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Short Communication

Antibody-mediated cell labeling of peripheral T cells with micron-sized iron oxide particles (MPIOs) allows single cell detection by MRI†

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ABSTRACT: Labeling cells with iron oxide is a useful tool for MRI based cellular imaging. Here it is demonstrated that peripheral rat T cells can be labeled in whole blood, *in vitro*, with streptavidin-coated micron-sized iron oxide particles (MPIOs), achieving iron concentrations as high as 60 pg iron per cell. This is 30 times the amount of labeling reported with ultrasmall particles of iron oxide (USPIOs). Labeling was mediated by use of a biotinylated anti-CD5 antibody, which is specific for peripheral T cells. Such labeling allowed the *in vitro* detection of single lymphocytes by MRI, using conditions well suited for *in vivo* animal work. Electron microscopic analysis demonstrated that MPIOs remained largely extracellular after labeling, with some evidence of intracellular uptake. Cell viability and early and late cytokine release studies showed no significant differences between labeled and unlabeled cells. Therefore, the use of MPIOs for achieving high iron concentrations for cellular MRI is potentially an effective new modality for non-invasive imaging of lymphocytes. Published in 2007 by John Wiley & Sons, Ltd.

KEYWORDS: MRI; iron oxide; T cells; contrast agents

INTRODUCTION

MRI is a useful tool for tracking lymphocyte migration in intact animals. Indeed, the earliest cellular MRI experiments involved MRI-based lymphocyte tracking (1). Currently, successful labeling of lymphocytes for MRI tracking makes use of citrate coated iron oxide nanoparticles (2), TAT peptide functionalized ultrasmall particles of iron oxide (USPIOs) (3) or SPIO-transfection agent complexes (4), achieving labeling ranges from ~0.5 to 2 pg iron per cell. Receptor-mediated endocytosis of SPIOs has been demonstrated for lymphocytes (5) as well as other immune cells, such as dendritic cells (6). Indeed, single SPIO labeled T cells have been detected *in vitro* by Dodd *et al*. (7).

Cell labeling with micron-sized iron oxide particles (MPIOs) has recently been demonstrated (8,9). MPIOs efficiently pack 0.1–10 pg of iron in an individual particle, allowing single cells labeled with only one or a few MPIOs to be detected by MRI (10). Labeling of adherent cells in culture is accomplished by simple co-incubation of MPIOs overnight with the cells, followed by washing to remove free MPIOs. This has been successfully implemented on a variety of cell types (11–15). Yet, while this labeling scheme is robust for adherent cells, labeling of cells in suspension, such as lymphocytes, with MPIOs has proven difficult.

Recently, it has been demonstrated that MRI can detect single cells if they are sufficiently labeled with iron oxide (15–17). Indeed, heavy labeling of cells with iron was required. Shapiro *et al*. (15) and Heyn *et al*. (17) achieved iron labeling of over 50 pg iron/cell. Since current lymphocyte labeling protocols do not provide iron contents at this level, it was hypothesized that cell labeling with MPIOs would achieve high levels of iron loading. The affinity of the MPIOs for the lymphocytes was established using a biotin–streptavidin system, mediated by a T cell antibody. Here we describe an antibody-mediated procedure for efficiently labeling peripheral T cells with MPIOs with sufficient iron to allow detection of single lymphocytes, *in vitro*, by MRI.

