-induced arthritis, Two-photon microscopy, Antigen presentation, Autoimmunity, T cell, Dendritic cell, Rheumatoid arthritis

Tárgyszavak

Proteoglikán indukált artritisz, Két foton mikroszkópia, Antigén prezentáció, Autoimmunitás, T sejt, Dendritikus sejt, Reumatoid artritisz

Abbreviations

Ab: antibody

Ag: antigen

APC: antigen-presenting cell

Apc: allophycocyanin

autoAb: autoantibody

autoAg: autoantigen

CIA: collagen-induced arthritis

CII: type II collagen

DC: dendritic cell

DDA: dimethyl dioctadecyl ammonium bromide

EGFP-LysM KI: enhanced green fluorescent protein-lysozyme M knock-in

GC: germinal center

IFN-γ: Interferon gamma

IL: Interleukin

JDLN: joint-draining lymph node

LN: lymph node

mAb: monoclonal antibody

MHC: major histocompatibility complex

OVA: ovalbumin (from chicken egg white)

PBS: phosphate-buffered saline

PG: proteoglycan (cartilage aggrecan)

PGIA: proteoglycan-induced arthritis

RA: rheumatoid arthritis

SCID: severe combined immunodeficient

TCR: T cell receptor

Teff: effector T cell

Tfh: follicular T helper cell

Tg: transgenic

Th: T helper

TPM: two-photon microscopy

Treg: regulatory T cell

TNF-α: tumor necrosis factor alpha

WT: wild type

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects 1% of the world's population. It is characterized by symmetrical deforming peripheral polyarthritis at early stages that progresses to a systemic illness with extraarticular organ involvement and chronic inflammation that causes joint destruction [1]. During the development of the disease, the presence of circulating antibodies (Abs) against immunoglobulins (rheumatoid factor, RF) and citrullinated proteins [2-4] can be detected in the serum years before the appearance of joint inflammation [5]. The presence of autoAbs in the serum indicates the importance of T cell-B cell interaction that takes place predominantly in the secondary lymphoid organs. By providing help to B cells, the involvement of autoreactive T cells in the pathogenesis of RA as well as in autoimmune animal models of RA has been well established, however, unanswered questions, such as the role of joint-homing T cells, remain. It is well recognized that T cells indirectly facilitate disease pathogenesis, however, some studies suggest a more direct involvement of joint-homing T cells by means of direct initiation and propagation of local inflammation [3, 6, 7].

Animal models of RA are superb experimental tools in demonstrating how T cells trigger joint inflammation, and thus can help to further our knowledge of disease mechanism and potential therapies [8-11]. In mouse models, including type II collagen (CII)-induced arthritis (CIA) and proteoglycan (PG)-induced arthritis (PGIA), major histocompatibility complex (MHC) II restricted antigen (Ag) presentation results in the generation of T cells and that cross-react with auto Ags such as mouse CII or mouse PG [12-16]. PGIA can be induced in wild type (WT) BALB/c mice by repeated intraperitoneal immunization with human articular cartilage PG in adjuvant [16-18]. As human PG specific T cells expand, arthritis develops after the animals' T and B cells start to respond to mouse cartilage PG as well [16, 17]. PGIA can be adoptively transferred from arthritic donors to syngeneic severe combined immunodeficient (SCID) mice by co-injection of T and B cells from arthritic donors and human PG without

adjuvant [19, 20]. The transferred cells can trigger arthritis upon re-activation with PG in vivo.

The role of T lymphocytes found in joints in the development of RA has been investigated for decades, but no definitive 'arthritogenic' T-cell populations have yet been identified [7]. Due to the low levels of T cell-derived cytokines in the rheumatoid synovial samples and the hypo-responsiveness of joint-infiltrating T cells to T cell receptor (TCR) stimulation, earlier studies could not find a direct role for T cells in the local inflammatory processes. Nonetheless, interferon gamma (IFN-γ) producing T helper 1 (Th1) cells and IL-17 producing Th17 cells are thought to have a major role in the development of synovial inflammation and Th17 cells are believed to contribute to cartilage and bone damage in RA [21, 22]. The inflamed synovium in RA patients contains more Th1 cells than Th17 cells [23, 24]. The reason for that might be the result of epigenetic instability of the Th17 cell chromatin structure, which is most important to phenotypic plasticity [25]. However, IFN-γ and IL-17 producing bifunctional Th1-Th17 cells have been identified the synovial fluid but not in the circulation of patients with RA [24-30]. Synovial fluid Th17 cells were also shown to produce both IL-17 and INF-γ ex-vivo. This could not be demonstrated with Th17 cells of the peripheral blood [23, 26]. The Th1 cell promoting cytokine IL-12 promotes conversion of peripheral blood Th17 cells to Th1 [26]. This might be one of the reasons of the increased ratio of Th1 to Th17 cells in the inflamed joints of patient with RA [23, 26]. PGIA in mice is a Th1 driven disease [31], as IFN-γ is necessary for the onset and progression of arthritis. Without IFN-γ the incidence and severity of arthritis are reduced and the absence of this cytokine also leads to weaker PG-specific IgG2a responses [32]. One advantage of using DDA (dimethyl-dioctadecyl ammonium bromide) over Freund's adjuvant (CFA) for immunization of mice for PGIA is that it induces a Th1 shift which leads to a more severe manifestation of the disease. High IFN-γ levels also reduce the number and percentage of activated T regulatory (Treg) cells [33]. Th2 shift induced by administration of Th2 type cytokines (IL-4 or IL-10) on the other hand lead to a less severe disease [15, 32], in part by inhibition of PG-specific IFN-γ responses and by reducing proinflammatory cytokine expression at target sites [31]. Unlike other mouse models such as collagen-induced arthritis (CIA), which is more Th17 driven [27], in

PGIA the lack of IL-17 have no effect on disease severity or inflammatory cell recruitment and PG-specific T cell and IgG2a Ab generation [28]. Moreover, the use of the Th17-shift inducing CFA adjuvant for immunization leads to a somewhat less severe disease manifestation [29].

With regard to other Th subsets, follicular T helper (Tfh) cells promote the development of high-affinity memory B cells and long-lived plasma cells in the germinal centers (GC) of lymph nodes (LN). Elevated number of Tfh has been noted in RA [34]. The failure of these cells to maintain self-tolerance and their role in autoimmunity have been thoroughly investigated [34-36]. In PGIA, mice lacking programmed death 1 ligand (Pdl1) showed increased autoreactive Th1 cell responses and elevated frequency and activation status of Tfh cells. This led to death of GC B cells in the spleen and worsening of arthritis symptoms [37]. Pdl1 regulates autoimmunity by suppressing autoreactive CD4+ T-cell responses. It also promotes B cell survival by restraining the expansion and activation of Tfh cells through interaction with programmed death 1 (PD1). Th1 to Tfh and dual Th1-Tfh developmental plasticity has been described in mouse models of other diseases [38] however it is yet to be demonstrated in RA or PGIA.

Treg cells can suppress effector T (Teff) cells, but the resistance of Teff cells to inhibition by Tregs might be one of the reasons for the loss of control and the perpetuation of autoimmune inflammation in RA. There are two known populations of Treg cells in human: the thymus derived natural and the peripherally induced Treg cells. Although they have shared and distinct properties, both have the ability to suppress Teff cells [23]. In PGIA depletion of Treg cells with anti-CD25 Ab led to severe arthritis in previously asymptomatic PG-immunized mice [39]. Also, administration of a B cell depleting anti-CD20 Ab (a rituximab analog) led to increased number of FOXP3$^+$ Treg cells and reduced disease severity in PGIA [33]. Depletion of these induced Treg population restored the severity of the disease to a level comparable with untreated mice [33]. In both mice and humans there is a high degree of reciprocal plasticity between Th17 and Treg cell subsets [23, 40-42]. Proinflammatory conditions in the inflamed joints and cytokine (mainly IL-2) consumption of Treg cells enhances Th17

development and a small portion of unstable peripherally induced Treg cells might also be converted into effector Th17 cells [23, 41, 42]. Treg cells have also been demonstrated to be able to gain control over Th17 cells by inhibiting IL-6 production in patients treated with tumor necrosis factor alpha (TNF-α) antagonists [43]. There are a few differences between human and mouse Treg cells: in mice, IL-2 promotes Treg cell development at the expense of Th17 cells, but in humans this is not the case [21]. In patients with RA, TNF-α inhibits the expansion of Treg cells and, as a result, anti-TNF biologic agents have been used to promote the expansion of these cells [44, 45]. In mice however, TNF promotes the expansion of Tregs [23, 46] and the anti-TNF therapy resulted in the suppression of their function [47].

The presence of large populations of innate immune cells such as granulocytes and macrophages in the rheumatoid joint indicates that T cell independent processes and non T cells also have important role in the joint pathology of RA. In serum or Ab transfer induced arthritis models, a T cell-independent transient arthritis develops following transfer of immune serum from arthritic donors or Abs to CII. Joint inflammation in these cases is characterized by massive influx of granulocytes into the joints of recipient mice, but a short disease course [8, 48]. A minor T cell population was also found in the synovial fluid of patients with rheumatic diseases, however, most of these cells were identified as Tregs [49]. The presence of these cells indicated a secondary recruitment of T cells to the site of inflammation. However, the prevailing view is that upon entry into the joints from the bloodstream, effector T cells can provide cytokine/chemokine stimuli to surrounding cells, and thus they actively trigger and maintain local inflammatory processes [7, 16, 50].

In recent years, two-photon microscopy imaging in vivo has greatly contributed to our understanding of the motility of T cells, and interactions between T cells and APCs in the lymph nodes [51-55]. The principle of two-photon microscopy [56, 57] is similar to the principle behind confocal microscopy in that fluorophores are excited by a laser beam to emit photons. Fluorophores can be excited by a continuous gas-phase laser beam or by a solid-phase non-continous laser beam. The chance of excitation by two photons is extremely low; however, it increases exponentially with increasing laser

power. The two-photon microscope (TPM) uses femtosecond laser pulses to excite fluorophores in the focal plane, but not in the surrounding tissues. This results in less background scatter and less overall tissue damage. Because TPM employs a far-red laser, it can penetrate deeper in the tissues than a confocal microscope. The emitted fluorescent signal is strengthened by photomultiplier tubes, which results in enhanced detection.

T cell recognition of Ags presented in the context of the MHC initiates signaling through the T cell receptor (TCR). Activation through TCRs requires prolonged physical contact between Ag presenting cells (APCs) and T cells as well as co-stimulatory signals, and the threshold of signaling is usually high. As a result, most of the two-photon imaging studies use genetically manipulated mice, as the TCR epitope repertoire is diverse and cognate T cells recognizing any single epitope are thought to be rare [53]. These genetically engineered mice express a TCR in a large population of their T cells, which is specific for a single epitope of a model Ag (e.g., ovalbumin (OVA), male-specific antigen (Dby), glycoprotein from lymphocytic choriomeningitis virus (LCMV) [58-64].

