Recruitment and proliferation of CD4\(^+\) T cells in synovium following adoptive transfer of adjuvant-induced arthritis

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Abstract

Adjuvant-induced arthritis can be transferred to naive Dark Agouti (DA) strain (DA.CD45.1) rats by thoracic duct (TD) lymphocytes. Disease can be re-induced in convalescent rats by further transfer of arthritogenic cells, suggesting that resolution of the adoptive disease is not due to active regulation. To examine whether resolution is due to exhaustion of effector cells, we transferred the disease to DA.CD45.1 recipients, using CD4\(^+\) T cells from DA.CD45.2 donors. At the height of the adoptively transferred disease, donor cells comprised only 5–10% of recirculating CD4\(^+\) T cells but they accounted for ~40% of the CD4\(^+\) T cells in synovium-rich tissues of the hind paws. Approximately 65% of the donor cells in the synovium expressed a marker of proliferation (Ki-67 antigen). Division of CD4\(^+\) T cells continued in shielded paws after suppression of the recirculating pool of lymphocytes by selective irradiation. Intravenously injected CD4\(^+\) TD T lymphoblasts from arthritic donors were recruited to normal paws and, in greater numbers, to paws of animals with existing arthritis. Survival of the \([\text{I}^\text{125}]\)iodo-deoxyuridine-labeled lymphoblasts was greater in animals with existing arthritis. We conclude that effector CD4\(^+\) T cells in target tissues can proliferate in response to autoantigens and exhibit enhanced survival. However, without a continuous supply, adoptively transferred effector cells do not produce autonomous local disease, due to limits to their lifespan and ability to replicate indefinitely.

Introduction

Adjuvant-induced arthritis (AA) is a polyarthritis that shares a number of features with rheumatoid arthritis (RA) in humans (1). Recent studies on the related disease oil-induced arthritis (OIA) suggest that both OIA and AA have genetic relevance as models for studying the pathogenesis of RA (2–4). In RA, the synovium contains large numbers of CD4\(^+\) T cells and activated dendritic cells (DCs), to the extent that it can assume the appearance of quasi-lymphoid tissue (5, 6). This raises the important question whether the inflammation in the synovium becomes autonomous in RA, sustained locally by activation, proliferation and differentiation of T cells in response to local arthritogens. In addition, there is evidence that activated T cells are recruited to rheumatoid synovium from the circulation (7), although it is not known what contribution these cells make to the disease process, their fate after recruitment, their origin or the proportion that is arthritogen specific.

Others and we have shown that polyarthritis can be transferred adoptively in the adjuvant-induced model in rats (8, 9), providing the opportunity to examine the fate of a single cohort of effector cells after intravenous injection into naive recipients. AA can be induced in susceptible strains of rats by immunization with CFA and the disease is mediated by T lymphocytes (1, 10, 11). In the Dark Agouti (DA) strain, immunization reproducibly generates effector cells that are competent to transfer disease (9). As few as 5 \(\times\) 10\(^7\) CD4\(^+\) T cells collected from the thoracic duct (TD) lymph during the late prodromal phase of the disease can transfer AA to naive syngeneic recipients, without the requirement for \textit{ex vivo
Effector T cells in adjuvant-induced arthritis

activation (9). The resulting synovitis is independent of components from the adjuvant (12) or concomitant transfer of antigen-presenting cells (APCs), suggesting that the donor CD4+ arthritogenic T cells are re-stimulated locally by an encounter with endogenous arthritogens (9, 13).

An interesting feature of adoptively transferred AA is that it is a self-resolving monophasic disease. This raises the question whether resolution is due to the activities of immunoregulatory cells, or whether it reflects the lifespan and replicative potential of the effector cells in the synovial microenvironment. To investigate the kinetics of effector T cells following transfer, we have bred a CD45.2 congenic of the arthritis-prone DA (CD45.1) strain and showed that it shares arthritis susceptibility with the parent strain. Availability of congenic donor cells and of a method to recover cells from synovium-rich tissues (SRTs) of the hind paws (14, 15) has allowed us to investigate the events that follow the arrival of arthritogenic T cells in the synovium. We describe the early recruitment of lymphoblasts after intravenous injection of purified arthritogenic CD4+ TD cells, the kinetics of donor and host T cell numbers in SRTs during the pathogenesis of adoptively transferred AA and the local proliferation of T cells in the synovium. The results have general relevance to understanding the behavior of effector T cells following their recruitment to a virgin target tissue.

Methods

Source of rats

PVG.CD45.2 rats were obtained from the Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia. SPF inbred DA.CD45.1 wild-type or DA.CD45.2 congenic (see below) rats were obtained from the Veterinary Services Division, Institute of Medical and Veterinary Science, Adelaide, Australia. All were females, aged 7–8 weeks at the commencement of experiments. During the experimental period, they were provided with standard rat food pellets and water ad libitum, and housed in conventional conditions at the Animal Care Facility, Institute of Medical and Veterinary Science. Experimental procedures, including observation of paws for arthritis, were performed under anesthesia (halothane or isofluorane plus nitrous oxide). Approval for the study was obtained from the Animal Ethics Committees of the Institute of Medical and Veterinary Science and the University of Adelaide.

Production of DA.CD45.2 congenic strain

To produce the DA.CD45.2 congenic strain, male PVG.CD45.2 rats were crossed with wild-type DA.CD45.1 rats and the F1 progeny were back-crossed to the DA.CD45.1 wild type. CD45.2-positive progeny were selected for further back-crossing, using flow cytometric screening of peripheral blood. Appropriate sex selection to the third back-cross was used to eliminate PVG X- and Y-chromosomes, and at this time, histocompatibility with DA wild-type rats was examined by skin grafting. Progeny of histocompatible parents were selected for further breeding. After the 12th back-cross, rats were intercrossed to obtain CD45.2/CD45.2 homozygotes and a SPF colony was produced at the Animal Care Facility, Institute of Medical and Veterinary Science, by caesarean derivation.