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MATERIALS AND METHODS

Cell labeling

Peripheral blood was harvested from adult Sprague-Dawley rats. Fresh, whole blood (5×10^6 T cells/ml) was incubated with various amounts of biotinylated rat anti-CD5 (Cedarlane Laboratories, Ontario, Canada), a pan T-cell antibody, for 1 h, both on ice and at room temperature, and with and without shaking. Antibody concentrations were 0×, .04×, .40×, 2×, 8×, 40× and 200× saturation of all CD5 receptors on each T cell, assuming 30 000 receptors/cell (18) and 100 kDa molecular weight for the antibody. After 1 h, green fluorescent, 1.63 μm streptavidin-coated MPIOs (1.0 pg iron/particle, Bangs Laboratories, Fishers, IN, USA) were added to the same whole blood and incubated for 1 h, either on ice or at room temperature, with or without shaking. MPIO concentrations were 0, 1, 5 and 50 beads/cell. Lymphocytes were harvested from the whole blood by standard Ficoll–Paque centrifugation. Briefly, whole blood samples were diluted 1:1 with PBS and layered over Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) at 1:1 ratio. Samples were centrifuged at 400 g for 30 min at 18°C. The white blood cell layer that forms following centrifugation was transferred to an Eppendorf tube and microcentrifuged at 3000 rpm for 5 min. The supernatant was removed and 1 ml ACK lysis buffer (Quality Biological, Inc., Gaithersburg, MD, USA) was added to lyse remnant red blood cells. After two washes with PBS, cells were filtered through 40 μm filters into tubes suitable for flow cytometry. This entire protocol was performed four different times for statistical averaging.

Functional analysis and imaging

Flow cytometry of freshly labeled cells was used to determine percent labeling. With one preparation, manual counting using both stereo and confocal fluorescence microscopy was also used to determine percent labeling and MPIO number per cell. All raw labeling data were corrected to account for the percentage of total lymphocytes that are T cells, which for rats is ∼72%. For one preparation, lymphocyte gates were sorted into two groups based on granularity. These cells were stained with DAPI to visualize nuclei and slides were prepared for confocal fluorescence microscopy. Viability was assessed by trypan blue exclusion tests.

For electron microscopic analysis, freshly labeled cells were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 at room temperature for 1 h, pelleted and encapsulated with low melting point agarose. The pellet was treated with 1% OsO4 in cacodylate buffer for 1 h and 0.25% uranyl acetate in acetate buffer at pH 5.0 overnight, dehydrated in a series of ethanol and embedded in epoxy resins. Thin sections were counterstained with uranyl acetate and lead citrate. Images were recorded with a CCD digital camera system (XR-100 from AMT, Danvers, MA, USA).

To assess extended cell viability following labeling, one sample was prepared at 2× saturation of CD5 receptors and 30 MPIOs per cell. Initial percent cell labeling was determined by manually counting cells with a stereo fluorescent microscope. Initial percent viability of labeled cells only was measured using trypan blue exclusion tests. Cells were then cultured for two days under 5% CO_2 in RPMI medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Fisher Scientific, Pittsburgh, PA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Fisher Scientific, Pittsburgh, PA, USA). After two days, labeled cell viability was again measured with trypan blue exclusion tests followed by a manual count of percentage lymphocytes with fluorescent MPIOs. All manual counting measurements were performed in triplicate.

To measure the effect of cell labeling on T cell function and response, ELISA-based cytokine release assays were performed, specifically for interferon-gamma (Pierce Biotechnology, Rockford, IL, USA) and interleukin-2 (BioSource International, Camarillo, CA, USA). Interferon-gamma production during early phases of the T cell stimulation constitutes one of the primary effector mechanism by which activated T cells mediate resistance to pathogens. Interleukin-2 is produced by activated T cells in an autocrine fashion and facilitates late proliferation of those cells in response to activation. Thus, any impairment in T cell function due to attachment of antibody or MPIO would directly compromise these signaling functions and would preclude the use of this labeling scheme. Activation of T cells through the T cell antigen receptor complex (CD3) was accomplished by incubating various numbers of labeled T cells in 100 μl of the above growth medium for two days in anti-CD3 coated plates. Both assays were performed at 2× saturation of CD5 receptors and 5 MPIOs per cell. Assays were performed over a wide range of cell numbers to simulate lymphocytes density *in vivo*.

For MRI, unsorted labeled and unlabeled lymphocytes were suspended at low density in 1% agarose containing 1 mM Gd-DTPA and imaged using 3D gradient echo at 11.7 T (Bruker Biospec, Billerica, MA, USA) using the following parameters: $TR = 30$ ms, $TE = 8$ ms, 100 μm isotropic voxel resolution over a 1.28 cm^3 field of view, acquisition time = 8 min. The Gd-DTPA was added to reduce the T_1 of the agarose to allow rapid imaging. The sizes (no. of pixels) of 12 hypointense contrast spots were measured using ImageJ software (NIH, Bethesda, MD, USA).