Studies focusing on T cell motility in the lymph nodes found that before meeting the Ag, T cells in both naïve WT and TCR transgenic (Tg) mice exhibit a random walk [58, 59]. However, a few hours after the injection of the Ag, TCR-Tg, but not WT T cells exhibited significantly reduced motility and non-random movement, indicating prolonged contacts between APCs (such as dendritic cells) and T cells. Another study described that T cell priming (with regards to motility) by Ag loaded dendritic cells (DCs) in TCR-Tg mice happened in three distinct phases [60]. The first 8 hours following entry to the lymph nodes, T cells made short-term interactions with DCs. During the next 12 hours, T cells became more stationary as the length of these interactions increased. On the second day, T cells disengaged from DCs and exhibited rapid, near random movement which indicated that T cells were proliferating or exiting the lymph nodes [60]. Other studies found that TCR-Tg T cells could establish long-term interactions very early after the presentation of Ag by DCs [65], and they were decelerated (arrested) as a result of these interactions in all phases of priming [66]. The controversial results of these

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studies made it impossible to establish an exact universal sequence of the motile behavior of T cells during primary of secondary exposure to antigen. However what all these studies have in common is that T cell interaction with APCs carrying Ag leads to the arrest of the cognate T cells ensuring its activation via long-lived interactions with the relevant APC, and that T cells exhibit high motility when they are ready to divide or leave the lymph node. When using T cells from genetically manipulated TCR-Tg mice, the response of each of these T cells is an isolated immunological event. In contrast, the response of Ag-experienced T cells from arthritic animals to in vivo Ag challenge is linked to the initiation of autoimmune arthritis. Understanding Ag induced changes in the motile behavior of these Ag-experienced T cells in the JDLNs may provide important information about the in vivo conditions of T cell activation and disease progression.

Aims of the study

In the studies included in the present thesis I have tried to find the answers for the following questions:

- Are T cells necessary for the development of PGIA at target sites (i.e., in the joints)?

- Is the availability of T cells critical for the development other animal models of arthritis? If so, how effective are T cell targeting therapies in suppressing experimental arthritis in animals or joint involvement in patients with RA?

- If the recruitment and Ag-induced activation of T cells within the lymphoid organs were necessary for the development of PGIA [67], we wanted to determine whether there was a difference in motility between Ag-experienced and naïve T cells in the joint draining lymph node (JDLN) and whether Ag-experienced T cells from immunized WT mice could mimic the motile behavior of T cells isolated from naïve TCR-Tg mice upon exposure to cognate antigen in a similar fashion, as reported by other studies [57].

- Is there a competition for access to antigen presenting cells between naïve and antigen experienced T cells?

- Does joint inflammation influence the motile behavior of T cells in the JDLN?

Materials and Methods

Antigen, mice, immunization, and assessment of arthritis

Cartilage from knee joints was obtained from consenting osteoarthritis patients who had undergone joint replacement surgery. The use of human cartilage for PG isolation was approved by the Institutional Review Board of Rush University Medical Center (Chicago, IL, USA). The procedures of PG isolation and deglycosylation have been described earlier in detail [10, 68]. For the first set of experiments we used adult female WT and SCID BALB/c mice (purchased from the National Cancer Institute, Frederick, MD, USA) and enhanced green fluorescent protein-lysozyme M 'knockin' mice (EGFP-LysM KI, purchased from the University of Missouri, Mutant Mouse Regional Resource Center, Columbia, MO, USA) which were back-crossed into BALB/c for 10 generations. All animal experiments were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center (Chicago, IL, USA)

In the second set of experiments we used adult female WT BALB/c mice (Frederick, MD, USA) as activated and naïve T cell donors, as recipients, and also for age matched controls. BALB/c mice expressing a human cartilage PG specific TCR Tg (PG-TCR-Tg mice) [69, 70] and recognizing the 5/4E8 immunodominant epitope located in the first globular domain of human PG [70, 71], were also used for in vitro T cell competition experiments.

WT or EGFP-LysM KI BALB/c mice were immunized with PG extracted from human cartilage as described [15, 16]. Each recipient mouse received 100 micrograms of sterile PG dissolved in 100 microliters of phosphate buffered saline (PBS) emulsified in 1 milligram of dimethyldioctadecyl-ammonium bromide (DDA; from Sigma-Aldrich, St. Louis, MO, USA) adjuvant dissolved in 100 microliters of PBS. The Ag emulsion was

injected intraperitoneally three times, once every three weeks [15, 16, 18]. Following the third injection, mice were inspected for symptoms of arthritis on the front and hind limbs twice a week, and the degree of inflammation was scored on a scale of 0-4 on each limb where 0 meant no signs of inflammation and 4 meant a very swollen limb with erythema. Each mouse received a severity score of 0-16 (a sum of the inflammation scores) as a measure of the severity of the disease [15, 72].

Separation, labeling and transfer of immune cells for in vivo imaging studies

Cells were isolated from the spleens and several JDLNs (brachial, axillary, inguinal and popliteal) of naïve or arthritic donor mice. T and non T cells (90% of which were B220 positive, for this reason, they will be referred to as B cells in the dissertation) were separated by using mouse T cell enrichment and T cell depletion kits (StemCell Technologies, Vancouver, BC, Canada), respectively [20, 67, 73]. With proper techniques, these separation kits provided distinct cell populations with a purity of 95%, or higher, which was confirmed by a flow cytometry (FACS Canto II flow cytometer and FACS Diva software, BD Flow Cytometry Systems, San Jose, CA, USA). As we mixed T cells and B cells in a 1:1 or a 1:3 ratio before transfer, the number of B cells was approximately the same or 3-times higher than the number of T cells in the transferred cell population. Since DCs can only be harvested from the lymphoid organs following digestion with collagenase (which we did not use), it is unlikely that we transferred a large number of dentritic cells (DCs) into the recipients. DCs were generated from bone marrow cells by a 10-day culture in the presence of granulocyte-macrophage colony stimulating factor [74, 75]. DCs were used as Ag-presenting cells (APCs) in select experiments. Total lymphocytes or T cells from arthritic mice were labeled with a red fluorescent cell tracker dye (CMTPX Molecular Probes, part of Invitrogen Corporation, Carlsbad, CA, USA), and cells isolated from naïve mice (or B cells in other experiments) were stained with a green fluorescent dye (CMFDA, Molecular Probes) [72]. Cell tracker dyes only stain viable cells as the conversion of these dyes requires esterase activity. In experiments in which imaging was performed on the inflamed joints of EGFP-LysM KI mice, no labeling was necessary, as these mice express green fluorescence in granulocytes [76]. After washing and incubation of cell tracker dye-labeled cells for 30

minutes at 37°C in culture medium containing fetal bovine serum, the cells were intravenously injected into the donor mice. Before the injection, the number of cells to be injected was determined by counting the cells following trypan blue staining. We conducted four different types of experiments. The number of injected cell types, and the injected Ag were different in each experiment:

To investigate T cell recruitment into the arthritic joints and their presence in the lymph node during the development of PGIA in SCID mice, we injected red fluorescent T cells and unstained or green fluorescent B cells (both from arthritic donors) at 1:1 to 1:3 ratios intravenously into SCID mice along with 50 micrograms of human cartilage PG without adjuvant at the time of the transfer to ensure re-stimulation of the injected cells [20, 72]. Using two-photon microscopy, we monitored the migration of the injected cells into the ankle joint, or into the popliteal lymph nodes, at 2 and 4 hours and then 1, 2, 3, 4, 7, 12 and 18 days following transfer. We imaged the ankles and the JDLNs of 3-8 mice at each time point. Mice were injected twice, first with unlabeled cells and, when the hind paws became arthritic, they received a second transfer with cells labeled with cell tracker dyes to monitor their migration into the inflamed ankles or into the popliteal lymph nodes. The inflamed ankles of EGFP LysM KI BALB/c mice [76] were imaged to specifically visualize granulocyte recruitment in their joints.

To compare the changes in the motile behavior of naïve and Ag-experienced T cells in response to a single dose of Ag, we injected 5×10^6 green labeled naïve T cells isolated from naïve mice, and 5×10^6 red labeled T cells from arthritic mice into naïve hosts. 2-3 hours following the cell transfer, mice were also given PG injected directly into the ankle joint. Control mice received either PBS or the irrelevant Ag OVA instead of PG. Two-photon microscopy imaging was conducted at various time points between 2 and 48 hours following the injection of 10 microliter PBS containing PG or OVA (20 microgram each), or PBS alone into the joint. 250 micrograms of L selectin blocker MEL-14 mAb [77] was also injected intravenously 2 hours before each imaging session to prevent new lymphocytes to enter the lymph node during the imaging session [60].

To study the motility of naïve and Ag-experienced T cells in the popliteal lymph nodes of arthritic hosts, we co-transferred green fluorescent T cells from naïve mice and red fluorescent T cells from arthritic donor mice into arthritic recipients. The lymph nodes of donor mice were imaged with two-photon microscopy at 4, 24, 48 and 72 hours post transfer. These mice also received MEL-14 mAb 2 hours before imaging.

To determine whether the differences in motility between naïve and Ag-experienced T cells were due to competition [64], we repeated the previous experiment, but this time we injected 10^7 naïve T cells without Ag-experienced competitors into arthritic recipients. These mice also received MEL-14 mAB 2 hours before imaging which was performed 4, 24, 48 and 72 hours after T cell transfer.

In a limited number of experiments, to investigate the interactions between T cells and DCs in the JDLNs, we injected fluorescent labeled bone-marrow-derived DCs (as APCs) along with PG into the ankle joints of arthritic recipients. We also injected fluorescent labeled T cells intravenously (which could be naïve or Ag-experienced) 24 hours after the intraarticular DC-Ag injection, The interaction of DCs with T cells in the JDLN was then visualized by two-photon microscopy as described below.

In vivo two-photon microscopy

Two-photon imaging was performed using Prairie Ultima two-photon imaging system (Prairie Technologies, Middleton, WI, USA).To reduce movement artifacts caused by breathing, mice were first anesthetized using a combination of intramuscular ketamine and xylazine for anesthesia induction, and isoflurane through a nose cone in the presence of 100% oxygen (Rodent Inhalation Anesthesia system, Protech International Inc., Boerne, TX, USA) for anesthesia maintenance. Using this combined method, mice had a regular respiratory rate of 5/min while their tissue oxygenation was maintained. As a proof of appropriate oxygenation, we found the transferred cells moving continuously throughout the imaging sessions for up to 5 hours.

The skin covering the lateral side of the ankle and the popliteal area was removed using microsurgical equipment, then the popliteal lymph node was brought to

the surface following a single cut and it was fixed using a clamp. As a result of the atraumatic technique, there was no bleeding. Mice were then fixed in the imaging chamber (Bioscience Tools, San Diego, CA, USA) using veterinary-grade super glue and adhesive strips. The imaging chamber then was filled with 37°C saline and the temperature was maintained throughout the imaging session using a heated pad (Fine Science Tools, Foster City, CA, USA). The ankle or the lymph node was exposed to a water-immersion objective ($\times$ 40; numerical aperture 0.8) of an upright Olympus BX51WI microscope (Olympus USA, Center Valley, PA, USA). The temperature of the objective was also maintained at 37°C using programmable temperature controllers (Bioscience Tools). The laser wavelength (Chameleon Ultra; Coherent Inc., Santa Clara, CA, USA) was set to 820 nm for two color imaging and 809 nm for three color imaging. During image acquisition, laser power gain and offset levels were set to minimize photo-damage and background noise. The emitted fluorescence was separated by filter cubes containing dichroic mirrors and specific wavelength filters (435 to 485 nm for blue, 500 to 550 nm for green, and 570 to 625 nm for red fluorescence) [78]. The emitted fluorescent light was detected by photomultiplier tubes (Hamamatsu, Hamamatsu City, Japan). With a help of a stage motor, we were able to slice along the z plane in 1-10 micrometer increments up to 500 micrometers deep into the tissue. 512x512 pixel serial images were captured using PrairieView (Prairie Technologies) image acquisition software. Then the images were used to produce 3 dimensional reconstructions of the tissue in case of serial images along the z plane (z-series) or to capture time lapse series (t-series) by acquiring images every 30 seconds at the same z plane within the T cell zone, usually 70 micrometers or more beneath the lymph nodes' surface [67]. Sometimes we also acquired 3 dimensional t series by scanning 2-10 x-y planes within a 100 micrometer thick segment of the lymph node. We used two channels for imaging only one color and three channels for simultaneous imaging of two colors, leaving one extra channel in each case for second harmonic generation and autofluorescent signals to visualize the tissue context [58].