Immunological reagents

The following mAbs were of mouse origin. mAbs R73 [anti-αβTCR (16)], W3/25 [anti-CD4 (17)], OX8 [anti-CD8 (18)] and OX33 [against the B cell-specific isoform of CD45 (19)] were used as neat culture supernatants from their respective hybridomas (gifts from Dr D. W. Mason and Dr A. N. Barclay, MRC Cellular Immunology Unit, Oxford, UK). The mAb MARK-1 [anti-κ light chains (20)] was produced by hybridoma cells provided by Dr H. Bazin (Université Catholique de Louvain, Bruxelles, Belgium). Anti-Giardia mAb 1B5 (G. Mayrhofer, unpublished results) was used as an IgG1 isotype control. Anti-Ki-67 (clone B56); anti-rat CD45.2 (mAb HIS41), either purified or FITC conjugated; PE- or biotin-conjugated mAb R73, FITC-conjugated mAb OX1 (anti-rat CD45); FITC-conjugated mAb 341 (anti-rat CD8); Cy-Chrome conjugated streptavidin were obtained from BD PharMingenTM (BD Biosciences, San Diego, CA, USA) and used at a concentration of 1 μg ml−1 (or at a dilution of 1:80 in the case of anti-Ki-67 when applied to cell smears). PE-conjugated HIS41 was obtained from eBioscience, San Diego, CA, USA. PE-conjugated goat anti-mouse Ig (GAM-Ig–PE), biotin-conjugated goat anti-mouse Ig (GAM-Ig–bi) and PE-Cy7-conjugated streptavidin were obtained from BD PharMingenTM (BD Biosciences, San Diego, CA, USA) and used at a concentration of 1 μg ml−1. Streptavidin-conjugated Alexa Fluor® 546 (S-AF-546) was supplied by Molecular Probes Inc., Eugene, OR, USA.

Induction of AA

AA was induced in 7-week-old rats by subcutaneous injection of 0.1 ml of CFA at the base of the tail (21). Essentially, all DA rats develop polyarthritis using this protocol, with inflammation appearing 9–10 days after inoculation. Although TD lymph is obtained at day 9 post-inoculation, rats receiving this treatment will be referred to nevertheless as ‘arthritic donors’. Untreated age- and sex-matched rats are referred to as ‘normal donors’.

Assessment of the severity of arthritis

Severity of polyarthritis was measured by allocating a score for each paw as follows: 0 (no evidence of arthritis), 1 (single focus of redness or swelling), 2 (two or more foci of redness or swelling), 3 (confluent but not global swelling) or 4 (severe global swelling). The ‘joint score’ for each rat is the sum of the scores obtained from the four paws (maximum score = 16).

Irradiation of rats

Rats were anesthetized with 0.07 ml Nembutal (Merital Australia Pty Ltd, Parramatta, Australia) and positioned in a Perspex tray so that their hind paws, including the ankles to the anatomical hairline, were shielded with 6-mm lead sheet. X-irradiated rats received a total dose (including back-scatter) of either 7.5Gy or 9.5Gy using a Philips 250/25 Deep X-ray instrument (Philips, Amsterdam, Denmark). The rats were re-positioned at half the dose so that they were irradiated equally.
from each side. Control rats were anesthetized but not irradiated.

Collection of TD lymph
Cannulation of the abdominal TD was performed as described previously (22). TD lymph was collected overnight at room temperature into flasks containing 5 ml of a solution of preservative-free heparin (25 U ml\(^{-1}\)) in PBS. Donor arthritic rats were cannulated on day 9 after injection of CFA (the day of anticipated onset of clinical disease).

Adoptive transfer of AA
In all cases, the cells used in adoptive transfer experiments were obtained from TD lymph. Early experiments used unseparated TD cells and AA was transferred using 2 \(\times\) 10\(^8\) washed cells from arthritic donors. The cells were injected intravenously via the lateral tail vein in 2 ml of RPMI plus 2% FCS (RPMI–FCS) over a period of 1 min. In later experiments, CD4\(^+\) T cells were prepared from TD lymph of either normal or arthritic donors, as described below. For adoptive transfer of AA using genetically marked TD CD4\(^+\) T lymphocytes, 1 \(\times\) 10\(^8\) purified CD4\(^+\) T lymphocytes from DA.CD45.2 donors were injected intravenously into each DA.CD45.1 recipient.

Production of purified CD4\(^+\) T lymphocytes
Lymphocytes were collected from TD lymph pooled from several donors and washed twice in RPMI–FCS by centrifugation (350 \(\times\) g for 7 min at room temperature). Purified CD4\(^+\) T cells were obtained by depletion of CD8\(^+\) T cells and B cells, as described previously (9). Briefly, cells were incubated with a mixture of neat hybridoma supernatants containing mAbs OX8 (CD8\(^+\) T cells), MARK-1 and OX33 (B cells) and 0.01 M sodium azide for 40–60 min on ice. After washing three times with RPMI plus 2% FCS plus 0.01 M azide (RPMI–FCS–Az), the cells were incubated with Pan Mouse IgG Dynabeads (Dynal, AS, Oslo, Norway) in RPMI–FCS–Az at a concentration of 1.0–1.5 beads per cell for 20–40 min on a rotator at 4°C. After removal of cells bound to beads by three cycles of a Dynal MPC-6 magnet, the cells remaining were washed twice in RPMI–FCS and allowed to reach room temperature before intravenous injection. Analysis by flow cytometry showed that <1% of the purified cells were stained with the depleting antibodies and ~95–97% could be labeled with mAb R73 or OX35 (anti-\(\delta\)TCR and anti-CD4, respectively).