RESULTS

Figure 1 is a chart of the labeling efficiency achieved with both different antibody and MPIO concentration. The *x*-axis is plotted logarithmically to allow the visualization of the wide range of concentrations used in this study. The

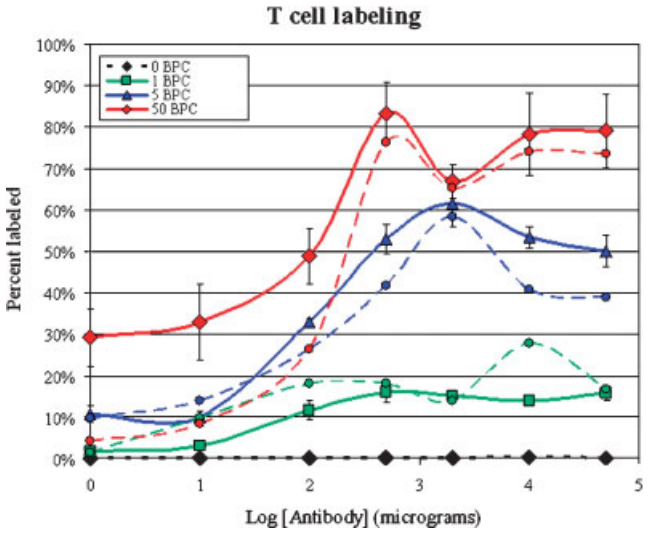

Figure 1. Plot of percentage T cell labeling vs antibody concentration. The red curves are for 50 MPIO beads per cell (BPC) in the labeling procedure, the blue curves are for five MPIO beads per cell and the blue curves are for one MPIO bead per cell. The black curve is for zero beads per cell. Solid lines are means of the flow cytometry measurements ($n = 4$); dashed lines are the manual counts ($n = 1$). Note that the *x*-axis is logarithmic.

results from flow cytometry are plotted together with the results from manual counting. Cell labeling improved with increasing antibody concentration, reaching a plateau at 2× saturation, for each MPIO concentration. Results from flow cytometry showed that, at 2× saturation, percentage T cell labeling was 16 ± 5, 53 ± 7 and $83 \pm 15\%$ for 1, 5 and 50 MPIOs/cell added, respectively. Manual counting of percentage cell labeling was 18, 42 and 76% for the same MPIO to cell ratios. There was a large difference between the flow cytometry and manual microscopy measurements at low antibody concentrations with 50 MPIOs/cell, with flow cytometry detecting highly increased cell labeling in these samples compared with manual counting. For example, for the samples with no antibody and 50 MPIOs/cell, flow cytometry detected a $29 \pm 7\%$ cell labeling, while manual counting only detected 4%. It is likely that a large number of free particles existed in these samples, which caused a high background count in the flow cytometry. Neither the temperature of the labeling reaction nor shaking the samples during labeling had any statistically significant effect on labeling efficiency. Viability as assessed by trypan blue exclusion test was >95%, even for the highest labeled cell populations.