Image processing and analysis

Images captured during imaging sessions were analyzed using Bitplane Imaris software (v. 6.1.3 or 7.2.3; Bitplane, South Windsor, CT, USA). They were first used to build time-lapse and 3 dimensional reconstruction videos, which were then adjusted to set optimal brightness, contrast, background and color intensity. Cell movements in the time-lapse series were analyzed using Imaris Track. Breathing movement artifacts were eliminated by applying drift correction, using tissue structure elements which moved together and employing the displacement filter built in Imaris Track. The computer rendered cell tracks were then reviewed and adjusted manually to exactly match the real cell paths. We were interested in the following parameters: displacement (D) – which is the distance between the start and end points of a particular cell path; average velocity – which is the path length divided by elapsed time (T) in micrometer/min; motility coefficient ($D_2/4T$: in μm_2/min) – which was calculated as the slope of the mean displacement plotted against the square root of time ($D/T_{1/2}$ in $\mu m/min_{1/2}$) [55, 58]; and the proportion of immotile cells as the percentage of all cells tracked. T cells were considered immotile if they were (i) visible for at least 10 min, (ii) their paths were confined to a 20 μm x 20 μm area within the x-y plane and (iii) their velocity did not exceed 4.5 $\mu m/min$ at any time during the imaging session [79]. The data was exported to and analyzed with Microsoft Excel. In each lymph node 111-216 cell tracks were analyzed using 3-5 mice at each time points post Ag injection.

Immunohistochemistry

Hindpaws and JDLNs of SCID mice following transfer of red CMTPX T cells and unstained B cells were dissected, embedded in OCT compound and snap-frozen. 8 micrometers thick frozen sections were cut using MICROM HM 550 cryostat (MICROM International, Walldord, Germany) and stored in -20 °C. Before immunostaining, cryosections were fixed in cold acetone and blocked using 5% normal goat serum and 5 micrograms/milliliter of anti-CD16/32 mAb (Fc Block; BD Biosciences, San Jose, CA, USA) in phosphate-buffered saline. For immune staining, Alexa Fluor 488-conjugated mAb (B220) against B cells, and Pacific Blue-conjugated mAb to CD35 (a follicular DC-

specific marker (BD Biosciences or eBioscience, San Diego, CA, USA) were used. Fluorescent cells were visualized with two-photon microscopy following post-fixation with 10% buffered formaldehyde.

MHC II-peptide tetramer binding assay

We used an allophycocyanin (Apc)-labeled human PG-specific tetramer (PG tetramer) composed of the human PG "5/4E8" peptide ([89]ATEGRVRVNSAYQDK [69-71]) linked to I-Ad (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-Ad (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 lymph nodes of naïve and PG-immunized (arthritic) WT BALB/c mice as described earlier. 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 [80]) 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. 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 earlier, 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 and 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 Ab or Fitc-rat IgG2b (isotype control) Ab (both from eBioscience). The

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percent of tetramer-binding (Apc+) CD4+ T cells in each sample was determined by flow cytometry (as described in the relevant section) after gating separately on red fluorescent (antigen experienced WT) and on unlabeled (naïve WT or naïve PG-TCR-Tg) cells within the Fitc-CD4+ population. Following preliminary dose optimization experiments, two replicate tetramer binding assays were performed.

Statistical analysis

Statistical analysis was performed with Microsoft Excels' Analysis ToolPak (Microsoft, Redmond, WA, USA) and SPSS software (v. 15, SPSS, Chicago, IL, USA). Depending on the homogeneity of variance, data was analyzed directly or transformed prior to analysis. Two groups were compared using Student's t test, or Mann-Whitney u test for non-parametric data. P values of less than 0.05 were accepted as statistically significant.

Results

Only a small number of T cells can be found locally in the joints during the development of PGIA following adoptive transfer of the disease to SCID mice

To investigate T cell recruitment before and during the development of PGIA, we injected a mixture of red (CMTPX) labeled T cells and unlabeled or green (CMFDA) labeled B cells (both isolated from arthritic BALB/c mice) into SCID mice and monitored the appearance of donor cells in the SCID ankle joints and JDLNs on days 1, 2, 3, 4, 7, 12 and 18 following cell transfer. While both red and green cells were visible at each time points in the JDLN (Figure 1B and 1D), we were unable to find these cells in the ankle joints in a consistent manner using two-photon microscopy (Figure 1A, 1C). Despite the fact that only inflamed arthritic ankles were imaged, only auto fluorescent macrophages (green-blue) and second harmonic generation (SHG) signals from collagen fibers (blue) [78] were detected in the synovial tissue (Figure 1A and C). The absence of donor T cells in the SCID joints was not due to technical problems because we were able to image T cells in the JDLN in vivo (Figure 1 B and D) and on immunohistochemistry slides (Figure 2A) and we could also visualize green fluorescent

neutrophil granulocytes in the ankles of EGFP-LysM KI mice upon induction of PGIA (Figure 2B and [67]). Occasionally, we found red donor cells moving in the synovial blood vessels of recipient mice at early time points up to 1 day post transfer (Figure 2D and E). However the morphology of these cells (i.e. shape, motile behavior [81], exclusion of cytoplasmic fluorescent dye by lobulated nuclei (Figure 2E) was more consistent with polymorphonuclear cells (granulocytes) rather than lymphocytes. Only a few percent of spleen cells of arthritic donor mice are neutrophils, but these cells are subject to preferential recruitment in synovial vessels as compared with lymphocytes [72, 81]. Because neutrophils do not live long in the recipients, donor cells visualized several days after transfer (Figure 2C) could more likely be lymphocytes.

Both red (T cells) and green (B cells) migrated readily to the JDLNs and occupied their separate territories (Figure 1B and D). We were able to visualize them consequently at each time point meaning that the intracellular fluorescence did not fade significantly during the time frame of the 18 days long experiment. The motile behavior of these cells was more consistent with lymphocytes (i.e. polarized shape, vigorous movement [55, 57, 59, 60]. In extended studies, immunohistochemistry and flow cytometry on the inflamed ankles of SCID mice also confirmed the dominating presence of Gr-1 positive neutrophil granulocytes and the very small proportion of T cells [67]. However, as noted earlier, the presence of Ag-specific T cells in lymphoid organs was found to be an absolute requirement for the development of PGIA in these mice [67]. Therefore, we focused our attention to studying T cell-APC interactions within the JDLNs by monitoring the in vivo motility of T cells using real time two-photon microscopy.

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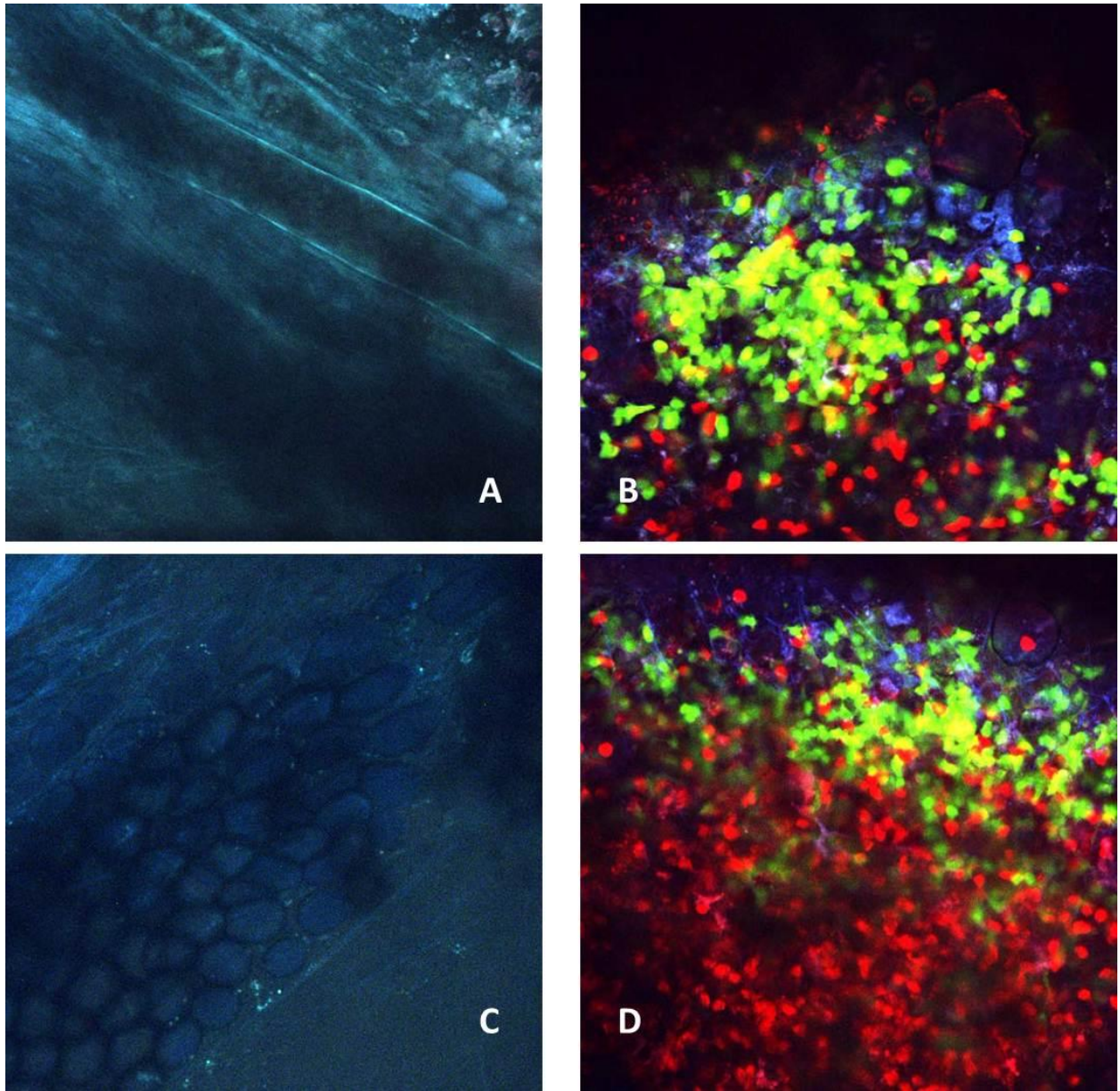
Figure 1. T and B lymphocytes separated from spleens of arthritic mice and injected into syngenic SCID mice can be visualized at any time point in the popliteal lymph nodes by two-photon microscopy (TPM), but are absent in the ankle joints of the recipient mice. (A) No red or green labeled cells are present with TPM in the ankle joint of a SCID mouse 2 days after transfer CMTPX (red) labeled T cells and CMFDA (green) labeled B cells from arthritic BALB/c donors. SHG signals from collagen fibers, the synovium and tissue macrophages are detected in the blue fluorescence channel in three-color acquisition. (B) Red T cells and green B cells occupy their respective