Radiolabeling and adoptive transfer of lymphoblasts
TD CD4\(^+\) T lymphocytes purified from pooled lymph were re-suspended in RPMI–FCS at a concentration of ~1 \(\times\) 10\(^7\) cells ml\(^{-1}\) and allowed to warm to 37°C. After addition of \([^{125}\text{I}]\)iodo-deoxyuridine (\([^{125}\text{I}]\)UdR) (1 \(\mu\)Ci ml\(^{-1}\)), incubation was continued at 37°C for 60 min (21). The cells were then washed three times in RPMI–FCS, divided into equal aliquots and transferred to either normal or arthritic syngeneic recipients. In the case of arthritic recipients, rats received the radiolabeled cells 12 days after inoculation of CFA. Each recipient received the equivalent of the overnight output from a single donor (~1 \(\times\) 10\(^8\) CD4\(^+\) T lymphocytes). The tissue distribution of radiolabeled cells in recipients sacrificed 24 h after cell transfer was determined radiometrically, as described previously (21). The radioactivity measured in each tissue site (less background) was expressed as the percentage of the total recovered radioactivity [counts per minute (c.p.m.) in organ \(\times\) 100/sum of the c.p.m. recovered in all tissues sampled]. The tissues selected for illustration in Results were the paws, small intestine, lung and thyroid. Other tissues included in the survey were the stomach, kidneys, uterus, liver, spleen, cecum, large intestine, thymus, lymph nodes (LNs) (mesenteric, cervical, inguinal, iliac and popliteal), proximal 3 cm of tail (including the adjuvant injection site in immunized rats),~1 g of quadriceps and the knee joints (21). The hind paws were removed just proximal to the ankle joint.

Collection of synovial biopsies for preparation of frozen sections
Inflamed tissues from rats with adoptively transferred arthritis were biopsied from the lateral aspect of the ankle joint, using a scalpel blade. The biopsies were embedded in OCT (Tissue-Tek, Sakura Finetek, Torrance, USA) immediately, snap frozen in isopentane cooled by liquid N\(_2\) and stored at –70°C until required.

Preparation of cells from recipient tissues for flow cytometric analysis
Tissues were collected from rats at specified times after transfer of genetically marked cells. Peripheral blood (~0.5 ml) was collected under anesthesia from a ventral artery in the tail into a syringe with the dead space loaded with heparin (5000 U ml\(^{-1}\)). A 22-gauge cannula was inserted into the abdominal aorta via a laparotomy incision and 1 ml of 100 U ml\(^{-1}\) heparin–PBS was injected. A second cannula was inserted into the inferior vena cava to collect the effluent and perfusion was commenced immediately with PBS at 4–6 ml min\(^{-1}\) for 2 min to wash blood from the hind limbs. Perfusion was continued for a further 8 min at the same rate, using RPMI containing 2% FCS, 12 \(\mu\)g ml\(^{-1}\) penicillin, 16 \(\mu\)g ml\(^{-1}\) gentamycin and 250 U ml\(^{-1}\) type 1 collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA). The rat was euthanized by opening the thorax and the popliteal and cervical LNs were removed and diced with fine scissors. Lymphocytes were expressed gently from the fragments using a loose-fitting homogenizer and the cells were re-suspended in RPMI containing 2% FCS, 12 \(\mu\)g ml\(^{-1}\) penicillin and 16 \(\mu\)g ml\(^{-1}\) gentamycin at 1 \(\times\) 10\(^7\) cells ml\(^{-1}\). Cells from the SRTs of the hind paws of the same rats were prepared by further digestion of the skinned and disarticulated paws with collagenase in vitro, as described elsewhere (14).

Flow cytometric analysis of lymphocyte sub-populations
Flow cytometry was used to assess the purity of CD4\(^+\) T cell preparations, to assess expression of cell-surface antigens and to measure the absolute numbers of host and donor cells in SRTs, LNs and blood. Approximately 1 \(\times\) 10\(^5\)–2 \(\times\) 10\(^5\) cells (cell suspensions) or 100 \(\mu\)l of heparinized whole blood (blood leucocytes) were incubated for 50 min on ice with mouse anti-rat mAbs [100 \(\mu\)l of neat culture supernatant containing 0.01 M azide or purified mAb diluted to 1 \(\mu\)g ml\(^{-1}\) in 100 \(\mu\)l of PBS plus 2% FCS plus 0.01 M azide (PBS–FCS–Az), all containing 10% normal rat serum (NRS)]. After washing twice in PBS–FCS–Az,
cells labeled with unconjugated mAbs were incubated with either GAM-Ig–PE or GAM-Ig–FITC (containing 10% NRS) for a further 1 h on ice in the dark. The cells were then washed twice in PBS–FCS–Az and in the case of cell suspensions fixed with 1% formalin (v/v) in PBS containing 2% glucose (w/v) and 0.02% azide (w/v). In the case of blood samples, the cells were re-suspended in residual buffer and red cells were lysed by vortexing for 5 s with 265 μl of 0.12% formic acid, followed by the immediate addition of 600 μl of cell-stabilizing solution (0.6% Na₂CO₃, 1.45% NaCl and 3.13% Na₂SO₄) and vortexing for a further 5 s before adding 3 ml of PBS–FCS–Az. After a second wash with PBS–FCS–Az, the cells were fixed as above.

For three-color analysis, cells were labeled directly with fluorochrome-conjugated mAbs and indirectly via unconjugated mAbs. Where applicable, cells (SRT or LN) were labeled first by the indirect technique. After completion of labeling with the secondary antibody (GAM-Ig–PE or GAM-Ig–FITC), the cells were washed twice in PBS–FCS–Az and incubated with 20 μl of neat normal mouse serum (NMS) for 15 min. Without removing the NMS, the cells were incubated with conjugated anti-rat mAbs (FITC-, R-PE- or Cy-Chrome™ as appropriate), and then washed and fixed as above. Control preparations, in which the primary antibody was replaced by washing buffer or by an isotype-matched mouse mAb of irrelevant specificity, were included in each analysis.

