MHC class II compartment, endocytosis and phagocytic activity of macrophages and putative dendritic cells isolated from normal tissues rich in synovium

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Keywords: antigen-presenting cells, endocytosis, MIIC, phagocytosis, synovial cells

Abstract

The endocytic and phagocytic activities of a population of MHC IIhi CD11c⁺ dendritic cell (DC)-like cells in synovium-rich tissues (SRTs) of normal rat paws were compared with CD163⁺ cells (putative macrophages) from the same tissues and pseudo-afferent lymph DCs, peritoneal macrophages and blood monocytes. Fifty percent of CD11c⁺ cells and 75% of CD163⁺ cells isolated from SRT internalized fluorescein-conjugated dextran (FITC-DX). Of these endocytic cells, half of those expressing CD11c, but only 30% of those expressing CD163, were surface MHC class II⁺ (sMHC II⁺). CD11c⁺ cells were more endocytic than monocytes or pseudo-afferent lymph DC, but some CD163⁺ cells (type A synoviocytes) were found to be highly endocytic. CD163⁺ cells from SRT were more phagocytic (25%) than the general MHC class II⁺ population (16%). Of phagocytic cells, 40% of CD163⁺ cells were sMHC IIvariable and they constituted 60% of all MHC class II⁺ phagocytic cells. Only 18% of phagocytic MHC II⁺ cells expressed CD11c and the most of these were MHC IIhi. In comparison, 60% of CD163⁺ peritoneal macrophages were phagocytic, while blood monocytes were poorly phagocytic. Intracellular MHC class II-rich compartments (MIIC) were prominent in sMHC IIhi cells in SRT but rare in CD163⁺ cells. Most MHC IIhi CD11c⁺ cells did not have a detectable MIIC.

Introduction

There have been outstanding advances in understanding the differentiation and functions of dendritic cells (DCs) in the past decade (1–11). Many of the advances have been achieved in vitro, using DCs produced by culture of bone marrow or blood monocytes with growth factors such as IL-4, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (4, 12, 13). It is clear from these studies that the functional characteristics of DCs are governed by the state of differentiation of the cells and that this, in turn, is responsive to the milieu of growth factors in which the cells are grown. It is to be expected, therefore, that resident DCs will be responsive to the special tissue microenvironments in which they complete their differentiation. Tissue-specific differences are observed between macrophages obtained from sites such as the peritoneal cavity and the alveoli of the lung (14–16). In order to understand the role of DC in the pathogenesis of inflammatory diseases that involve discrete tissue locations, it is prudent, therefore, to study DCs obtained directly from the tissues of interest. One such site is the synovium, the seat of inflammation in polyarthritides such as rheumatoid arthritis (RA).

DCs are potent antigen-presenting cells (APCs) that present both endogenous and exogenous antigens to naïve T cells (1, 2). Tissue DCs capture antigens by multiple pathways, including fluid-phase endocytosis (3), receptor-mediated endocytosis via a range of receptors such as the mannose receptor (3) and FcRs (4) and phagocytosis of particulates such as microbes, apoptotic cells and necrotic cells (1, 5, 6). MHC class II molecules play a central role in the presentation of these exogenous antigens to T cells (7) and DCs are unique as stimulators of naïve T cells (8). Immature DCs that are
Endocytosis and MIIIC in putative dendritic cells

Counterparts in vivo from the MIIIC to the plasma membrane (11). The exact event that follows activation of these immature DCs is the redistribution of MHC class II–peptide complexes on the surface of DCs (10, 11). An important event that follows activation of these immature DCs is the redistribution of MHC class II–peptide complexes from the MIIIC to the plasma membrane (11). The exact counterparts in vivo of these monocyte-derived DCs have not been investigated thoroughly and we have found putative immature DCs in rat synovium that express high levels of surface MHC class II molecules (sMHC II) (16a), while others have found similar cells in lung (17) and small intestine (18).

The macrophage is another cell in normal and rheumatoid synovium. Macrophages are resident cells, whose immediate precursors are blood monocytes. Macrophages are key participants in innate immunity, by virtue of their high phagocytic and endocytic activity (19). They can be induced to express MHC class II molecules and co-stimulatory molecules by IFN-γ at sites of T cell-mediated inflammation, allowing them to stimulate antigen-specific T cells (20, 21). Type A synoviocytes are specialized macrophages that line synovial spaces (22). These cells phagocytose cellular debris and other waste in the joint cavity and they have been shown to possess antigen-presenting ability in some circumstances (22).

It is apparent that monocytes, macrophages and at least some DCs are derived from a common bone marrow progenitor (23). Because these cells share a number of functional and phenotypic features, it is difficult to distinguish the immature and mature members of the two lineages. We have described recently (16a) the morphological and phenotypic characteristics of a population of indeterminate cells in rat synovium-rich tissue (SRT) that contains precursors of DCs. These cells express some surface markers that distinguish them from blood monocytes and macrophages but further functional characterization is necessary to confirm their commitment to the DC lineage. In the present study, we analyze the endocytic and phagocytic activities of the indeterminate cells and compare these functions and the MIIIC in blood monocytes, peritoneal macrophages, type A synoviocytes and DC from pseudo-afferent mesenteric lymph.