territories (i.e., the inner T-cell zone and marginal lymphoid follicles, respectively) in the JDLN in the same mouse on this TPM image. Collagen in the LN capsule and stroma and subcapsular macrophages generating SHG are seen in blue. (C) No red (CMTPX) or green (CMFDA) labeled cells are visible on this TPM image in the inflamed ankle joint of a SCID mouse 12 days after cell transfer in a similar experiment. TPM image of the inflamed ankle of a SCID mouse 12 days after transfer of red-labeled T cells and green labeled B cells from arthritic donors. Although this joint was heavily inflamed, no red fluorescent T cells (or green fluorescent B cells) were found by in vivo TPM imaging. SGH signals from collagen and autofluorescent macrophages are seen in blue. (D) Large number of T cells and B cells visible in the JDLN 12 days after cell transfer in the same experiment. The TPM images shown are representative samples of ankle and LN images from six SCID mice at each time point.
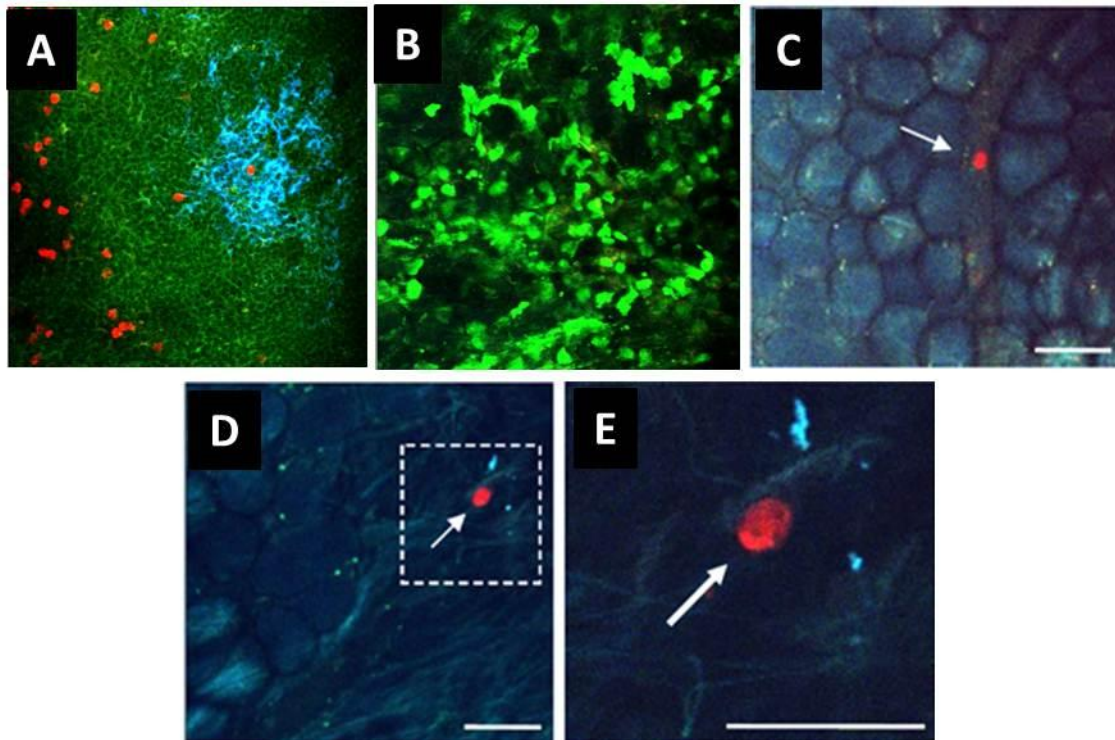

Figure 2. The absence of T cells in the ankle is not due to technical issues with imaging. (A) Immunohistochemistry of a popliteal lymph node from a SCID mouse transferred with red florescence-labeled T cells (red) and unlabeled B cells from arthritic BALB/c donors. The frozen section of the lymph node was immunostained with an Ab against

B220 to visualize B cells (green) and against CD35 (blue) to identify follicular DCs. Transferred T cells were mainly localized outside the B cell zone, but some of them appeared inside B-cell follicles in the vicinity of follicular DCs. In sum, we were able to visualize the transferred T cells in the JDLN in vivo and in tissue sections, whereas T cells could not be found in the ankle joint before or after the development of PGIA. (B) TPM image of an inflamed ankle of an EGFP-LysM KI mice 2 days after onset of PGIA show large population of green fluorescent granulocytes in the synovial tissue and cavity. In these mice, EGFP is knocked in the Lysozyme M locus thus neutrophil granulocytes (and some DCs) express this protein at high levels. EGFP-LysM KI mice used for this experiment were backcrossed into the BALB/c background and were immunized to develop PGIA. (C) This SCID joint was imaged 4 days after the transfer of CMTPX (red)-labeled T cells and unlabeled B cells from arthritic donors. A single red cell (arrow) is seen within the shadow of a blood vessel. Serial images showed tethering-rolling movement of this red cell along the vessel walls, indicating that it was located intravascularly. The presence of donor cells in the recipients' joints was not typical, and was even less frequent at later time points (days 7-18) after transfer. Scale bar, 50 µm. (D-E) Donor cells are occasionally found in the synovial tissue or vessels after transfer of T cell-enriched fractions. (D) A red fluorescent cell (arrow) is visible in the synovial tissue 1 day after i.v. transfer of CMTPX-labeled T cells, along with CMFDA (green)-labeled B cells, from arthritic donors to a SCID mouse. (E) High-magnification imaging of the midsection of the same immobile red cell (boxed in panel d) revealed the presence of cytoplasmic "holes" corresponding to nuclear lobes. This raised the suspicion of this red cell being a polymorphonuclear leukocyte from the donor spleen cell preparation.

Naïve and Ag-experienced T cells exhibit different motility characteristics in the JDLN of naïve hosts after encountering a single dose of intraarticularly injected PG

First we hypothesized that there could be differences between the motile behavior of Ag-experienced and naïve T cells upon encountering PG as the relevant Ag in the JDLN of a naïve host because of the difference in the number of T cells with

TCRs able to respond to the Ag (quantitative) and because of the difference in affinity of the TCR of individual cells (qualitative) between the two injected cell groups [63]. To test this we injected $5x10^6$ green labeled naïve T cells isolated from naïve mice, and $5x10^6$ T cells from arthritic mice to a naïve host and monitored their motile behavior with two-photon microscopy in the JDLN in response to in vivo Ag challenge (i.e., injection of PG into the ankle joint). This setting ensured the priming of the rarer naïve cognate T cells and the re-stimulation of the Ag-experienced T cells by the animals' own APCs. Similarly to other models investigated with two-photon microscopy [54, 60, 63], both cell groups exhibited high motility during the first two hours following the administration of antigen (Figure 3A and 3B). By 4 hours following Ag exposure, each cell group exhibited reduced cell motility. Two hours later, T cells regained some of their mobility but they stayed below their baseline motility level. However, Ag-experienced T cells showed significantly reduced motility as compared with naïve T cells (Figure 3A and 3B). At the 4 hours' time point the percentage of immotile cells was also significantly higher in the Ag-experienced population than in the naïve (Figure 3B open bars). This indicates that Ag presentation is most efficient in the JDLN 4 hours after intraarticular Ag administration leading to the arrest of T cells dominantly in the Ag-experienced population.

Other studies reported that cognate T cells may become arrested for several hours following an encounter with APCs [60, 66]. To investigate this in our disease model, we extended the time frame of imaging post Ag injection to 48 hours. In this experiment we conducted imaging 4, 8, 24 and 48 hours post Ag injection. We observed significantly reduced motility (Figure 3C and 3D) and more constrained cell tracks among Ag-experienced T cells compared to their naïve counterparts as indicated by the differences in their motility coefficients (Figure 3D) and displacement plots (Figure 3E, left-side panel). The proportion of immotile cells was also much higher in the Ag-experienced T cell group (Figure 3D) and Figure 4A and 4B). The motility pattern of naïve T cells rather resembled a random walk [55, 57, 60]. The difference in the motility parameters of the two T cell groups was clearly the result of the preferential recognition of PG by the Ag-experienced group of T cells. The same experiments using OVA as

irrelevant Ag or PBS not lead to the above-described differences (Figure 3E, middle and right-side panels).

Eight hours post Ag injection and beyond, however, both T cell groups regained their original motility (Figure 3C and D and Figure 4). These results suggest that a single encounter with PG in the recipient mouse leads to a short-term presentation of the Ag and to short term interactions between the T cells and the antigen presenting cells. Although the APCs are not visible in this study, we theorize that the differences in the motility parameters of the Ag-experienced and naïve T cell groups were due to the higher number of cognate cells and their higher affinity to MHC-PG peptide complexes in the Ag-experienced population.

A Naive ○ Ag-experienced ●

B Naive ☐ Ag-experienced ▨ (bars); Naive ○ Ag-experienced ● (lines)

C Naive ○ Ag-experienced ●

D Naive ☐ Ag-experienced ▨ (bars); Naive ○ Ag-experienced ● (lines)

E 4h after PG injection / 4h after OVA injection / 4h after PBS injection
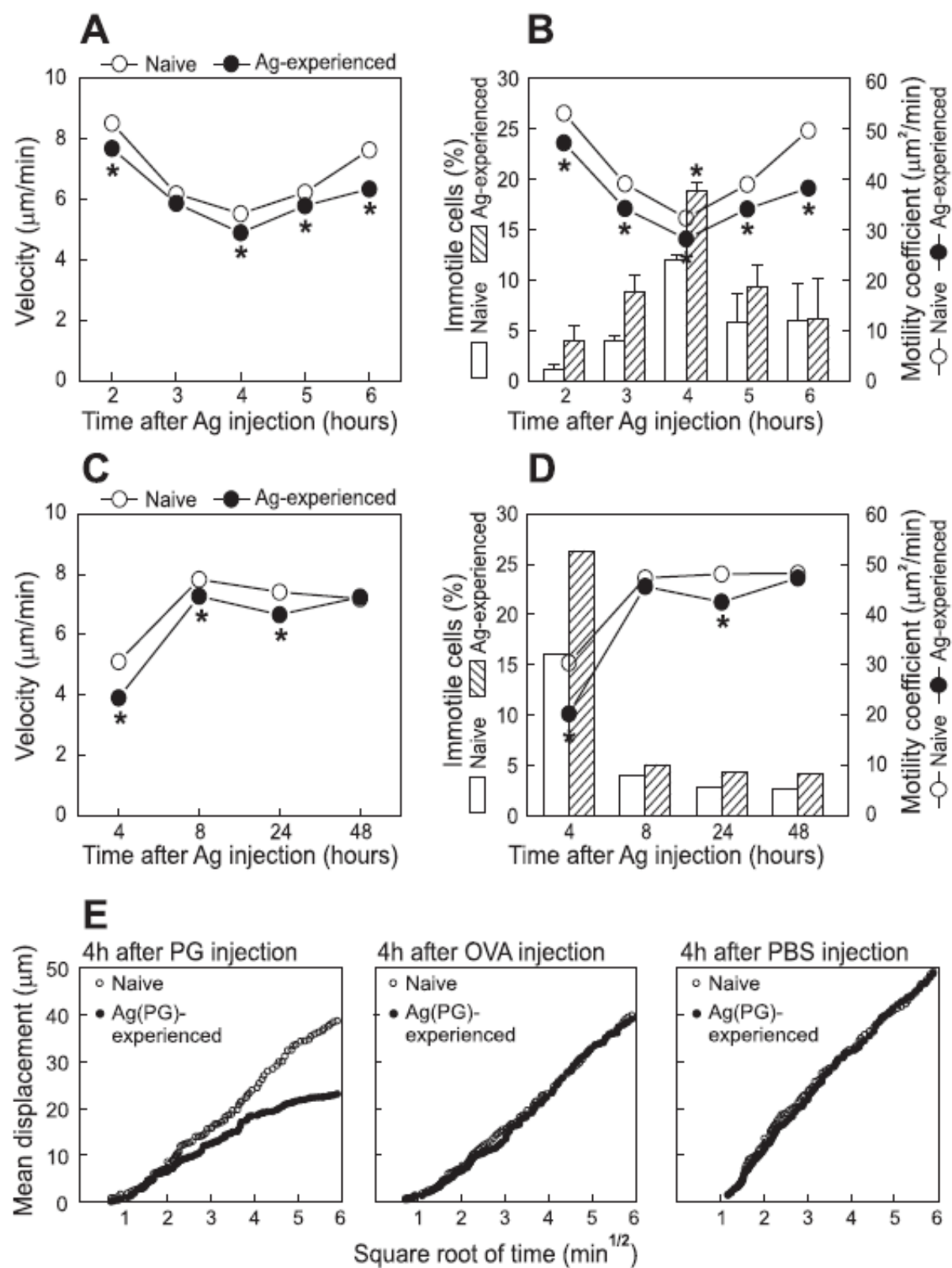Naive ○, Ag(PG)-experienced ●