To label cells expressing the Ki-67 antigen, SRT cells or LN cells (0.5 × 10⁶–2.0 × 10⁷) were vortexed briefly and re-suspended in 5 ml cold fixative containing freshly made 3% PFA in PBS, pH 7.4. After fixation for 20 min, the cells were washed twice in PBS–FCS–Az and held overnight in the same buffer at 4°C. For permeabilization, the cells were re-suspended at 1 × 10⁷ ml⁻¹ in cold, freshly made saponin buffer, containing 1% saponin (Sigma Chemical Co., St Louis, MO, USA) and 5% BSA (Trace Biosciences, New Zealand) in PBS, pH 7.4. For the four-color analysis, aliquots (100 μl) of the re-suspended cells were transferred to tubes containing appropriate amounts of the primary antibody (anti-Ki-67 or control mAb) diluted in NRS. The cells were then labeled by the indirect technique as described (see above, three-color analysis), except that the secondary antibody (GAM-Ig–FITC) used to detect the anti-Ki-67 mAb was diluted in saponin buffer, and the fourth color was achieved using R73–biotin and streptavidin PE–Cy7.

Labeled cells were analyzed using a COULTER® EPICS® XL-MCL flow cytometer and CYTOMICS™ RXP software. Cell aggregates were excluded by gating on events with a constant peak height versus peak area ratio. In the case of cells from SRTs, events include cells of hematopoietic origin (lymphocytes, macrophages, polymorphonuclear leucocytes, etc), cells of non-hematopoietic origin (fibroblasts, endothelial cells, etc) and assorted debris. A ‘lymphocyte’ gate was defined by examining the forward and side scatter of LN cells (see Fig. 4A and Results). Analysis was confined to events within this lymphocyte gate, from a total of ∼5 × 10⁵ events. To estimate the absolute numbers of cells, a standard number (5 × 10⁵) of FITC–polystyrene beads (CalibRITE™, BD Biosciences) was added to each tube. The absolute number of cells per tube, as represented in a specified electronic gate, was calculated as follows: absolute number of cells per tube = number of cells (cytometer gate)/number of beads (cytometer gate) × (5 × 10⁵).

Preparation of cell smears

Cytopsin preparations were prepared on glass slides subbed with gelatin (5 g l⁻¹)–chrome alum (0.5 g l⁻¹), using SRT cells prepared from rats 9 days after receiving CD4⁺ TD T cells from arthritic DA.CD45.2 donors. Cytopsins were prepared using cytocentrifugation (Cytopsin 4, ThermoShandon, ThermoElectron Corporation, Runcorn, UK), by applying 5 × 10⁵ cells to each slide.

Immunocytochemistry

Enumeration of Ki-67⁺ donor cells or Ki-67⁺ T cells was achieved by dual fluorochrome immunofluorescence. Air-dried cell smears and frozen sections were fixed in acetone at 4°C for 10 min and washed in three changes of cold PBS. To detect CD45.2 (donor cells) and the Ki-67 antigen simultaneously, 50-μl aliquots of antibodies (mAbs HIS41 and B56, respectively) diluted in 10% NRS were applied to each slide in the dark for 50 min in the following order (with 3 × PBS washes after each incubation, except where indicated): mAb B56 (1/80), GAM-Ig–bi (1/50), 10% NMS (20 min, one wash) and S-AF-546 (1/200) + mAb HIS41–FITC (1/50) + 10% NMS. To detect T cells and the Ki-67 antigen simultaneously, the order of incubations was as follows: mAb B56 (anti-Ki-67), GAM–FITC, 10% NMS and mAb R73–PE conjugate (anti-rat αβTCR). Stained cell smears or frozen sections were mounted in Dako® fluorescent mounting medium and stored in the dark until microscopic examination. Control slides were included, where each of the antibodies or fluorochrome conjugates was replaced separately by washing buffer or by an isotype-matched mouse mAb of irrelevant specificity.

Confocal microscopy

Cells were visualized by epifluorescence using a Bio-Rad MRC-1000UV confocal laser scanning microscope, allowing individual cells to be assessed as positive or negative for surface and/or nuclear labeling. Cells were examined at random by tracking from the top left to bottom right of each slide. Each cell that was HIS41⁺ or R73⁺ was scored as positive or negative for expression of the Ki-67 antigen.

Statistical analyses

Differences in the numbers of cells shown in Figs 5(A and B) and 6(A, F and G) were analyzed using one-way analysis of variance (ANOVA), with log transformation of the outcome and appropriate post hoc comparisons. Comparisons between control and X-irradiated rats shown in Fig. 9 were made using a paired t-test. The data in Fig. 10 were analyzed by one-way ANOVA—some tissues showed heterogeneity of variance and were analyzed by weighted ANOVA, with the weighting proportional to the reciprocal of the variance. Comparisons were made between recipients and between donors, although only the donor comparisons are shown.

Results

Kinetics of onset and resolution of adoptively transferred AA

Figure 1(A) illustrates the arthritis that follows adoptive transfer of 2 × 10⁵ TD lymphocytes from DA donors 9 days after
inoculation of CFA to naive syngeneic recipients. Clinical joint scores reach a peak 8–10 days after transfer and are sustained until about day 16, before gradually declining. Beyond approximately day 24, acute inflammation subsides and only fibrotic lesions remain. To examine whether the subsidence of inflammation is due to the development of immunoregulatory mechanisms that suppress the activity of the transferred effector cells, we administered a second bolus of $2 \times 10^8$ TD lymphocytes (collected from donors 9 days after inoculation of CFA) to the convalescing animals at day 50 after the first transfer. A similar number of TD cells from the same pool were also administered to age-matched naive recipients. As shown in Fig. 1(B), the course of arthritis was essentially identical in the convalescent rats and the naive recipients. This suggests that the subsidence of inflammation in the convalescent rats was not due to induction of long-lived immunoregulatory cells but could indicate exhaustion of the arthritogenic cells from the first inoculum. In the experiments that follow, disease was transferred using purified CD4+ T cells from arthritic donors (9).