**Methods**

**Animals**

Inbred specific pathogen-free female Dark Agouti rats were obtained at 8–10 weeks of age from the Gilles Plains Animal Resource Centre (Adelaide). The rats were maintained at the animal facility of the Institute of Medical and Veterinary Science, and had free access to standard food pellets and water. Experiments were conducted according to a protocol approved by the institutional Ethics Committees of the Institute of Medical and Veterinary Science and the University of Adelaide.

**Reagents**

The mAbs and the fluorescent conjugates have been described previously (16a). Briefly, mouse mAbs specific for rat CD45 (OX1) and MHC class II (OX6) were used as neat hybridoma culture supernatants. The following purified mouse anti-rat mAbs were purchased and used at the concentrations recommended by the manufacturer: BA2 (CD11c) and ED2 (CD163) were obtained from Serotec, Oxford, UK. PE-conjugated mAbs OX1 and OX6, and biotin-conjugated OX6, were purchased from PharMingen (San Diego, CA, USA) and used at the recommended concentrations. Isotype-matched control mAbs were 1B5 (IgG1, anti-giardia; G.M., unpublished results) and ID4.5 (IgG2a, anti-Salmonella typhimurium) (24). Secondary antibodies were FITC-conjugated goat anti-mouse Ig (GAM-Ig) and biotin-conjugated GAM-Ig (PharMingen). The biotinylated antibodies were detected by streptavidin–PE (Southern Biotechnology Associates, Birmingham, AL, USA), streptavidin–FITC or streptavidin–Cy-Chrome (PharMingen) conjugates.

**Isolation of mononuclear cells from hind paws**

Single-cell suspensions were obtained from the SRTs of rat hind paws as described previously (16a). Briefly, cells were obtained by vascular perfusion of the hind limbs with collagenase and further enzymatic digestion of the disarticulated hind paws in vivo. The digested SRTs were disrupted mechanically to yield cell suspensions for flow cytometric analysis of endocytic and phagocytic activity and the distribution of MHC class II.

**Preparation of synovial cells**

Cells lining the synovium of the knee joint were obtained by lavage, using complete medium (CM) containing 250 U ml⁻¹ of collagenase as described previously (16a). To study endocytic activity in vivo, 50 μl of fluorescein-conjugated dextran (FITC-DX; 1 mg ml⁻¹ in PBS) was injected into the knee joint cavity. The synovial lining cells were harvested 1 h later by the same method.

**Collection of mesenteric pseudo-afferent lymph DCs**

Mesenteric lymphadenectomy and thoracic duct cannulation were performed as described previously (25).

**Preparation of peritoneal cells and peripheral blood monocytes**

Peritoneal cells and PBMCs were prepared as described previously (16a).

**Flow cytometry**

Dual-color immunofluorescence staining was performed using a combination of indirect and direct techniques, as described previously (16a). Briefly, cells (1–2 × 10⁵) were incubated with saturating concentrations of primary antibody for 1 h, washed twice, incubated with FITC–GAM-Ig for 1 h and washed twice. Free valences of GAM-Ig were blocked with undiluted normal mouse serum (NMS) for 15 min and the cells were then incubated with a PE-conjugated secondary antibody for 1 h, washed and fixed with 1% PFA.

The labeled cells were analyzed using a Beckman Coulter EPICS XL-MCL flow cytometer and Coulter Expo 32 software. In all studies, an electronic gate was established from the plot of forward versus side scatter of light that is known to include the population of DCs found in rat pseudo-afferent lymph...
(16a). This is referred to herein as the ‘DC gate’. Information was obtained from at least 10 000 events within this gate.

**Cell sorting**

Cells were stained by the indirect technique with antibodies against rat MHC class II, CD11c or CD163 (FITC, indirect), maintained at 4°C and sorted at a rate of 3000 events s⁻¹ using a FACStarplus FACS and CellQuest software (Becton Dickinson, San Jose, CA, USA). The sorted cells were used for investigation of intracellular MHC class II molecules.

**Analysis of fluid-phase endocytosis by flow cytometry**

Endocytic uptake of a fluid-phase marker was measured by dual- or three-color flow cytometry, using FITC-DX (40 000 kDa; Molecular probes, Eugene, OR, USA) as described by Sallusto et al. (3). Briefly, SRT cells, peripheral blood monocytes, peritoneal macrophages or mesenteric pseudo-afferent lymph DCs were incubated in CM containing 10% FCS plus 1 mg ml⁻¹ FITC-DX for 90 min at 0°C (to measure non-specific adherence) or at 37°C (to measure energy-dependent uptake). After washing with ice-cold PBS containing 1% FCS and 0.01% NaN₃, the cells were labeled with PE conjugates by either direct or indirect techniques, as described above. For labeling with two antibodies, the cells were allowed to take up FITC-DX as described above and then labeled first with mAbOX1 (CD45, hybridoma culture supernatant), mAb ED2 (CD163) or mAb B A2 (CD11c) (purified antibodies) for 1 h on ice. They were then incubated with biotin-conjugated GAM-IgG, followed by streptavidin–Cy-Chrome. After blocking free valencies of the GAM-Ig by incubation with NMS, the cells were incubated with PE-conjugated mAb OX6 (MHC class II). Active uptake of FITC-DX by cells at 37°C was estimated (see above) as the proportion of cells with higher fluorescence than observed in cells incubated with the marker at 0°C, or the difference in mean fluorescence intensity (MFI) between samples incubated at 37°C and 0°C.