Figure 3. *Changes in the motile behavior of co-transferred naïve and antigen (Ag)-experienced T cells over time in the popliteal lymph node of naïve hosts in response to intraarticular Ag 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 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 intraarticular 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 immotile naïve (open bars) and Ag-experienced (cross-hatched bars), and their motility coefficients are shown at each time point. The results represent the means ± 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 intraarticular PG injection. (C) Velocity and (D) the percent of immotile 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 intraarticular 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.*
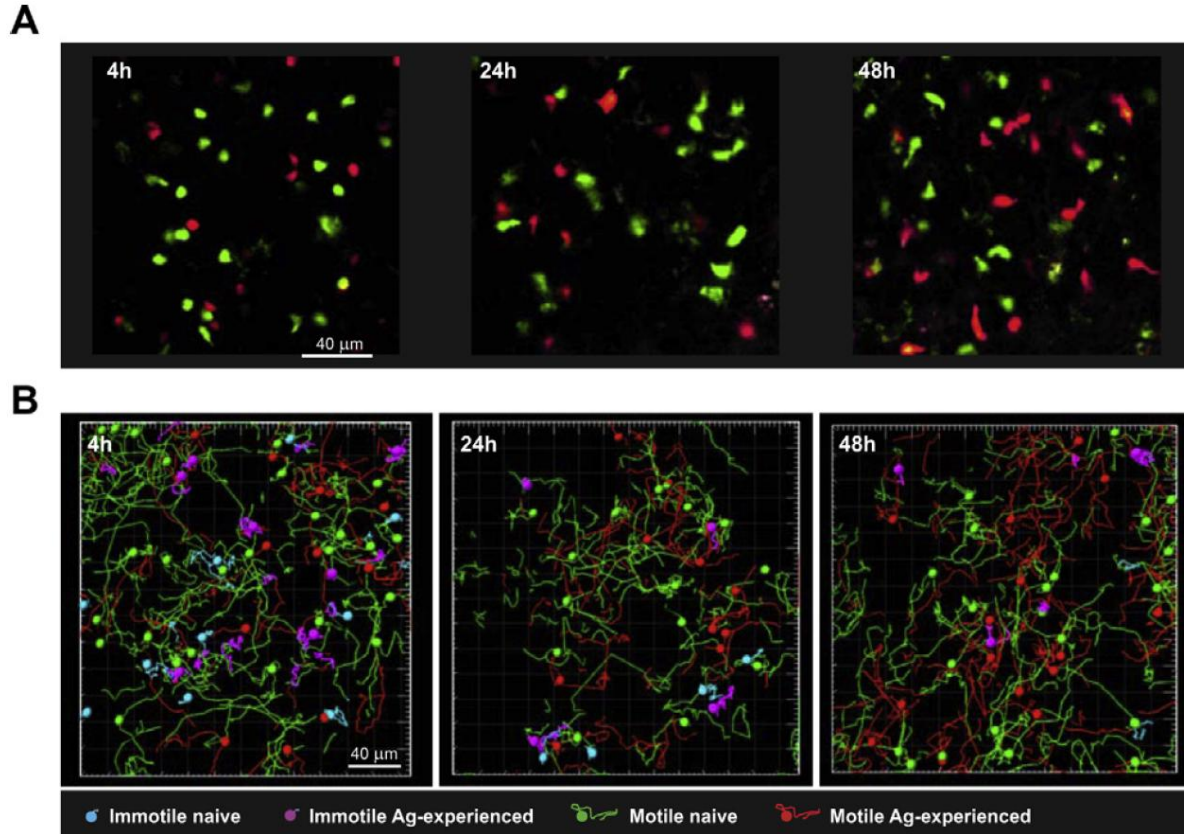
Figure 4. *Representative TPM images and illustration of the motility of co-transferred naïve and antigen (Ag)-experienced T cells in the popliteal LN of the naive host between 4 and 48 h after intraarticular antigen injection. (A) Representative images of naïve (green fluorescence-labeled) and antigen-experienced (red fluorescence-labeled) T cells in the LN at 4, 24, and 48 h after intraarticular injection of PG into naïve recipient mice. The images (taken from the long-term experiment shown in Figure 3C and D) show a large proportion of immotile (round) T cells in each population at 4 h, 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.*

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Ag-experienced T cells engage in long-lasting interactions with APCs in the JDLN when transferred into arthritic recipients. Naïve T cells do not show similar behavior.

We wanted to determine whether a transfer of the same number of Ag-experienced and naïve T cells into an arthritic host would lead to a more profound arrest in the joint-draining lymph node - following PG injection into the ankle joint - than in the naïve host. This was suggested by a previous study that demonstrated that APCs isolated from mice with PGIA present to Ag-experienced T cells much more efficiently than antigen presenting cells from naïve mice in vitro [16]. We conducted imaging at the same time points following cell transfer (i.e. at 4, 8, 24 and 48 hours), but the overall reduced motility among the Ag-experienced T cells prompted us to add an even later time point at 72 hours (Figures 5A and 5B). By that time the motility of the visualized cells increased substantially. The arrest of naïve T cells was also more profound at 4 hours compared to their arrest in the naïve host (Figures 3 and 5) and nearly equal proportions of the two T cell groups were arrested at this early time point (Figure 5B). The naïve cells however disengaged from the APCs by 24 hours and became very motile by 72 hours after PG injection (Figure 5). Their behavior suggested that either they were only transiently engaged with antigen presenting cells in the arthritic JDLNs or they had limited access to these cells because they were outcompeted by their Ag-experienced counterparts [64].
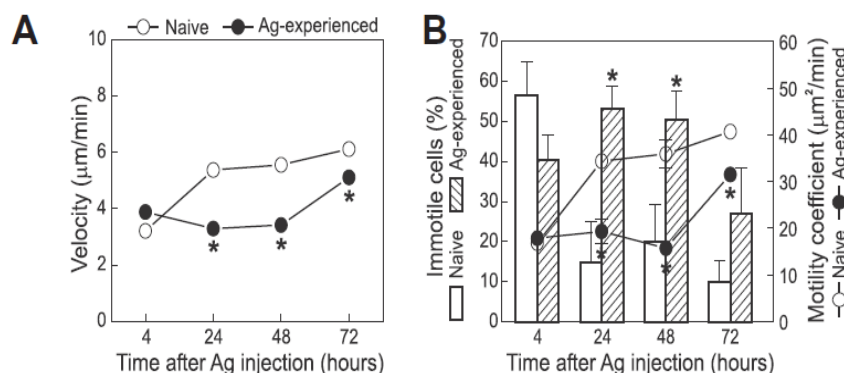
Figure 5. *Motility of co-transferred naïve and antigen (Ag)-experienced T cells in the popliteal LN of the arthritic host in vivo. The (A) velocity and (B) the motility coefficients with the percentage of immotile cells are shown for the co-transferred naïve (open circles/open bars) and Ag-experienced (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 Figure 3 (C and D).*

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

T cells are re-circulating among lymphoid organs then die in a few days unless they encounter both cognate Ag and co-stimulatory signals sufficient to initiate an activation program. Because the number of APCs with proper MHC and co stimulatory molecules [63] for any given Ag is low, T cells have to compete with each other for access to APCs in the lymphoid organs. Ag-experienced T cells clearly have the advantage in this competition over their naïve counterparts because of their higher numbers and higher affinity to the relevant Ag. In the experiments described above, the concentration of the intraarticularly injected PG was probably very low in the JDLN thus making ideal circumstances for T cell competition [82]. In the experiment described below, we sought to determine whether this competition can be demonstrated in vitro with a MHC tetramer assay using 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: [89]ATEGRVRVNSAYQDK [69-71]) within the core protein of the PG molecule, and a non-specific I-Ad-CLIP tetramer as a control. The binding of the PG tetramer could be clearly demonstrated even at suboptimal (non-saturating) dose of the tetramer (Figures 6A and 6B) and table 1). The number of binding naïve T cells was higher in the absence of the PG experienced T cells (Figure 6A, middle left and bottom panels respectively) which indicated that they were outcompeted by them for tetramer binding. The specificity of PG tetramer binding could be confirmed by demonstration of further competition between antigen-experienced T cells and naïve PG-TCR-Tg T cells (Figure

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5B), which express a TCR specific for the exact 5/4E8 epitope represented by the PG peptide in the tetramer [69, 70]. No competition was demonstrated at higher (saturating) doses of the PG tetramer and it was also not observed when T cells were incubated with the control CLIP tetramer at any dose (table 1).

This experiment demonstrates that T cells compete for binding of an antigenic peptide when the availability of this epitope is limited. And that the binding capacity of T cells is proportional with the relative number (and possibly the TCR affinity) of cognate T cells in the competing populations. Also this experiment shows that the differences in T cell motility between the Ag-experienced and naïve T cell populations are likely due to competition for access to antigen presenting cells presenting PG epitopes.
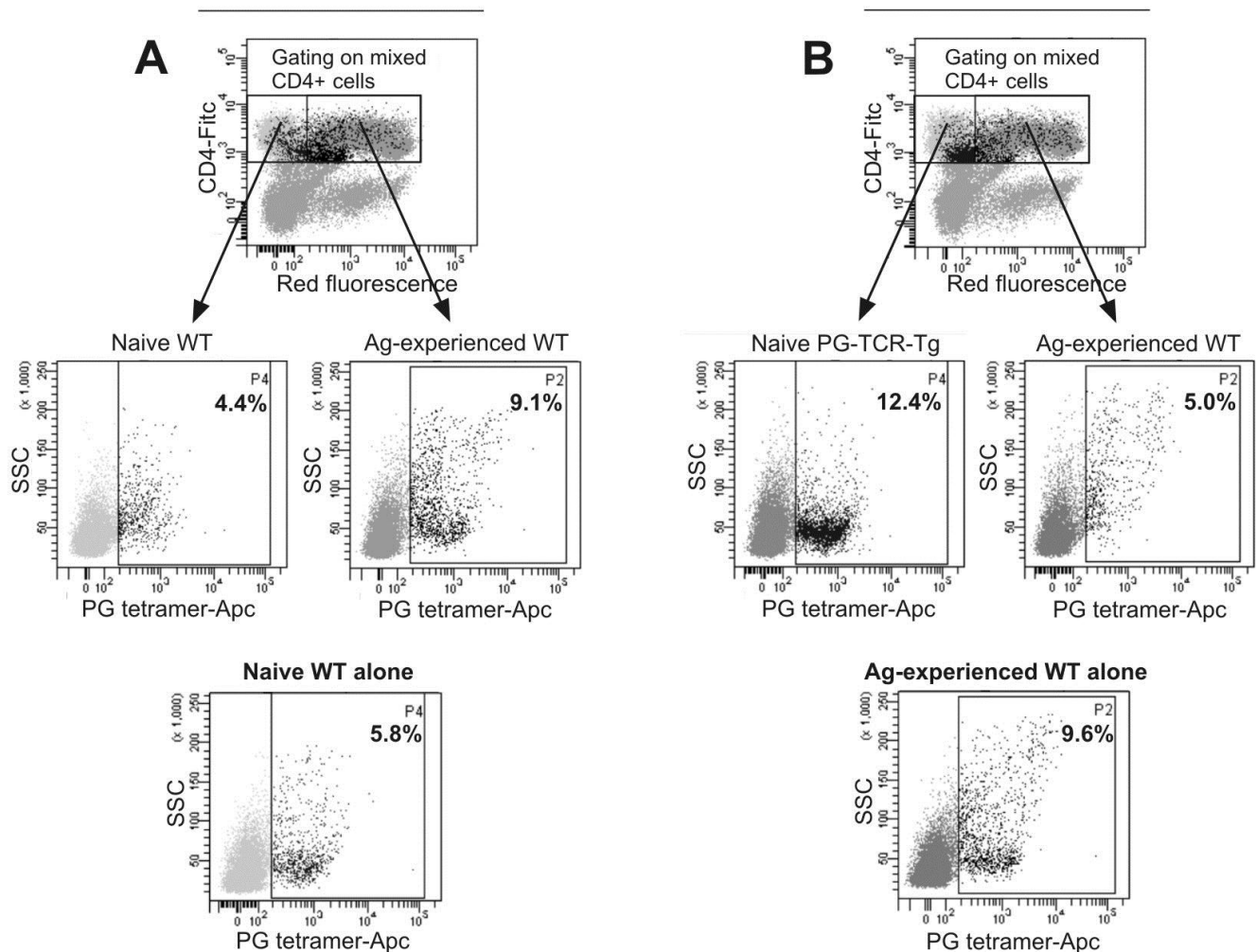
Figure 6. *Flow cytometry scatter plots demonstrate competition between T cell populations for MHC-PG peptide tetramer binding in vitro at a suboptimal dose (0.6 μg for 1.2 million cells) of the tetramer. (A) The percent of naïve T cells that bind the I-Ad-PG peptide tetramer (middle left panel) is lower when they are mixed with Ag (PG)-experienced T cells (middle right panel) than if incubated alone (bottom panel) with the tetramer. (B) 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 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 (Table 1). The data shown are from one of two replicate experiments with similar results.*