CD45 congenic arthritis-prone DA rats

To facilitate tracing of donor T cells, we produced CD45 congenic DA rats. The DA.CD45.2 and DA.CD45.1 wild-type animals demonstrated similar susceptibility to actively induced AA (Fig. 2). After transfer of $1 \times 10^8$ CD4+ T cells purified from TD lymph from either normal or arthritic DA.CD45.2 donors, serial sampling of blood from the recipients showed that the absolute numbers of donor cells from either source declined by ~1.9% per day between day 3 and day 24 (data not shown).

Cells from the SRTs of the hind paws of rats

The SRTs of the hind paws (ankles, small joints of the paws and the synovial sheaths of tendons) are targets of the disease process in adoptively transferred AA. Collagenase digestion of SRTs from a pair of normal hind paws produced $4 \times 10^6$ viable cells, consisting of both CD45+ and CD45−/C255 cells (Fig. 3A). This number was unchanged by adoptive transfer of TD lymph CD4+ T cells prepared from normal donors (Fig. 3A). However, following transfer of CD4+ T cells from arthritic donors, the total number of cells recovered from the paws increased between day 6 and day 9, coinciding with the escalation of inflammation in the paws (Fig. 3B). Approximately half of the viable cells recovered from the paws at this stage of the disease expressed CD45 (Fig. 3A and Fig. 4B). Many of the CD45+ cells had the light scatter characteristics of macrophages and polymorphonuclear leukocytes (Fig. 4A) but some expressed the $\alpha\beta$ TCR (Fig. 4B).

Donor and host CD4+ T cells in the SRTs of the hind paws after adoptive transfer of arthritogenic TD lymphocytes

A ‘lymphocyte gate’ (Fig. 4A) contained essentially all the T cells in the SRT preparations. After transfer of DA.CD45.2 CD4+ T cells, cells of donor or host origin within this gate were detected by staining with mAb HIS41 (anti-CD45.2), in a cocktail with mAb OX35 (anti-CD4) and mAb R73 (anti-$\alpha\beta$ TCR) (Fig. 4C). Addition of FITC-conjugated CaliBRITE™ beads to samples allowed calculation of the absolute numbers of lymphocytes in each SRT preparation. CD4+ T cells were detected in low numbers in normal SRTs ($<1 \times 10^4$ cells per pair of hind paws) and this number was essentially unaltered following adoptive transfer of CD4+ T cells purified from TD lymph of normal donors (Fig. 5A).
Evidence of local cell division and localization of donor CD4+ T cells in the synovium

To estimate the proportion of the donor cells in cycle in the SRTs, collagenase digests were prepared from the hind paws of two rats 9 days after adoptive transfer of arthritogenic TD CD4+ T cells. Cytospin preparations of the pooled cells were stained for dual-color immunofluorescence with mAb HIS41 to identify donor cells and mAb B56 to detect Ki-67 antigen in cycling cells (Fig. 7A). Counts of Ki-67+ cells revealed that ~66% of the donor cells were in active cell cycle. In contrast, when duplicate slides were dual stained to detect all αβTCR+ cells (mAb R73), ~55% of the total T cells (host plus donor) expressed the Ki-67 antigen (Fig. 7B). It can be calculated from these data that ~49% of host R73+ cells (CD4 plus CD8) were also in cell cycle.

To obtain direct estimates of the numbers and proportions of host and donor CD4+ T cells that are in cell cycle, Ki-67 antigen was detected by flow cytometry. Four-fluorochrome analysis was performed at day 6 and days 8–9 after adoptive transfer of arthritogenic CD4+ TD cells, labeling the fixed and permeabilized SRT cells with mAb B56 (Ki-67, FITC), followed by mAbs HIS41 (CD45.2, PE), OX35 (CD4, Cy-Chrome) and R73 (PE–Cy7). The results from a representative preparation are shown in Fig. 8, where it can be seen that a large proportion (~75%) of the CD4+ T cells in SRTs express Ki-67, compared
with cells from cervical LNs from the same animals (~17%). The proportions of donor and host Ki-67+ cells were approximately equal at day 6 (85 and 82%, respectively) and day 8–9 (80 and 71%, respectively), as summarized in Table 1. The proportions of Ki-67+ cells estimated by flow cytometry in SRT cells from rats examined 8–9 days after adoptive transfer were higher than those obtained by immunofluorescence microscopy (see above).

Because host cells represented ~95% of the total CD4+ T cells in the recirculating pool, it was reasoned that many of the cycling host-derived cells in the inflamed paws could have been recent recruits from the ambient pool of activated T cells. Therefore, the ambient pool was suppressed 5 days after transfer of the arthritogenic CD4+ T cells by subjecting the animals to whole-body irradiation, while shielding the hind paws. In four experiments, each with one irradiated and one control rat, the rats received either 7.5Gy or 9.5Gy of X-irradiation, doses that we have found to suppress adoptively transferred arthritis and reduce circulating CD4+ T cells to ~1% and 0.5% of control values, respectively (data not shown). This severe purging of lymphocytes from the tissues external to the hind paws caused moderate attenuation of the disease indices in the hind paws (joint scores, 6.0 unirradiated and 4.3 ± 0.5 irradiated). Rats were sacrificed either 3 days (7.5Gy, 1 rat; 9.5Gy, 2 rats) or 6 days (7.5Gy, 1 rat) after irradiation, SRT digests were prepared from the shielded hind paws and the cells were labeled for analysis by flow cytometry (see above). The proportions of donor and host CD4+ T cells in the SRT preparations and the proportions in cell cycle were estimated. Because the doses of irradiation used reduced circulating lymphocyte numbers by equivalent amounts, the results from all irradiated rats are combined into a single ‘irradiated’ group.