**Phagocytosis of latex beads in vitro**

Fluoribrite carboxylated fluorescent latex beads (Fluoribrite carboxy YG, 1 µm diameter; Polysciences, Warrington, PA, USA) were used as particle tracers to analyze phagocytosis by cells from SRT, peritoneal macrophages or blood monocytes. When three-color flow cytometry was required, 0.431-µm carboxylate-modified blue fluorescent beads (Sigma–Aldrich, St Louis, MO, USA) (catalog no. L3280) (26) were substituted for Fluoribrite beads. Before use, the beads were washed three times in sterile cold PBS by centrifugation and resuspended in sterile PBS. To measure phagocytosis, 10 µl of packed beads suspended in 0.3 ml of CM containing 20% FCS was added to 10⁶ cells in Teflon tubes (NUNC Brand Products, Denmark). After incubation for 90 min at either 37°C (energy-dependent uptake) or 0°C (non-specific adherence), cells were washed three times with ice-cold PBS containing 1% FCS and 0.01% NaN₃ to remove free beads. The cells were then labeled to detect expression of CD45, MHC class II, CD163 (monocytes/macrophages) (27) or CD11c (DCs) by dual- or three-color labeling (as described above). In the case of three-color labeling, cells that had been incubated with blue fluorescent beads were labeled with mAbs against CD45, CD163 or CD11c. They were then washed, incubated with biotin-conjugated GAM-Ig plus streptavidin–FITC and free valences of the GAM-Ig were blocked with NMS. After further washes, the cells were stained with PE-conjugated mAb OX6, fixed with 1% PFA and analyzed by flow cytometry.

**Confocal microscopy**

Smears were prepared by cyto centrifugation from cells after in vitro endocytosis or phagocytosis and staining with antibodies for flow cytometry (see above). They were analyzed by confocal microscopy using a Bio-Rad MRC 1000 confocal laser scanning microscope. Images were taken using a ×60/1.4-numerical aperture lens with ×2.5, ×3 or ×4 optical zoom. Optical slicing of cells labeled with two fluorescent labels (FITC plus PE or Cy-Chrome) allowed each color to be imaged in the median slice of each cell, with subsequent merging of images.

**Detection of sMHC II plus intracellular MHC class II molecules**

To visualize cell sMHC II and intracellular MHC class II molecules separately, cells were incubated first with mAb OX6, followed by FITC-conjugated GAM-Ig. After fixation in 3% PFA, the cells were permeabilized for 30 min in PBS containing 1% saponin and 5% BSA and blocked with NMS as described previously (16a). Intracellular MHC class II molecules were then stained with biotin-conjugated OX6 and the biotinylated antibody was detected using streptavidin–Cy-Chrome. Confocal microscopy was used to examine MHC class II distribution in cytospin preparations of labeled cells, as described above.

**Results**

In a previous study (16a), we deduced that precursors of mature DCs were contained within the population of cells in SRT from normal rat hind paws that expresses CD11c and high levels of MHC class II molecules (MHC class II²⁺). This deduction was based on morphology, absence of the macrophage marker CD163 and in vitro response to GM-CSF. We examine herein the activity of these cells in endocytosis and phagocytosis and the distribution within the cytoplasm of MHC class II molecules. Net energy-dependent uptake of fluid-phase marker (FITC-DX) and particulate marker (FITC-labeled or blue fluorescent latex beads) at 37°C was estimated in each case by comparison with uptake at 0°C.

**Flow cytometric analysis of endocytosis by cells in SRT**

Uptake of soluble FITC-DX by endocytosis was assessed in vitro at either 0°C or 37°C. Analysis was performed by flow cytometry, using a light scatter gate based on the characteristics of DCs in mesenteric pseudo-afferent lymph (16a). The gross uptake of FITC-DX at 37°C by cells expressing CD45, CD163, MHC class II or CD11c is shown in Fig. 1(A–D). Net energy-dependent uptake of FITC-DX was observed in ~70% of the CD45⁺ cells in SRT (derived from Fig. 1A) and about two-thirds of these cells (box in upper right quadrant) displayed high endocytic activity. There was an ~20-fold difference in MFI between populations with high (MFI 307)
and low (MFI 14.9) levels of endocytic activity. Approximately 75% of the CD163⁺ cells exhibited net uptake of FITC-DX (derived from Fig. 1B) and of these, ~30% displayed high endocytic activity (box in upper right quadrant). Net uptake of FITC-DX was observed in ~70% of the sMHC II⁺ cells (derived from Fig. 1C), one-third displaying high endocytic activity. Of the CD11c⁺ cells, ~50% displayed net uptake of FITC-DX (derived from Fig. 1D) and of these, ~15% took up large amounts of FITC-DX (box in upper right quadrant). CD45/C255 cells endocytosed modest amounts of FITC-DX (Fig. 1A).