Supplementary Table 1. Percent of I-A^d-peptide tetramer binding CD4$^+$ cells in single and mixed cultures of naïve WT, Ag-experienced (Ag-exp.) WT, and naïve PG-TCR-Tg T cells at different doses of tetramers

Cell cultures	Naïve WT alone	Ag-exp. WT alone	Naïve PG-TCR-Tg alone	[a]**Naïve WT** with Ag-exp. WT	[a]**Ag-exp. WT** with Naïve PG-TCR-Tg
Treatment					
I-A^d-PG tetramer (Ag-specific)					
0.3 µg	1.3	2.7	4.8	**0.7**	**0.9**
0.6 µg	[b]5.8	[b]9.6	13.7	[b]**4.4**	[b]**5.0**
1.2 µg	6.2	10.4	15.3	**6.1**	**10.0**
2.4 µg	6.5	10.3	15.8	**6.7**	**10.9**
I-A^d-CLIP tetramer (non-specific)					
0.3 µg	0.4	0.6	0.2	**0.3**	**0.5**
0.6 µg	2.9	3.0	2.7	**2.8**	**3.3**
1.2 µg	3.4	2.9	3.3	**3.0**	**3.2**
2.4 µg	3.0	3.5	2.7	**3.2**	**3.7**

[a]Values shown for mixed cells are from **one** of the two cell populations. [b]Flow cytometry plots are shown in Fig. 6.

When there is no competition, arrest of naïve T cells can be demonstrated in the JDLNs of arthritic hosts.

To investigate the effect of the lack of competition on the in vivo motility of the naïve T cells in similar setting, we injected only naïve T cells into arthritic hosts. Recipient mice received intraarticular PG and intravenous MEL-14 before imaging similar to previous experiments. The total number of injected naïve T cells equaled the combined number of injected T cells in the in vivo experiments described above. We conducted imaging at 4, 8, 24, 48 and 72 hours post transfer and PG injection. Naïve T cells arrested and remained immotile for the first 24 hours (Figures 7A and 7B). Their motility coefficient increased and the percentage of immotile cells dropped in an almost linear fashion between 24 and 72 hours (Figure 7B). This suggested that when naïve T

cells had to compete only with the endogenous Ag-experienced T cells in this setting, they indeed had better access to Ag than in the presence of transferred Ag-experienced competitors [64].
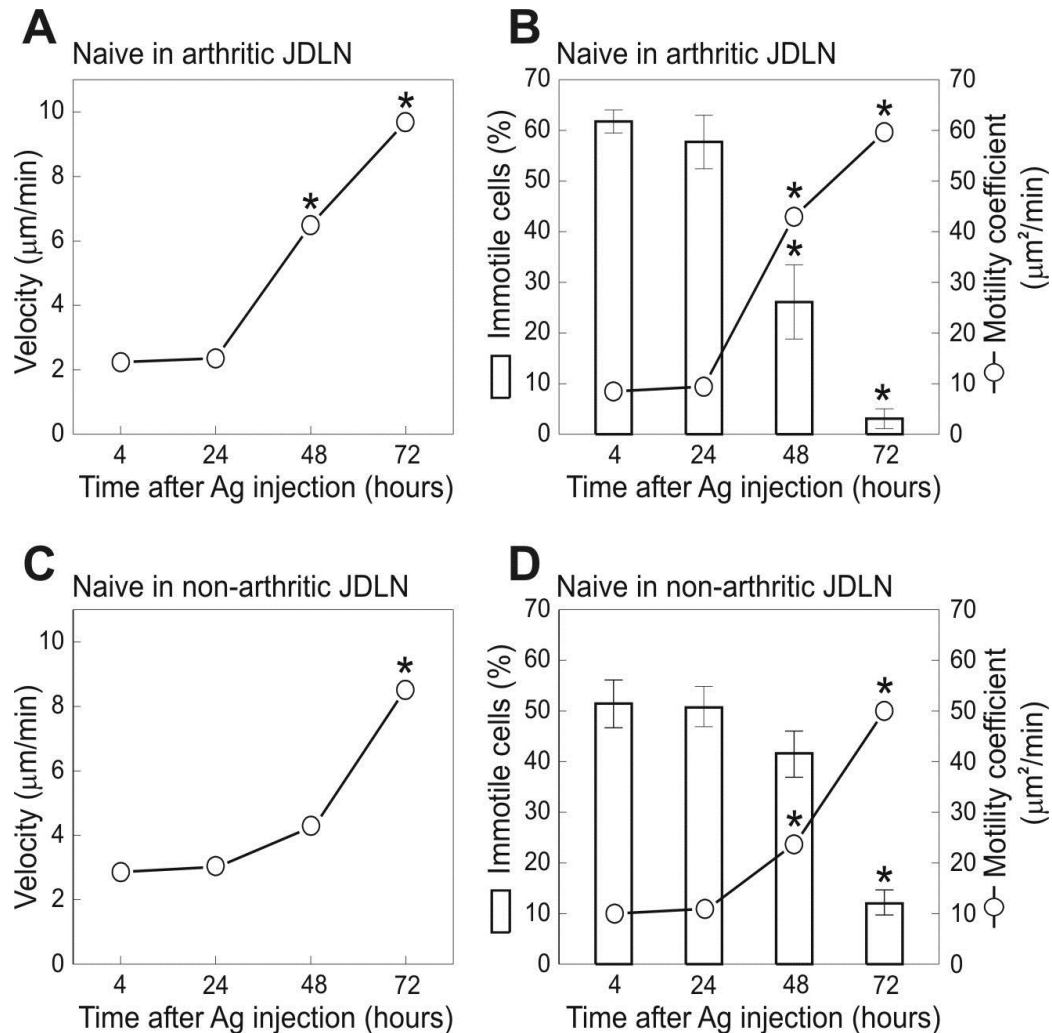
Figure 7. *Motility of transferred naïve T cells in the popliteal LNs of arthritic hosts over time in response to Ag injection into inflamed (A and B) or non-inflamed (C and D) ankle joints. (A) Velocity and (B) percent of immotile cells (open bars) and their motility indices (open circles) are shown 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 Figure 3. Asterisks indicate statistically significant changes over time relative to the starting time point (4 hours).

The motile behavior of naïve T cells in the JDLN is modulated by joint inflammation

In this experiment we wanted to determine whether the presence of inflammation in the ankle joint changes the motile behavior of naïve T cells in the ankle joint-draining lymph node. To do this we repeated the experiment described in the previous topic but we imaged the joint-draining lymph node that drained a not visibly inflamed ankle joint. We found that although the initial velocity of the T cells in the non-arthritic JDLN was slightly higher, T cells in arthritic JDLN regained their motility sooner (compare Figures 7A and 7B with Figures 7C and 7D). It is hard to determine purely on the basis of T cell motility whether the described differences are a result of less efficient priming as compared to priming in inflamed JDLN or the consequence of the absence of the effects of inflammatory mediators in the joint-draining lymph node. However these findings suggest that inflammation (in this case, arthritis) has a modulating effect on the dynamics of antigen presentation in lymph nodes draining inflammatory sites.

Ag-experienced T cells make contacts with transferred, recently migrated DCs but they engage in long lasting interactions with endogenous (LN-resident) APCs

In a limited number of experiments, we co-transferred fluorescence labeled DCs and Ag (injected into the ankle joints) with naïve or antigen experienced T cells (injected intravenously) into arthritic mice to visualize their interaction [54] by two-photon microscopy. By 24 hours a number of DCs cells migrated into the JDLNs and they were visible in the T cells zones of the lymph nodes (Figure 8). Both naïve and Ag-experienced T cells made contacts with these labeled DCs, however these contacts were mainly temporary (i.e. were lasting for less than 10 minutes.) The same T cells however were observed to engage in long term interactions with autofluorescent spots we thought to be endocytic vesicles of mice's own resident APCs (Figure 8). From these