The numbers of CD4+ T cells recovered from pairs of hind paws in the irradiated group (range, 2.1 × 10⁴–30.2 × 10⁴) were lower than those in unirradiated controls (range, 7.4 × 10⁴–72.6 × 10⁴), although the ranges overlapped. The remaining cells appear, therefore, to have been resident in the paws at the time of irradiation. The proportions of donor cells are shown in Fig. 9(A). Irradiation of the body, with shielding of the hind paws, did not eliminate either donor or host CD4+ T cells, but it had a disproportionate effect on the host-derived cells. The proportion of donor-derived CD4+ cells in the irradiated group (mean, 64.6 ± 2.7%) was significantly greater than that in the control group (mean, 45.7 ± 5.1%; Fig. 4A).
In situ detection of Ki-67+ cells in the tissues of the hind paws

To verify that the CD4+ cells in the hind paws were indeed located in synovial tissues, biopsies of synovium were obtained from the ankle region and examined using dual fluorochrome immunofluorescence. Cells expressing the α/β TCR (red) and the donor CD45.2 allele (green) were detected in frozen sections of the inflamed synovium, together with T cells that were of host origin (Fig. 7C). Staining with mAb B56 (red) showed that many of the donor cells (green) in the synovium expressed Ki-67 (Fig. 7D). Differential counts of cells of donor origin showed that of 102 cells counted, 63% expressed the Ki-67 nuclear antigen. This figure agrees well with those obtained from isolated SRT cells, using immunofluorescence on stained smears or flow cytometry on cell suspensions (see above).

Recruitment of CD4+ T lymphoblasts to the synovium

To examine the nature of the donor cells that colonize the synovial tissues of the hind paws during the first 24 h after adoptive transfer, we labeled the lymphoblasts in CD4+ cells purified from the TD lymph of arthritic rats during the course of adoptively transferred arthritis. Cells from SRTs were prepared from the TD lymph of arthritic (9 days post-inoculation with adjuvant) or normal DA.CD45.2 donors. SRTs were prepared from pairs of hind paws (see Methods) at the times indicated (n = 3 rats at each time). Absolute numbers of donor- and host-derived CD4+ lymphocytes were determined from flow cytometric data by inclusion of FITC-conjugated CaliBRITETM beads in the analysis. Donor cells were identified as described in Fig. 4(C). (A) The number of donor and host-derived CD4+ T cells recovered from the SRTs of the hind paws of individual DA.CD45.1 recipients. Some normal control rats (‘day 0’) did not receive any cells (n = 2). The increase in numbers of host and donor CD4+ T cells from day 6 to day 9 was significant (*P < 0.05). (B) CD4+ T cells of donor origin are shown as the percentage of total CD4+ T cells in the SRTs. The percentage of donor CD4+ T cells at day 3 was significantly greater after transfer from arthritic donors, compared with normal donors (*P < 0.05). In the case of transfers from arthritic donors, there was a significant increase from day 3 to day 6 (**P < 0.05).

P = 0.016), suggesting that irradiation had reduced the ambient traffic of host-derived lymphoblasts to the tissues, as expected. Nevertheless, host-derived cells were not eliminated and it is noteworthy that the proportions of Ki-67+ cycling cells were similar in the remaining host- and host-derived CD4+ T cells (Fig. 9B and C) and that the proportions in each were not changed significantly by irradiation (P = 0.408, donor cells; P = 0.370, host cells). The similarity in the proportions of Ki-67+ donor and host-derived cells in the shielded paws suggests not only that the remaining host-derived population is independent of the ambient pool but also that it may be responding to locally presented autoantigens or to other stimuli in the inflamed synovium.

In situ detection of Ki-67+ cells in the tissues of the hind paws

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significance ($P = 0.051$), it is similar in magnitude to the difference (12%) between the amounts of radionuclide recovered from the paws of arthritic recipients (14.7%) compared with normal recipients (2.7%). Taken together, the results suggest that there is greater survival of the transferred CD4+ T lymphoblasts in the paws of recipients with arthritis.

**Discussion**

Understanding T cell-mediated inflammation, in the context of autoimmune disease, requires knowledge of how the activities of effector T cells are regulated in the target tissues and, in particular, whether local presentation of antigen affects the longevity and proliferative behavior of these cells. An influential view exists that effector T cells have very restricted ability to proliferate in target tissues (23, 24), although this is based mainly on the use of in vitro activated T cells and acute models of T cell recruitment. In a chronic disease such as RA, where cells may be recruited constantly to the affected synovium (7), even modest levels of local proliferation could amplify the pathogenic impact of the effector cells. We, therefore, examined the behavior of a single cohort of CD4+ T cells in synovium during the pathogenesis of adoptively transferred AA. Our findings do not support the conclusion that effector T cells lack the capacity to proliferate in target tissues. Importantly, we used naturally activated TD CD4+ T cells, thus avoiding the possible effects of in vitro stimulation on the recruitment, function and fate of the effector cells in vivo (25). Adoptive transfer of AA in DA strain rats is independent of exogenous antigens, suggesting that the disease-causing T cells are reactivated locally in synovial tissues by endogenous arthritogens, the nature of which is unimportant in the context of these studies.

Our finding that arthritis can be re-induced in convalescing rats by adoptive transfer of a second bolus of arthritogenic TD lymphocytes indicates that the decline of inflammation following the first bolus is not due to the development of long-lived immunoregulatory mechanisms. Our resulting hypothesis was that adoptive disease is terminated by exhaustion of the effector cells and that in the actively induced disease the arthritis is maintained by successive cohorts of arthritogenic cells that are released from LNs in response to the adjuvant. To investigate the behavior of donor- and host-derived T cells after adoptive transfer, we developed a CD45.2
nuclear staining). Staining) in synovium and three expressing Ki-67 antigen (B56, red)
(D) Five donor T cells (HIS41, green membrane green) T cells in synovium. Three donor-derived T cells are shown
antigen appears yellow. (C) Total (R73, red) and donor-derived (HIS41, green) T cells in synovium. Three donor-derived T cells are shown
green (green plus red). (D) Five donor T cells (HIS41, green membrane staining) in synovium and three expressing Ki-67 antigen (B56, red nuclear staining).