The surface antigen phenotype of the MHC class II⁺ cells that internalized FITC-DX was studied by three-color flow cytometry. Cells containing FITC-DX (gated as in Fig. 2A, to exclude passive adherence) were analyzed further for co-expression of MHC class II and CD45, CD163 or CD11c (the plots in Fig. 2B–D). Of the CD45⁺ cells that had taken up FITC-DX, ~40% expressed sMHC II⁺ (Fig. 2B), 28% at high levels. While a majority of CD163⁺ cells internalized FITC-DX (Fig. 1B), only 30% of these cells expressed sMHC II molecules (18% at high levels) (Fig. 2C). In contrast, 50% of the CD11c⁺ cells that contained FITC-DX expressed high levels of sMHC II molecules (Fig. 2D). A subset of the CD11c⁺ cells (35%) did not express MHC class II molecules and these may be the recently emigrated CD11c⁻ MHC class II⁻ blood monocytes (16a). The CD163⁺ MHC class II⁺ cells were more endocytic (MFI 33.6) than the CD11c⁺ MHC class II⁺ (MFI 5.5) (data not shown).

Flow cytometric analysis of endocytic activity of synoviocytes

Some of the CD163⁺ cells from SRT are type A synoviocytes (16a). Type A cells in the synovial intima are known to take up foreign materials, such as latex particles or HRP, from the joint cavity (28, 29). To compare the endocytic activity of synovial lining cells and cells from SRT, we examined uptake of FITC-DX by cells obtained from the knee joint by lavage (Fig. 3). Dual-color flow cytometric analysis showed that ~94% of the CD45⁺ lavage cells were highly fluorescent after incubation with FITC-DX in vitro (Fig. 3A). Although most of the CD45⁺ lavage cells (fibroblast, type B synoviocytes) took up FITC-DX, most of these cells were considerably less fluorescent than the CD45⁺ cells. Approximately 94% of these cells expressed high endocytic activity (Fig. 3B). The profile of CD163 expresion and uptake of FITC-DX by type A synoviocytes was similar to that of some CD163⁺ cells from SRT (Figs 1B and 3B). Cells lavaged from the knees varied in their expression of MHC class II molecules (Fig. 3C).
Abbreviations: MHC, major histocompatibility complex; FITC-DX, fluorescein isothiocyanate-dextran; SRT, spleen-rather-than-thymus; DC, dendritic cell.

Approximately 92% of the MHC class II+ synoviocytes took up FITC-DX at 37°C in vitro at levels greater than control cells at 0°C (data not shown). Many (65%) showed high levels of endocytosis. Similar results were obtained in vivo, when FITC-DX was injected into the joint and cells were lavaged 1 h later (data not shown).

Comparison of endocytic activity of cells from SRT, peripheral blood monocytes, pseudo-afferent lymph DCs and peritoneal macrophages

Endocytosis is characteristic of immature DCs and macrophages (30). However, DCs are known to become less active in endocytosis as they mature (3). In contrast, the ability of monocytes to take up and process antigens has been found to increase during their maturation into DC in vitro (12, 31). We compared, therefore, the endocytic activity of SRT subsets with monocytes, macrophages and afferent lymph DCs, using dual-color flow cytometry.

First, we compared the cells that express CD11c (Figs 1D and 4A and B). Approximately 25% of CD11c+ blood mononuclear cells (monocytes) took up FITC-DX (Fig. 4A), but at levels that were considerably lower (MFI 21.8) than the uptake by CD11c+ cells from SRT (MFI 64). Very few of these cells (box in the upper right quadrant of Fig. 4A) took up as much
FITC-DX as the most endocytic CD11c+ cells from SRT (Fig. 1D). Of the CD11c+ cells in partially purified pseudo-afferent lymph (Fig. 4B), ~30% took up moderate levels of FITC-DX (MFI 26) but few were as active in endocytosis as some CD11c+ cells from SRT (box in right upper quadrant).

We then compared the endocytic activity of the CD163+ cells from SRT with monocytes and macrophages (Figs 1B and 4C and D). We have shown previously that blood monocytes express low levels of CD163 (16a) and, as expected, they displayed moderate levels of FITC-DX uptake (MFI 50), compared with uptake by some of the CD11c+ cells from SRT (Fig. 1B) or in knee joint lavage fluid (Fig. 3B). Uptake by the CD163lo sub-population was comparable with that of blood monocytes (Fig. 4C).

Confocal microscopy of endocytic activity
Endocytic activity was examined further, using cytospin smears prepared from the SRT cells that had been labeled for flow cytometric analysis (see above). Uptake of FITC-DX by sub-populations of cells expressing either CD163 or CD11c was observed by dual-color confocal microscopy. As seen in Fig. 5(A), internalized FITC-DX was distributed relatively uniformly in the cytoplasm of most of the CD163+ cells from SRT. In contrast, FITC-DX was taken up into multiple small vesicles in the cytoplasm of CD163+ synovial lavage cells, whether exposed to the marker in vitro (Fig. 5C) or in vivo (data not shown). Within CD11c+ cells, FITC-DX was in small cytoplasmic vesicles and larger irregular-shaped structures (Fig. 5B).

Flow cytometric analysis of phagocytosis by cells from SRT
Phagocytic activity was studied by flow cytometry, comparing the uptake of fluorescent microbeads at 37°C or 0°C. The proportion of fluorescent events after incubation at 37°C (adherent beads) was subtracted from the proportion of fluorescent events after incubation at 37°C (adherence plus phagocytosis), to yield the proportion of cells that had actively internalized fluorescent beads. Using 1-μm FITC-labeled beads, it was found by dual-color flow cytometry that ~10% of the CD45+ cells prepared from SRT had taken up one or more...
beads per cell after incubation for 90 min at 37°C (data not shown). The FACS was used to sort CD45+ cells into increments of fluorescence intensity (corresponding to multiples of phagocytosed beads). Analysis of cells in cytopsins prepared from these fractions by confocal microscopy confirmed that cells in each successive increment of fluorescence intensity contained an additional bead (data not shown).