investigations we conclude that further imaging between visualized host APCs (i.e., DCs expressing fluorescent proteins [61, 83]) and transferred T cells will be necessary to better understand the interactions between these cells in PGIA.
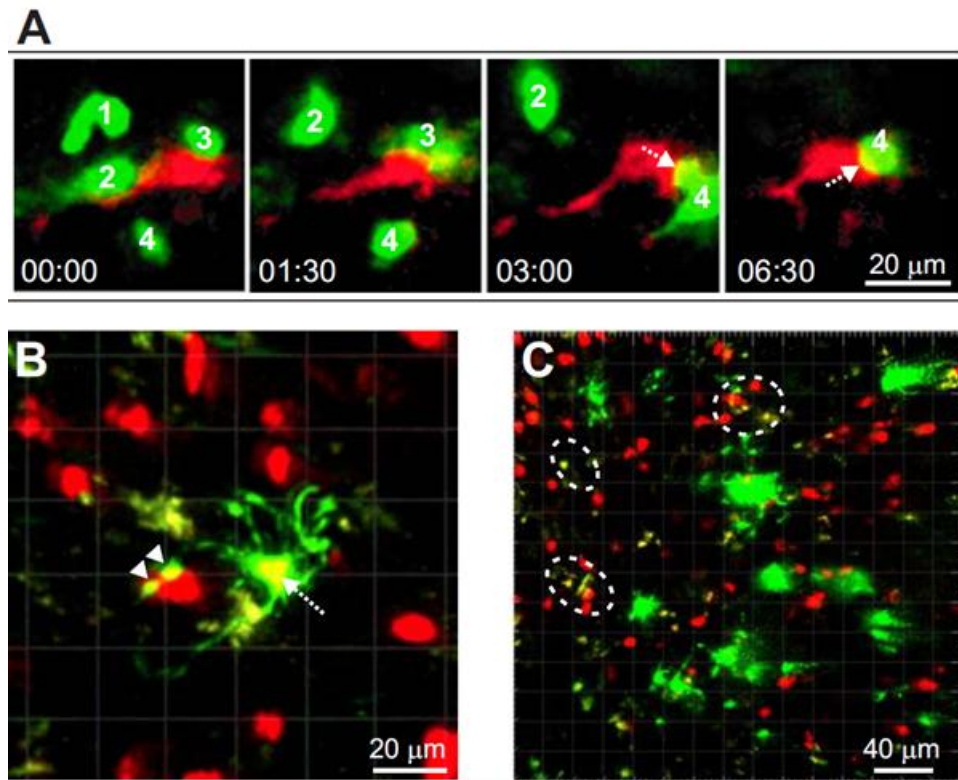
Figure 8. *Interactions between dendritic cells (DCs) and transferred naïve (A) or Ag-experienced (B and C). T cells in the popliteal lymph node (LN) 24 hours after intraarticular injection of DCs and antigen into arthritic hosts. (A) Time-lapse images of interactions of naïve T cells (green fluorescence-labeled) with an intraarticularly delivered DC (red fluorescence-labeled) that had migrated into the popliteal LN. At the beginning of image recording, the DC is in contact with T cells #2 and #3, but not with #1 or #4. After 3 min, the DC shows engagement only with T cell #4, which lasts for over 3 min. The area of overlapping yellow fluorescence (arrow) may indicate the beginning of immunological synapse formation between the DC and the T cell. (B) A DC (green fluorescence-labeled), which appears in the JDLN 24 hours after intraarticular injection, exhibits a probing movement with its elaborate dendrites that frequently land on the surface of nearby Ag-experienced (red fluorescence-labeled) T cells. As in panel*

A, areas of overlapping yellow fluorescence (arrowheads) may depict immunological synapses. Yellow fluorescent dots within the body of the DC (broken arrow) likely represent autofluorescent endocytic vesicles. (C) A still image of a 3D time-lapse series illustrating the transient nature of interactions between the transferred Ag-experienced T cells (red fluorescence) and recently migrated DCs (green fluorescence) in the JDLN 24 hours after intraarticular injection of DCs and Ag into an arthritic host. T cells contact the DCs for short periods of time, but they preferentially arrest on, and engage in longer-term (>10 min) interactions with, LN-resident APCs (likely represented by yellow spots of autofluorescent endocytic vesicles within the white dotted circles).

Discussion

In autoimmune diseases, autoreactive T cells either directly attack the affected organs or tissues, or they provide support to B cells to produce pathogenic autoAbs. In animal models of multiple sclerosis, massive infiltration of the central nervous system by encephalogenic T cells was demonstrated with different methods, including two-photon microscopy [84, 85]. In animal arthritis models the presence of CD4 positive T cells in the inflamed joint was described on multiple occasions, but few studies commented on the small size of this cell population relative to other leukocytes in the joint [72, 81, 86, 87]. The presence of CD4 positive (both Th1 and Th17) T cells has been demonstrated in the inflamed joints in patients with RA [3, 7, 88-90]. Because of the presence of these cells in the target organs it has been believed that influx of activated T cells was necessary for the initiation and progression of the disease. To find out whether these suggestions apply to PGIA, we monitored the migration of fluorescence-labeled antigen experienced T cells into the ankles of mice during the adoptive transfer of PGIA. We used two-photon microscopy to visualize T cell recruitment in joints and JDLNs, which method was applied for the first time in an autoimmune model of RA.

Our TPM images clearly demonstrated that both T and B cells isolated from the spleens and lymph nodes of donor mice could be found in the lymph nodes of the recipient mice and occupied their territories in the T or B cell zones. Whilst expression of L-selectin (homing receptor) adhesion molecule and the CC chemokine receptor

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CCR7 are required for lymphocyte entry into the lymph node [91, 92], positioning of newly-arrived lymphocytes within T and B cell zones is determined by the expression of the CXC chemokine receptor CXCR5. Cells expressing CXCR5 (B cells and follicular helper T cells) migrate to the B cell zone, whilst CXCR5 negative cells can be found in the T cell outside the B cell follicles [93, 94].

To monitor T cell influx into the joint we conducted two-photon imaging of the ankles of SCID recipient mice at several time points before and after the disease onset [95]. Interestingly we were unable to find T cells in the joint at any stage of the disease in a consistent manner with two-photon microscopy. Flow cytometry revealed a small population of CD4+ T cells in the synovial fluid [67]. This might have been either due to the flow cytometer's capacity to analyze larger number of cells, or to the T cells ability to flip directly into the fluid from the rich network of blood vessels located underneath the synovial lining [81]. These cells contributed to less than 1% of the whole population of the cells found in the synovial fluid the majority of which were neutrophils [67]. Similarly to our findings, Holmdahl and colleagues and other studies on murine CIA in DBA/1 mice [96], and PGIA in BALB/c mice [72, 81], described low proportion of joint-homing T cells, suggesting that the paucity of these cells in the arthritic joints is characteristic for both CIA and PGIA.

Despite that PGIA cannot be induced by serum transfer [97], non-T cells such as neutrophils are essential and directly involved during the development of the disease. Elimination of circulating neutrophils resulted in prompt abrogation of arthritis in both PGIA [98] and a serum/Ab transfer induced model or RA [48]. When T cells were depleted prior to transfer from arthritic donor to SCID recipients, arthritis did not develop in the SCID mice, indicating that the presence of T cells in the recipient was necessary for disease development [67]. In SCID mice transferred with T cell-depleted donor population, mouse PG-specific serum autoAbs were also missing despite adequate pool of transferred B cells, which indicated the necessity of T cell help for the production of autoAbs by B cells. The fact that these mice also did not develop arthritis shows that PG-specific Abs play an essential role in the development of the disease. This has been confirmed by other disease models or RA [7, 73, 99, 100]. In these models serum Abs

against murine CII or other autoAgs were able to induce transient arthritis when injected into naïve mice [7, 8, 100, 101]. Deposition of immune complexes containing autoAgs is a known phenomenon in RA and serum/Ab induced arthritis models [102-106]. The Abs leaking from synovial blood vessels [106] may associate with autoAgs (such as PG or CII) found locally in the joints. The immune complexes formed this way can activate the complement system, leading to the recruitment of Fc receptor-bearing phagocytic cells (neutrophils and monocytes) from the circulation. These cells then infiltrate the tissues and cause swelling [8, 103, 105, 107, 108].

Despite that neither serum nor T cell or B cells are alone are capable of transferring arthritis in PGIA [20, 97] during adoptive transfer, a mild transient synovitis has been observed after co-injection of serum and T cells. This transient arthritis could be prolonged by repeated administration of immune serum, but serum transfer with B cells did not lead to similar results [73]. These observations suggest that although arthritogenic Abs and T cells might synergize to initiate inflammation, the presence of B cells (supported by T cells) in the lymphoid organs is necessary for arthritis development and the establishment of chronic disease.

Then what is the reason for the effectiveness of T cell targeting approaches, using anti-CD4 Abs, genetic manipulation or T cell depletion [67] in preventing arthritis in the PGIA [109], CIA [110] and K/BxN [8] models, and the failure of T cell targeting strategies in RA? There, despite the availability of T cell targeting therapies since the 1990s, clinical trials using anti-CD4, anti-CD5, anti-CD7 and anti-CD52 mAbs have not been able to demonstrate a definite clinical efficacy or connection between Ab-induced T-cell depletion and disease status [111]. The only method of T cell inhibition that showed promise was blockade of signaling through the co-stimulatory molecule CD28 using a CTLA4-Ig fusion protein known as abatecept [112]. Indirect T cell blockade through administration of B cell inhibitor anti-CD20 mAb (rituximab) might decrease the activated phenotype of peripheral and tissue-resident T cells through elimination of Ag-presenting B cells [113]. However, the results are controversial: administration of rituximab might enhance the number and function of T cells [113], but another study found that rituximab therapy did not influence the frequency of Treg cells in RA [114].

Rituximab was also described to deplete the CD20$^+$ subset of T cells with proinflammatory properties. Depletion of a proinflammatory T cell subset might contribute to the beneficial effect of B cell targeting therapies of RA [115].

What might be the reason for this discrepancy between the efficacy of T cell depletion therapies between murine arthritis models and RA? Firstly, RA is characterized by 'waxing and waning' of disease symptoms and a persistence of polyclonally or oligoclonally expanded T cells including self-reactive populations. In most rodent models however, joint inflammation is preceded by a robust and single-Ag focused T cell response, hence they can be described as monophasic. Only a part of this response is directed against self. By deleting T cells or knocking out molecules essential for the effector function of T cells, the development of arthritis is easily preventable. Also in animal models, T cells show an early involvement in arthritis thus anti-CD4 therapies are most effective if used as a prophylaxis and administered before the appearance of clinical symptoms [110]. Administration of anti-CD4 treatment in the CIA model after the disease onset did not suppress the disease or eliminate the CII-reactive pathogenic population of T cells [110]. This might in part explain the why this treatment proved inefficient when used in established RA. Secondly, in the majority of animal models, counter-regulatory mechanisms mainly involving innate immune cells such as joint-infiltrating myeloid-derived suppressor cells [75] kick in early during the course of inflammation. By effectively reducing T cell responses and inflammation in a reasonably short period of time, these innate immune cells mask the potentially beneficial effects of T cell targeting therapeutics administered after disease onset. The success of abatacept mediated blockade of T cell co-stimulation [112] indicates that it may be more effective to indirectly influence T cells rather than depletion or neutralization. Adoptive transfer of CD25$^+$ Treg cell inducing bone marrow cells suppressed ongoing CIA [116] and PGIA [39]. This indicates that by increasing the number of either in vivo induced or transferred ex-vivo-induced autologous Treg cells could be effective in the management of RA. B cell depletion by administration of anti-CD20 Ab proved to be highly effective in RA not only by reducing T cell dependent autoAb production in PGIA [33] and K/BxN [42] models, but also by diminishing PG-specific T cell responses and by increasing Treg cell activity in PGIA [33]. These animal

studies suggest that B cell depletion via rituximab in RA probably has an indirect effect on T cells by shifting the balance between pathogenic and suppressive subsets in favor of suppressive Treg cells. In conclusion, the reason why T cell depletion therapy failed in RA whilst it proves to be effective in mouse arthritis if administered before the disease onset might be due to differences in disease course and in the potency and persistence of autoreactive T cells. These results suggest that the key to further success in the treatment of RA lies in therapies that restore or stabilize the number and suppressive function of Tregs and other regulatory cells.