**Fig. 7.** Detection of T cells and Ki-67 antigen expression in cytospin preparations of cells from SRTs (A and B) and in tissue sections (C and D) prepared from DA.CD45.1 recipients 9 days after adoptive transfer of CD4+ T cells prepared from TD lymph of arthritic (9 days post-inoculation with adjuvant) DA.CD45.2 donors. mAbs R73 (direct immunofluorescence), HIS41 (direct immunofluorescence) and B56 (indirect immunofluorescence) detect the αβTCR, CD45.2 and Ki-67 antigen, respectively. (A) Donor cells (HIS41, green) and cells expressing Ki-67 antigen (B56, red). (B) T cells (R73, red) and cells expressing Ki-67 antigen (B56, green). One T cell expressing Ki-67 antigen appears yellow. (C) Total (R73, red) and donor-derived (HIS41, green) T cells in synovium. Three donor-derived T cells are shown
(green plus red). (D) Five donor T cells (HIS41, green membrane staining) in synovium and three expressing Ki-67 antigen (B56, red nuclear staining).

**Table 1.** Proportions of donor- and host-derived CD4+ T cells in SRTs that express Ki-67 antigena

<table>
<thead>
<tr>
<th>Origin of CD4+ T cells</th>
<th>Time after adoptive transferb</th>
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<tbody>
<tr>
<td></td>
<td>Day 6 (n = 3)</td>
</tr>
<tr>
<td>CD45.2+ (donor)</td>
<td>85 ± 12</td>
</tr>
<tr>
<td>CD45.1+ (host)</td>
<td>82 ± 8</td>
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aApproximately 1 × 10^8 CD4+ T cells prepared from TD lymph of arthritic (9 days post-inoculation of adjuvant) DA.CD45.2 rats were transferred to DA.CD45.1 recipients, and at the times indicated, cell suspensions were prepared from SRTs of the hind paws for analysis by four-color flow cytometry. The cells were labeled to detect CD45.2, αβTCR, CD4 and Ki-67 antigen as described in Methods. Percentages of CD4+ T cells of donor or host origin that express Ki-67 antigen (mean ± standard deviation).

Congenic that shares susceptibility to AA with the wild-type DA.CD45.1 strain. T cells from both normal and arthritic DA.CD45.2 donors survive in DA.CD45.1 recipients for periods comparable with the rate of turnover of recirculating lymphocytes in normal rats (26, 27).

SRTs in normal paws contained a small numbers of T cells that were in physiological transit through the tissues (23, 28, 29) and the numbers were not changed significantly after adoptive transfer of TD CD4+ T cells from normal donors. In contrast, after adoptive transfer of TD CD4+ T cells from arthritic donors, the numbers of total viable cells recovered from the paws increased, in parallel with the clinical course of the acute polyarthritis. By day 9, half of the cells recovered did not express CD45, indicating that the inflammatory process involves hyperplasia of resident stromal cells as well as recruitment of cells of hematopoietic origin. These changes were noted as early as 3 days after transfer but the increase did not reach statistical significance until day 9. Nevertheless, small numbers of cells expressing CD45.2 were detected on day 3 and day 6 after transfer. This was consistent with the observation that [125I]UdR-labeled CD4+ T lymphoblasts were recruited to the SRTs within 24 h of transfer and with our earlier findings using unseparated TD lymphoblasts (21).

The early recruitment of donor lymphoblasts to the synovium may explain transient mild inflammation that usually occurs during the first 48–72 h after adoptive transfer of arthritogenic TD cells (9). These ‘pioneer’ cells could condition the synovium for participation in the inflammatory response that follows (30). This ‘conditioning effect’ might include production of chemokines, up-regulation of vascular endothelial adhesion molecules or induction of more efficient antigen presentation by DCs and/or macrophages. It is clear that very small numbers of donor T cells (~1.5 × 10^3 per paw at day 3 after adoptive transfer) are sufficient to initiate the early stages of synovitis. Even at the height of the disease (day 9 after transfer), CD4+ T cells constituted only 1.2% of total cells (2.4% of the CD45+ cells) in the SRTs.

The increase in CD4+ T cell numbers in the inflamed paws involved cells from both donor and host. The proportions of host-derived CD4+ and CD8+ T cells (~90% CD4+) reflect the CD4+:CD8+ T cell ratio in blood (~4:1, data not shown). Donor T cells in the SRTs reached a peak at day 9 after adoptive transfer and then declined as the inflammation subsided. Although donor
T cells accounted for only 4% of recirculating CD4+ T cells (LNs and blood), they represented 17% of the total CD4+ T cells in SRTs at day 3 and reached 40% at day 9 after cell transfer. This local enrichment in SRTs is consistent with a local antigen-specific process. In our earlier studies, we showed that after transfer of TD lymphoblasts from arthritic donors, recruitment into synovium commenced within minutes of injection and continued for at least 24 h as cells were released back into the circulation from extra-articular sites of initial lodgment (31). There was evidence that lymphoblasts from arthritic donors were retained in the synovium, compared with transient residence by those from normal donors. However, while the short-term recruitment and retention of lymphoblasts might be responsible for the pioneer cells that were present in the first days after adoptive transfer, it is unlikely to be the sole explanation for the
accumulation of donor cells that was observed to accelerate from day 6 and reach a peak 9 days after cell transfer.