Approximately 25% of the CD163+ cells in SRT showed active uptake of FITC-labeled beads after incubation for 90 min at 37°C, compared with only 16% of all cells that expressed MHC class II (Fig. 6). To identify the phenotypic characteristics of the phagocytic MHC class II+ cells, we used 0.431-μm blue fluorescent beads and analyzed the phagocytic cells by three-color flow cytometry (Fig. 7). Cells containing fluorescent beads were gated (Fig. 7A) and examined for co-expression of MHC class II molecules and either CD45, CD163 or CD11c. Approximately 40% of the CD45+ cells that had taken up fluorescent beads expressed sMHC II molecules and all of the phagocytic MHC class II+ cells expressed CD45 (Fig. 7B). Approximately 60% of the phagocytic MHC class II+ cells expressed CD163, and these cells expressed MHC class II molecules at varying levels (Fig. 7C). MHC class II molecules were expressed by ~40% of the phagocytic CD163+ cells. Only 18% of CD163+ phagocytic cells expressed high level of MHC class II. CD11c+ cells constituted only 18% of the phagocytic MHC class II+ cells in SRT and most of these expressed high levels of MHC class II molecules (Fig. 7D). A small population of phagocytic CD11c+ MHC class II+ cells may represent either monocytes or sMHC II+ immature DCs. The CD163+ MHC class II+ population was found to be more phagocytic (MFI 40) than the CD11c+ MHC class II+ population (MFI 6.5).

**Phagocytosis of FITC-labeled beads by peritoneal macrophages and blood monocytes**

The phagocytic activity of cells from SRT was compared with peritoneal macrophages and blood monocytes. Clear quantitative differences were observed between the phagocytic activities of cells from SRT and peritoneal macrophages. More than 60% of CD163+ peritoneal macrophages took up FITC-labeled beads after incubation for 90 min at 37°C, compared with 25% of CD163+ cells from SRT. Moreover, peritoneal macrophages internalized, on an average, a larger number of beads during the 90-min incubation period than SRT cells (Figs 6B and 8B). Confocal microscopy of peritoneal cells labeled with anti-CD163 showed numerous fluorescent beads in the cytoplasm, indicating that the FITC staining detected by flow cytometry represented beads that had been internalized (Fig. 8C). In contrast, blood monocytes exhibited minimal phagocytosis of the FITC-labeled beads under the conditions of incubation (data not shown). Thus, phagocytic monocyte-like cells in SRT preparations (see above) are different from blood monocytes.

**Surface and intracellular expression of MHC class II molecules by cells from SRT**

We have shown previously (16a) that permeabilization of cells from SRT increased the detection of CD45+ cells that express MHC class II molecules from ~40 (intact cells) to ~60% (permeabilized cells). This increase was due mainly to an increase in the proportion of MHC class II+ cells (from 12 to 23%) of the CD45+ cells. The proportion of MHC class II+ cells was not increased substantially by permeabilization, although the intensity of staining increased slightly. We attributed the effect of permeabilization to providing access by antibodies to an intracellular compartment(s) of MHC class II molecules.

We have now verified this interpretation by comparing the detection of MHC class II molecules on fresh cells, cells fixed with 4% paraformaldehyde and cells fixed and then permeabilized. Live cells were labeled to detect sMHC II molecules (indirect, FITC) and aliquots were then fixed. One aliquot was analyzed directly by flow cytometry. A second aliquot was permeabilized and incubated with biotinylated antibody to detect intracellular MHC class II molecules (biotin–streptavidin–FITC). Some cells were fixed before labeling sMHC II molecules, to examine the effect of fixation on recognition of the molecules by mAb OX6. Flow cytometric analysis indicated that fixation before staining did not change the percentage of sMHC II+ cells detected by mAb OX6. Flow cytometric analysis indicated that fixation before staining did not change the percentage of sMHC II+ cells detected by mAb OX6 (data not shown). As shown in Fig. 9(A), cells expressing sMHC II molecules constituted ~7.5% of the total events in the gated population. After permeabilization, the proportion of MHC class II+ cells increased to 15% of the events in this gate, due mainly to the increase in MHC class II+ cells (Fig. 9B).

Next, we assessed the distribution of MHC class II molecules by fluorescence confocal microscopy. Live cells...
stained with mAb OX6 (anti-MHC class II) showed heteroge-
nous surface staining (Fig. 9C). When cells were stained to
detect both surface (indirect, FITC) and intracellular (biotin–
streptavidin–Cy-Chrome) MHC class II molecules, expression
of cytoplasmic MHC class II molecules (cMHC II) was also
heterogenous. Cells with the following phenotypes were
detected: sMHC II + cMHC II/C255, sMHC II + cMHC II + and
sMHC II/C255 cMHC II +. Most of the cells that were sMHC II hi
(green) were found to contain cMHC II (red), although a few
had no detectable MHC class II molecules in the cytoplasm.
Of the sMHC IIlo cells, most contained either no intracellular
MHC class II molecules or relatively small amounts (Fig. 9D).
Importantly, some cells were detected that expressed no
detectable sMHC II but had cMHC II.