Focusing our attention to T cell-T cell and T cell-DC interactions and how these interactions affect the motile behavior of T cells we found, that Ag-experienced T cells from genetically unmanipulated but PG-immunized mice, exhibited similar behavior to naïve TCR-Tg cells in response to encountering Ag presenting cells that carry cognate Ag in the lymph nodes. The differences in motility between the Ag-experienced and naïve T cells were clearly detectable in both arthritic and naïve recipients following co-transfer of these cells. These findings are consistent with our theory that repeated immunization with PG in the presence of adjuvant considerably increases the number of Ag-experienced T cells expressing specific TCRs. The high number of these cells in PGIA allowed proper analysis of motility in the context of presentation of PG in the lymph nodes of naïve and arthritic recipient mice. The higher affinity of the TCRs of PG-experienced memory cells to MHC-PG peptide complexes (as suggested by the PG tetramer binding experiment) and the higher expression of co-stimulatory and adhesion molecules, as compared to naïve T cells, facilitate the establishment of long-lived contacts between APCs and Ag-experienced T cells. These long-lived contacts limit the exposure of the naïve T cells to the same APCs when co-transferred as proved by our in vivo and in vitro experiments. However the arrest of these naïve cells is much more robust when transferred into the arthritic lymph node. This can be explained by the increased frequency and higher Ag presenting potency of the APCs in the arthritic lymph node. However other factors for example new peptide epitopes (including autoepitopes) generated in the host mice upon repeated immunization and/or inflammatory joint destruction [15, 16] might contribute to the more robust arrest. When these Ags presented to naïve T cells, self-tolerance can be broken in BALB/c mice

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during the induction of PGIA, as the arthritic mice show reactivity with self PG [16] and other Ags like citrullinated proteins and self IgGs which are not related to PG. These reactivities are known to arise due to intra- and intermolecular epitope spreading in both BALB/c mice with PGIA of humans with RA [17, 117-120].

APCs in the lymph nodes of mice with PGIA are likely able to present a large repertoire of antigenic peptides with diverse specificities. Cognate T cells for these peptides may exist in the transferred naïve and Ag-experienced populations. The fact that Ag-experienced T cells preferentially engage in long-lasting interactions with endogenous APCs and that they only transiently contact recently emigrated, cultured and intraarticularly injected exogenous dendritic cells in the lymph nodes of arthritic recipient mice supports this theory.

Last, we found that T cells initiate long term contacts less frequently in the absence of inflammation (arthritis) at the site of Ag injection. The biological significance of this phenomenon and whether it is restricted to PGIA needs further investigation.

Summary

In my PhD study, I characterized the trafficking and motility of T cells in the joint and JDLN in PGIA. My new findings are as follows:

- Development of PGIA does not involve significant influx of T cells into the joints.

- I monitored activation-related T cell motility in vivo in genetically unaltered mice for the first time in a complex autoimmune inflammatory disease.

- The motility of Ag-experienced T cells is similar to the motility of TCR transgenic T cells described by other research groups using model antigens.

- T cells are preferentially arrest in JDLN of mice with PGIA.

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

- The effectiveness of T cell depletion therapies in murine models of RA can be explained by the elimination of T cells before disease onset (which is possible in experimental arthritis but not in RA), and by the different course of the disease in animal models and RA.

Animal models of RA are essential for in vivo investigation and analysis of T cell recruitment, T cell-APC interactions and competition between Ag-experienced and naïve T cells for Ag-bearing APCs in the lymphoid organs. These investigations are not accessible for in vivo studies in humans. The poor availability of these cells in the distal joints and the relatively limited amount of synovial fluid makes these models less ideal to study function of these cells at target sites. Additional studies are needed to refine our understanding of T-cell pathology in autoimmune arthritis and to further enhance the value of preclinical animal studies in predicting the outcome of T-cell-focused therapeutic interventions in RA.

Összefoglalás

A PhD értekezésemben a T sejtek vándorlását, valamint mozgási tulajdonságait vizsgáltam a bokában és a bokát drenáló nyirokcsomókban. Eredményeim:

- A PGIA kialakulása nem jár együtt jelentős számú T sejtek bokába történő vándorlásával.
- A T sejtek aktivációjával kapcsolatos motilitási változásokat vizsgáltam in vivo, genetikusan nem módosított egerekben, első alkalommal egy komplex autoimmun gyulladásos betegségben.
- Az Ag-felismerő T sejtek mozgási tulajdonságai hasonlóak a TCR transzgenikus egerekből izolált T sejtekéhez.
- A T sejtek mozgása jelentősen lelassul a PGIA-s egerek gyulladt ízületeit drenáló nyirokcsomókban.
- A naív T sejtek motilitását befolyásolja a gyulladás és a környezetükben lévő sejtek.
- A T sejt depléciós terápiák hatékonyságát egérmodellekben részben a T sejteknek a betegség előtti eliminálása, részben a betegség monofázisos lefolyása magyarázza.

Az RA állatmodelljei nélkülözhetetlenek a T sejtek migrációjának, a T sejt és APC közötti interakciók, valamint a naív és Ag-felismerő T sejtek közötti kompetíció in vivo vizsgálatához. További tanulmányok szükségesek ahhoz, hogy jobban megértsük a T sejtek szerepét az RA-ban, és hogy növeljük a T sejtek ellen irányuló terápiás stratégiákat vizsgáló állatkutatások értékét.

Links to online two-photon videos

http://arthritis-research.com/content/12/2/R44/additional

Supplemental Video 1. Localization and motility of fluorescent T and B cells in the popliteal LN of a SCID mouse following their transfer from arthritic BALB/c mice. Donor T cells were labeled with CellTracker Red (CMTPX) and non-T cells (~90% B cells) with CellTracker Green (CMFDA), and mixed at a ratio of 1:2 prior to injection into the SCID mouse. The popliteal LN was subjected to TPM imaging on day 7 after cell transfer. Z series of images were acquired from the LN in vivo. Autofluorescent subcapsular sinus macrophages (light blue) are seen in the top layer of the cortex. B cells (green) localize in the more superficial, while T cells (red) in the deeper areas of the LN. Most T and B cells exhibit a polarized shape and 'random' movement. Imaging depth: 87 µm; time elapsed: 9.5 min.

http://www.sciencedirect.com/science/article/pii/S000887491200161X

Supplementary video 4: (mmc4) Movie 1. Motility of co-transferred naïve (green fluorescent) and antigen-experienced (red fluorescent) T cells in the popliteal LN 4 hours after injection of cognate antigen (PG) into the ankle joint of an arthritic recipient mouse. The majority of T cells in both the naïve (green) and antigen-experienced (red) populations exhibits round shape and limited motility. Only a few motile cells are seen among the naïve population.

Supplementary video 5: (mmc5) Movie 2. Motility of co-transferred naïve (green fluorescent) and antigen-experienced (red fluorescent) T cells in the popliteal LN 72 hours after injection of cognate antigen (PG) into the ankle joint of an arthritic recipient mouse. At this late time point, both naïve (green) and antigen-experienced (red) T cells exhibit high motility.

Supplementary video 6: (mmc6) Movie 3. Time-lapse movie of interactions between recently migrated dendritic cells (DCs) and antigen-experienced T cells in the popliteal LN of an arthritic mouse 24 hours after injection of the DCs and antigen into the ankle joint. The green fluorescence-labeled DCs that recently migrated to the LN from the ankle joint exhibit a probing movement by dynamically extending their dendrites in all directions. However, only a few T cells maintain a transient contact with the dendrites of any of the DCs (a briefly appearing white arrowhead "V" points to an example of such a contact). These antigen-experienced T cells preferentially engage with resident APCs that are most likely represented by the yellow autofluorescent endocytic vesicles. Autofluorescent vesicles are frequently seen in transferred DCs (Fig. S1B), and cells having yellow autofluorescence can be identified as endogenous APCs by positive staining with anti-MHC Class II antibody in JDLN tissue sections (not shown). Such endogenous APCs are visible in this movie within areas indicated with white dotted circles.

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Publications

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List of publications related to the dissertation

1. **Kobezda, T.**, Ghassemi-Nejad, S., Mikecz, K., Glant, T.T., Szekanecz, Z.: Of mice and men: How animal models advance our understanding of T-cell function in RA.
   Nat. Rev. Rheumatol. Epub ahead of print (2014)
   DOI: http://dx.doi.org/10.1038/nrrheum.2013.205
   IF:9.745 (2012)

2. **Kobezda, T.**, Ghassemi-Nejad, S., Glant, T.T., Mikecz, K.: In vivo two-photon imaging of T cell motility in joint-draining lymph nodes in a mouse model of rheumatoid arthritis.
   Cell. Immunol. 278 (1-2), 158-165, 2012.
   DOI: http://dx.doi.org/10.1016/j.cellimm.2012.08.003
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3. Angyal, A., Egelston, C., **Kobezda, T.**, Olasz, K., László, A., Glant, T.T., Mikecz, K.: Development of proteoglycan-induced arthritis depends on T cell-supported autoantibody production, but does not involve significant influx of T cells into the joints.
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7. **Kobezda, T.**: The role of the foot in the mechanism of shock absorption.
   Biomech. Hung. 2 (1), 31-38, 2009.

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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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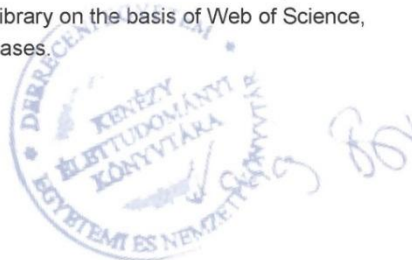
Presentations and Posters

- In vivo imaging of T cell motility in the JDLN of genetically unmanipulated mice in a model of rheumatoid arthritis.
  Tamas Kobezda, Sheida Ghassemi-Nejad, Colt Egelston, Tibor T. Glant and Katalin Mikecz.
  American College of Rheumatology Annual Scientific Meeting, Chicago, 2011
  Arthritis and Rheumatism, 63 (10S), 286, 2011

- Genome-wide methylation profiling studies of lymphocytes in arthritic mice
  Tamas Kobezda, Katalin Olasz, Katalin Mikecz, Tibor T. Glant and Tibor A. Rauch
  American College of Rheumatology Annual Scientific Meeting, Chicago, 2011
  Arthritis and Rheumatism, 63 (10S), 752, 2011

- Arthritogenic T cells regulate the homeostatic expansion of antigen specific B cells and these B cells or antibodies essential for the development of arthritis
  Katalin Kis-Toth, Mariann Radacs, **Tamas Kobezda**, Willem van Eden, Katalin Mikecz and Tibor T. Glant
  American College of Rheumatology Annual Scientific Meeting, Chicago, 2011
  Arthritis and Rheumatism, 63 (10S), 688, 2011

- Myeloid derived supressor cells present in the synovial fluid of mice with proteoglycan-induced arthritis are potent supressors of dendritic cell maturation and T cell proliferation
  Julia Kurko, Colt Egelston, Timea Besenyei, Beata Trynisewska, **Tamas Kobezda**, Tibor A. Rauch, Katalin Mikecz and Tibor T. Glant
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  Arthritis and Rheumatism, 63 (10S), 393, 2011

- Dancing with the stars: In vivo imaging of immune cells in the joints and JDLN of mice with PGIA

  Tamas Kobezda, Sheida Ghassemi Nejad, Colt Egelston, Tibor T. Glant and Katalin Mikecz

  Midwest Connective Tissue Workshop, Chicago, 2010

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  Annual Meeting of the American Association of Immunologist, Seattle 2009.

- Introduction of the structure of the foot on plastination preparation and the dynamics of the foot

  Tamas Kobezda

  Hungarian National Scientific Circle of Students, Budapest, 2007

- In vivo examination of analgetic and antiinflammatory effects of TRPV1 receptor antagonists

  J. Szilvási, **T. Kobezda**, T. Kark

  Scientific Circle of Students, Debrecen, 2007

- The effect of AMG9810 in various pain tests and on capsaicin-induced neuropeptide release

  T. Kark, A. Toth, J. Szilvasi, **T. Kobezda**, A. Szalai, J. Nemeth, R. Porszasz

  Congress of European Neuropeptide Club, Santorini Island, 2007

- Introduction of the structure of the foot on plastinated preparation: the dynamics of the foot

  T. Kobezda

  Scientific Circle of Students, Debrecen, 2006

Appendix