The alternative explanation is that antigen-specific donor cells proliferated in response to arthritogens, either locally in SRTs or at other sites such as the local LNs. The latter explanation was not supported by the lack of donor cell enrichment in the popliteal LNs. Direct evidence of local proliferation of donor-derived cells was obtained by observing expression of Ki-67, a nuclear antigen that is up-regulated in cells that are actively cycling (32, 33). The majority of donor T cells in preparations of SRTs harvested on day 9 after adoptive transfer (~66% in cytosin preparations and 80% by flow cytometric analysis) expressed nuclear Ki-67 antigen. A similar proportion (63%) of the donor cells in frozen sections of synovium obtained at this time also expressed the Ki-67 antigen, confirming that the results from enzyme digests of SRTs were representative of synovial tissues in situ. Together, the results suggested that the enrichment of donor cells observed in the SRTs was at least in part the result of local cell proliferation.

Nevertheless, similar proportions of host-derived CD4+ T cells in SRTs were also in cell cycle. We have shown previously that lymphoblasts in ambient TD lymph from normal donors are recruited into inflamed synovium (21), suggesting this as a possible source of the Ki-67+ host-derived cells. Since host-derived CD4+ T cells in the recirculating pool (blood and LNs) outnumbered donor-derived cells by ~20:1, it is a reasonable expectation that non-specific recruitment of donor- and host-derived T blasts from the ambient pool would reflect this ratio. On this basis, the almost equivalent numbers of Ki-67+ donor- and host-derived cells in SRTs provides additional support for the hypothesis that arthritogenic donor cells have been expanded selectively by endogenous arthritogens. To assess whether some Ki-67+ CD4+ T cells in SRTs were recent immigrants from the circulation, we used X-irradiation to ablate all lymphocytes, except for those in the shielded hind paws.

Following irradiation with hind paw shielding, there was no change in the proportion of the donor-derived cells in SRTs that expressed Ki-67 antigen. Furthermore, the relative proportions of donor-derived to total CD4+ T cells were increased from 46% in the unirradiated controls to 65% in the irradiated animals. These findings indicate that donor cells already in the paws at day 5 after transfer had continued to proliferate for a further 3 days, without supplementation from external sources. The preponderance of donor-derived cells in the SRTs provides strong support for selective expansion as an explanation, because in the circulation, donor cells still constituted only 5–10% of the small number of CD4+ T cells that remained after irradiation (data not shown). Nevertheless, a moderate reduction in severity of arthritis in the shielded paws after irradiation indicated that the full impact of the adoptively transferred disease is partially dependent on continuing recruitment of radio-sensitive cells from the circulation. Furthermore, the numbers of donor CD4+ T cells recovered in SRTs from the shielded hind paws were usually less than those from unirradiated controls, suggesting that local cell division and continuing recruitment of donor-derived T cells occur simultaneously during the effector phase of adoptively transferred AA. If there is continuing recruitment, it is not clear whether it involves blasts that are generated elsewhere or memory cells that are stimulated into cell cycle in the SRTs by local APCs.

Clues regarding the role of cognate antigen in determining the retention and longevity of CD4+ lymphoblasts can be obtained from inspection of the data concerning [125I]without accumulation in the thyroids from normal and arthritic recipients of [125I]IUdR-labeled TD CD4+ T cells. As shown previously for unseparated TD lymphocytes (21), the recruitment of purified TD CD4+ T lymphoblasts from arthritic donors into inflamed synovium was 3- to 4-fold greater than recruitment of cells from normal donors. The relative amount of [125I]IUdR-labeled CD4+ T lymphoblasts from normal donors was the small intestine, a clue that during T cell-mediated inflammation, the synovium can acquire the ability to conscript naive T cells. While it is possible that the host-derived cells might have been activated non-specifically by a homeostatic response to radiation-induced lymphopenia (36) or by growth factors produced by the arthritogenic T cells, this seems unlikely because these phenomena would be expected to affect host- and donor-derived cells equally. A more exciting possibility is that whole-body irradiation has revealed a population of proliferating host-derived CD4+ T cells in the SRTs that is independent of new lymphoblasts recruited from the circulating ambient pool. One possibility is that these host-derived CD4+ cells are immunoregulatory cells and that they could be responsible for...
the observed resolution of the adoptively transferred disease. However, if this was the case, the cells (or their effects) must be short lived because they do not modify the course of a second round of adoptively transferred disease. More interestingly, the immunological microenvironment of the diseased synovium could release naive autoreactive T cells from control mechanisms that normally maintain peripheral tolerance. While a single adoptive transfer did not lead to sustained disease, it is possible that in a chronic disease such as RA, a constant supply of effector cells could ignite a recursive process in the synovium that leads to de novo activation of T cells and the establishment of local autonomy.

Conclusion
Transfer of a cohort of effector cells produces a period of inflammation that is limited by the eventual exhaustion of the arthritogenic population. Adoptively transferred AA can, therefore, be considered as one cycle in a continuous process that commences at the end of the prodrome of the actively induced disease. The inflammation in actively induced disease is sustained by successive cohorts of arthritogenic CD4+ T cells and by local proliferation of these cells after they have been recruited to the synovium. It is not clear whether the proliferating donor-derived cells in synovium were the progeny of lymphoblasts in the original inoculum or whether they originated from memory cells that were recruited into the SRTs by the activities of pioneer lymphoblasts. Our previous studies suggest that adoptively transferred disease is initiated by engagement of effector cells with endogenous arthritogens in the synovium (9, 31) and circumstantial evidence presented herein suggests that survival of CD4+ T lymphoblasts from arthritic donors is enhanced by their encounter with cognate synovial antigens. We suggest that arrival of effector CD4+ T cells in synovium enhances presentation of endogenous arthritogens, thus promoting survival and proliferation of the cells and facilitating the local activation of additional T cells.

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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>adjuvant-induced arthritis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>c.p.m.</td>
<td>counts per minute</td>
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<tr>
<td>DA</td>
<td>Dark Agouti</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>GAM-Ig-bi</td>
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<tr>
<td>[125I]IUdR</td>
<td>[125I]ido-deoxyuridine</td>
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<td>S-AF-546</td>
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<td>thoracic duct</td>
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References