Intracellular MHC class II molecules in subsets of
cells from SRT and comparison with monocytes and
macrophages

Immature DCs are known to contain cMHC II (32) and we also
found abundant intracellular MHC class II molecules in DCs
from pseudo-afferent lymph (Fig. 10D). We compared,
therefore, cMHC II in sub-populations of cells prepared from
SRT. Cells that expressed either high or low levels of sMHC II
were sorted by FACS (Fig. 10A), fixed, permeabilized and
stained for detection of cMHC II as described above. Confocal
microscopy of cytospin preparations revealed that ~70% of
sMHC II hi cells contained cMHC II, while the remainder had no
detectable cMHC II (Fig. 10B). In contrast, of the cells that
expressed low levels of sMHC II, most did not contain cMHC II
(Fig. 10C).

We also separated the CD11c+ (Fig. 11A) and CD163+ (Fig.
11D) sub-populations of cells from SRT (FITC). The cells were
then permeabilized, stained to detect MHC class II molecules
(Cy-Chrome) and cytospin preparations were examined by
confocal microscopy. Most of the CD11c+ cells (Fig. 11B)
expressed high levels of sMHC II but did not contain a detect-
able cMHC II (Fig. 11C). However, it is noteworthy that some
CD11c+ cells expressed both sMHC II and cMHC II. Of the
CD163+ cells (green), only a minority contained intracellular
MHC class II molecules (red) and most did not express either
sMHC II or intracellular MHC class II molecules (Fig. 11E).

Because monocytes are recognized to be precursors for both
DCs and macrophages (13), we analyzed the distribution of
MHC class II molecules in blood monocytes. Cytospin
preparations of cells stained to detect sMHC II and intracellular
MHC class II molecules separately (see above) revealed that
most monocytes do not express MHC class II molecules,
although a small sub-population expresses variable levels at the cell surface. Some of these MHC class II+ cells were found to contain cMHC II (Fig. 12A). Most peritoneal macrophages expressed sMHC II, although the level of expression was variable and a minority displayed an intracellular compartment (Fig. 12B). Thus, on the basis of distribution of MHC class II molecules, most of the CD163+ cells in SRT resemble monocytes and peritoneal macrophages. Expression of sMHC II was heterogeneous on cells lavaged from knee joints and some of these cells contained cMHC II (Fig. 12C). The small number of CD163+ cells from SRT that contain cMHC II may, therefore, be type A synoviocytes.

Discussion
DCs and T cells play vital roles in the pathogenesis of RA (33–39) and animal models of polyarthritis (40–43). Competent DCs in the synovium may present arthritogens to autoreactive
Fig. 9. Flow cytometric and confocal microscopic analysis of expression of surface and cytoplasmic expression of MHC class II molecules by cells prepared from the SRTs of normal rat hind paws. (A) Freshly prepared cells labeled indirectly with either negative control mAb 1B5 (shaded histogram) or anti-MHC class II (mAb OX6) (open histogram). (B) Cells labeled with either mAb 1B5 or mAb OX6 as above and then fixed and permeabilized. The cells were then re-incubated with either mAb 1B5 (shaded histogram) or with mAb OX6 to demonstrate total cellular MHC class II molecules (open histogram). (C and D) Confocal microscopic analysis of the distribution of MHC class II molecules (surface, FITC [indirect]; cytoplasmic, biotin–streptavidin–Cy-Chrome) in cells from SRTs. (C) Expression of sMHC II molecules by freshly isolated cells, stained indirectly with mAb OX6. Expression of MHC class II is heterogeneous, with some cells showing intense staining (arrow) and some stained weakly (arrowhead). (D) Detection of both sMHC II (green) and intracellular (red) MHC class II molecules, showing cells with high level of sMHC II that also stain for intracellular MHC class II (arrows) and cells with lower levels of sMHC II that stain weakly for intracellular MHC class II molecules (arrowhead). Original magnification: ×336.

Fig. 10. Analysis of intracellular MHC class II molecules in sub-populations of cells prepared from SRTs of normal rat hind paws and in pseudo-afferent lymph dendritic cells. (A) Freshly isolated cells from SRTs were stained with mAb OX6 (see legend of Fig. 9). Cells that expressed either high or low levels of sMHC II molecules were sorted, processed to stain intracellular MHC class II molecules (see legend of Fig. 9) and prepared by cytospin. (B) Many cells that expressed high levels of sMHC II molecules also exhibited an intracellular MIIC. (C) Most of the cells that expressed low levels of sMHC II molecules contained either little or no cMHC II molecules. (D) Most mesenteric pseudo-afferent lymph DC, stained in the same way, have abundant sMHC II molecules and an intracellular MIIC. Original magnifications: B and C, ×210; D, ×336.
T cells and lead to local cell-mediated inflammation. Development of autoimmunity could be initiated by dysregulation of either cell types, and the resulting immunologically mediated inflammation could become chronic due to the potential for recursive interactions between activated T cells and activated DCs. Therefore, as a prelude to studies on their interactions with arthritogenic T cells, it is important to identify and characterize DCs and DC precursors in normal SRT. In a companion paper (16a), we have identified cells in normal SRT that have an indeterminate surface antigen phenotype and the capacity to produce DC in response to GM-CSF. This population has now been characterized functionally, in terms of endocytic and phagocytic behavior, possession of an antigen processing MIIC and expression of sMHC II molecules. The results assist in differentiating the indeterminate population from macrophages and blood monocytes, where monocytes share expression of CD11c with DCs and expression of CD163 with macrophages.

Endocytosis is a characteristic of immature DCs (3), and it is an important mechanism for the capture of soluble antigens. Our results indicate that CD11c+ cells from SRT can be subdivided into three sub-populations on the basis of uptake of FITC-DX (Fig. 1D). The most numerous (sub-population one) shares with blood monocytes intermediate levels of endocytosis (Fig. 4A) and expression of low levels of CD163 (Fig. 4C). However, unlike blood monocytes, most of the...
CD11c+ cells that took up FITC-DX also expressed MHC class II molecules (the majority at high levels). Despite this difference, these small cells have greater resemblance to monocytes than to the endocytic DCs in pseudo-afferent lymph (Fig. 4B). Sub-population two took up FITC-DX in amounts that were an order of magnitude greater than either monocytes or peritoneal macrophages (Fig. 4D). The third sub-population (sub-population three) did not exhibit any endocytic activity, and in this respect, these CD11c+ cells resemble a sub-population of the DC prepared from pseudo-afferent lymph (Fig. 4B). Cells in this population may represent more mature DCs that have ceased uptake of exogenous antigens.

Therefore, on the basis of endocytic activity, we suggest that sub-population one represents recently migrated monocytes that have commenced differentiation toward the phenotype of immature tissue DC, sub-population two consists of immature DC, while sub-population three consists of cells with maturity comparable with some migratory DC in afferent lymph. This sequence is consistent with the increase in endocytic activity described during the differentiation of peripheral blood monocytes to immature DC in response to GM-CSF and IL-4 in vitro (12) and the endocytic activity of population two is consistent with the known role of resident DC in sampling both exogenous antigens and auto antigens (44, 45).

Interestingly, some CD163+ cells from SRT (Fig. 1B) took up ~10 times as much FITC-DX as peritoneal macrophages (Fig. 4D). These cells may represent type A synoviocytes because CD163+ cells obtained by knee joint lavage exhibited similar levels of endocytosis (Fig. 3B) and we have identified CD45+ cells with similar morphology and phenotype previously in joint lavages (16A). Others have shown that type A synoviocytes take up both particulate and colloidal materials from the joint space (28, 46). The type A synoviocytes from knee joints appear to express MHC class II molecules constitutively (Fig. 3C), suggesting that in addition to possible functions in turnover of synovial fluid macromolecules and uptake of infectious agents, these cells may be competent to process and present exogenous antigens and/or auto-antigens (22).

DCs have also been shown to phagocytose particulate matter (5, 47), apoptotic cells (6) and tumor cells (46), but their phagocytic activity is relatively weak compared with macrophages (49). Furthermore, monocyte-derived immature DCs have been reported to be less phagocytic than monocyte-derived macrophages (50). Therefore, the level of phagocytosis is an additional characteristic that can assist in defining these cell types (49). We found that blood monocytes exhibited minimal phagocytosis of FITC beads (providing a clear identifying phenotype for this common precursor of the macrophage and DC lineages), while resident peritoneal macrophages (Fig. 8) exhibited vigorous phagocytosis (providing a functional phenotype for fully differentiated macrophages). With respect to DC differentiation, phagocytosis can provide an indication of maturity because it is confined to relatively immature DCs (47).

It is significant, therefore, that among cells in SRT, many that expressed CD163 took up fluorescent latex beads (Figs 6B and 7C). However, they were less phagocytic than resident peritoneal macrophages (Fig. 8B) and the proportion of phagocytic cells was also smaller (Figs 6B and 8B). This may reflect differences in the differentiated characteristics of macrophages from these different tissue microenvironments. Many of the phagocytic cells from SRT did not express MHC class II molecules, consistent with the overall phenotype of CD163+ cells from this tissue (16A). However, some phagocytic cells expressed high levels of MHC class II molecules (Fig. 6D) and when phagocytic cells were examined separately (Fig. 7), many that expressed high levels of MHC II molecules also expressed CD163. From studies on synovial lavage cells (16A), the latter are likely to be type A synoviocytes. While most of the phagocytic cells in SRT appear to be macrophages, some phagocytic cells expressed CD11c (Fig. 7D) and these may represent immature DCs. At this stage, we have not studied simultaneous uptake of FITC-DX and blue fluorescent beads to relate activity in fluid-phase endocytosis to phagocytosis within the CD163+ and CD11c+ subsets.

The MIIC is an essential feature of immature tissue DCs that are in a phase of antigen acquisition (32). In contrast, an MIIC is not essential for the scavenging and degradative functions of resident macrophages (51), although these cells can express MHC class II molecules in response to IFN-γ and thus function as APC (20, 21). The weak endocytic activity and the absence of either sMHC II or cMHC II that we have observed in rat blood monocytes suggest that monocytes are not equipped to take up or present exogenous antigens. However, the significance of the small subset of monocytes that expresses sMHC II (and in some cases, cMHC II) is less clear (see below).

The indeterminate cells from SRT are small cells with monocyteid morphology that express high levels of sMHC II. Approximately 70% of these cells contain a detectable MIIC (Fig. 10B). Of the MHC class IIhi cells, ~40% express CD11c (data not shown) and confocal microscopy of sorted CD11c+ cells from SRT indicates that a few contain a detectable MIIC (Fig. 11A–C). Thus, most of the CD11c+ cells resemble blood monocytes, but differ by expressing sMHC II. They also resemble the minority of sMHC II+ monocytes in blood (see above), some of which also contain cMHC II. The relationships between these subsets of monocytes in blood is not known—the sMHC II+ monocyteid cells could be a distinct subset of DC precursors or, as discussed elsewhere (16A), they could represent precocious differentiation of monocytes toward indeterminate cells of the type that we described in SRT. The morphology and expression of MHC class II and CD11c of indeterminate cells in SRT is consistent with an origin from either activated monocytes or from a dedicated circulating precursor. Some difficulties arise in attempting to link the CD11c+ and CD11c– sub-populations of indeterminate cells in SRT with the predominantly CD11c+ DC in pseudo-afferent mesenteric lymph and further studies are needed on the possible lineage relationships between these cells. We have detected a small sub-population of indeterminate cells that has no detectable sMHC II but possesses an MIIC. This sub-population may represent the in vivo counterpart of the immature monocyte-derived DCs that have been described in vitro (7). Evidence of this population was also found in our flow cytometric analysis of permeabilized SRT cells (16A). Importantly, however, most of the indeterminate cells in normal synovium express high levels of both sMHC II
and cMHC II. These findings show that the orthodox DC ‘life cycle’ (7, 11) requires modification to accommodate the presence of DC-like cells in tissues that are not exposed to inflammatory or microbial ‘danger signals’.

In conclusion, most of the cells that expressed CD163 did not contain cMHC II and most were actively phagocytic. Interestingly, some of the CD163+ cells (type A synoviocytes) were highly endocytic, suggesting that they may be involved in scavenging debris and macromolecules from the synovial fluid. Under normal circumstances, these macrophages may be adapted for degradation of endocytosed material, rather than for presentation of antigenic self-peptides. In contrast, the indeterminate cells have some characteristics of monocytes (but express high levels of MHC class II molecules) and some characteristics of DCs (although some do not express CD11c). Phenotypically, the closest relative of many indeterminate cells appears to be the small sub-population of MHC class II+ monocytes in blood, suggesting that they may represent progression from monocytes to DCs under the influence of local tissue environmental factors. Overall, the indeterminate cells display endocytic and phagocytic properties that are consistent with the behavior of immature DC and many contain cMHC II (consistent with an MIIC). The monocyteid cells may represent early DC; the cells with abundant sMHC II and cMHC II may be immature tissue DCs that are actively sampling local antigens, while those indeterminate cells with abundant sMHC II but little cMHC II may be DCs that are about to migrate to the regional lymph nodes. This differentiation pathway resembles the differentiation pathway from monocytes to DCs in vitro (4, 12, 13, 52, 53), with the important difference (discussed above) that cells with both MIIC and sMHC II occur in normal tissues, independently of overt signals from inflammatory cytokines or ‘danger signals’ of microbial origin. This suggests that normal tissue DCs are competent to process and present self-antigens in situ, and this is consistent with our findings in adaptively transferred adjuvant arthritis (AA) (41). The presence of these cells could have important implications for both maintenance of self-tolerance and for presentation of self-antigens in autoimmunity. We suggest that local influences in tissue microenvironments (connective tissues versus synovial intima) within un-inflamed synovium direct the differentiation of recently arrived blood monocytes toward DCs and macrophages, respectively (16). These differentiation pathways, and the nature of the recruited monocytes (54), could be modified by the presence of activated T cells (47) and by local inflammation and we are now in a position to examine these possibilities during the pathogenesis of both actively induced and adoptively transferred AA.

Acknowledgements

This work was supported by Project Grant 250426 from the National Health and Medical Research Council of Australia and by a Grant from the Research Committee of the Royal Adelaide Hospital. M. M. was an Eileen Urquhart Research Fellow of the Arthritis Foundation of Australia. Assistance with confocal microscopy was given generously by Meredith Wallwork, Adelaide Microscopy, University of Adelaide. We are grateful to Sandy Macintyre and Alan Bishop, Flow Cytometry Unit, Department of Haematology, Institute of Medical and Veterinary Science, Adelaide, South Australia, for their advice and technical assistance in cell sorting.

Abbreviations

AA adjuvant arthritis
APC antigen-presenting cell
CM complete medium
cMHC II cytoplasmic MHC class II
DC dendritic cells
FITC-DX fluorescein-conjugated dextran
GAM-Ig goat anti-mouse Ig
GM-CSF granulocyte macrophage colony-stimulating factor
MFI mean fluorescence intensity
MIIC MHC class II-rich compartments
NMS normal mouse serum
RA rheumatoid arthritis
sMHC II surface MHC class II
SRT synovium-rich tissue

References

16a Moghaddami, M., Cleland, L. G. and Mayrhofer, G. 2005. MHC II+ CD45+ cells from synovium-rich tissues of normal
Endocytosis and MiIC in putative dendritic cells