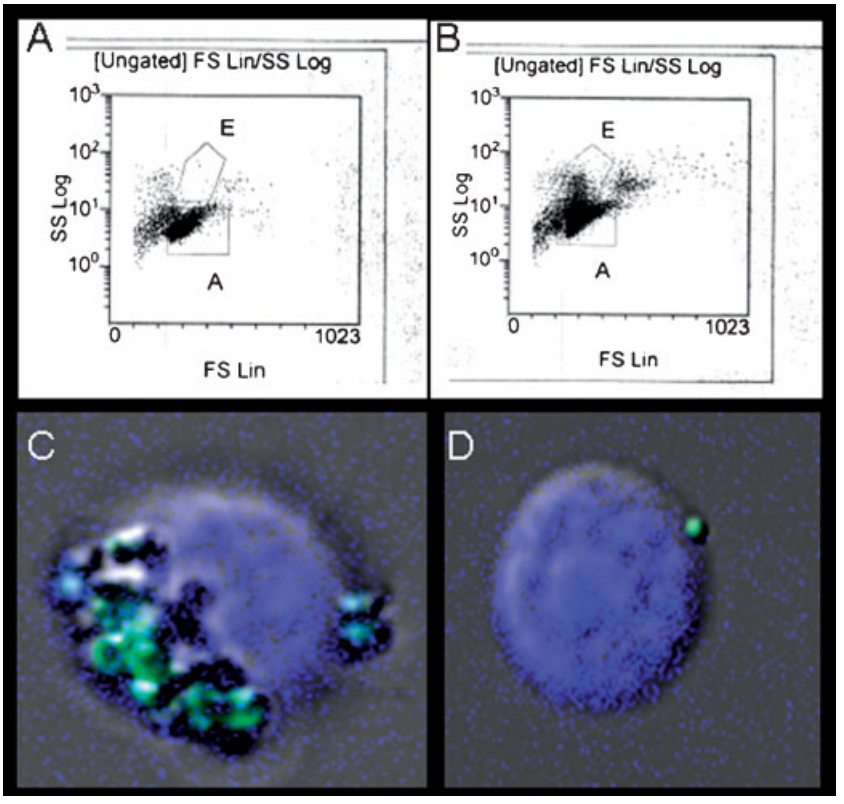

Figure 2. Example flow cytometry reports for samples without (A) and with MPIO labeling (B). This report is from cells labeled with 0.4× saturating amounts of antibody and five MPIOs per cell. Gate A is the less granular T cells, gate E is the more granular T cells. (C, D) Confocal microscope images of labeled cells from 2× saturating antibody and 50 MPIOs per cell. (C) Representative labeled cell from gate E, MPIOs are green, nucleus is stained blue with DAPI. (D) Representative cell from gate A. Each T cell measures approximately 10 μm in diameter.

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Figure 2 shows the heterogeneity in number of incorporated MPIOs per T cell. Flow cytometry revealed two populations of labeled lymphocytes, both equal in cell size, but one appearing more granular than the other. Figure 2(A) is a flow cytometry report for cells stained with antibody only, revealing a single, compact lymphocyte population in gate A. Labeling with particles did not change the linear dimensions of the cells, but it can be seen in Fig. 2(B) that the lymphocyte population extended into gate E, displaying increased granularity. Following a sort of the lymphocyte gate based on granularity for a sample incubated with 2× saturating antibody and 50 MPIOs/cell, confocal fluorescent microscopy revealed that the more granular appearing cells had many more incorporated MPIOs (range 16–56 MPIOs/cell, mean 35.0 ± 11.4) than the less granular appearing population (range 1–5 MPIOs/cell, mean 2.5 ± 1.3). These two cellular subsets were equally populated for the highest labeling conditions (43 vs 46%, more granular vs less granular).

Electron microscopy was performed to determine the cellular location of MPIOs. Figure 3 shows several T cells with particles attached to the surface of the cells (A–F, greater than 90%) and one with particles endocytosed (G–I). The particles appear heterogeneous in the density of dark iron oxide inside. This may be a real reflection of the iron oxide content of the particles or an oblique cut on the side of a particle. The surface attached particles often appear attached to cellular extensions, as in the case of (A–C) or in concave areas of the cell, perhaps as the cell tries to endocytose the MPIO (D–F). Figure 3(G) shows one cell which has endocytosed three MPIOs, with expansions in Fig. 3(H–I). While these MPIOs are clearly within the cell, they are close to the edge of the cell, probably due to the low amount of cytoplasm in a T cell. No attempt was made at this stage to determine the percent of MPIOs present extra- or intracellularly.

Cellular viability and the percentage of fluorescent cells were assessed. For this, cells were labeled with 2× saturation of the CD5 receptors and 30 MPIOs per cell.

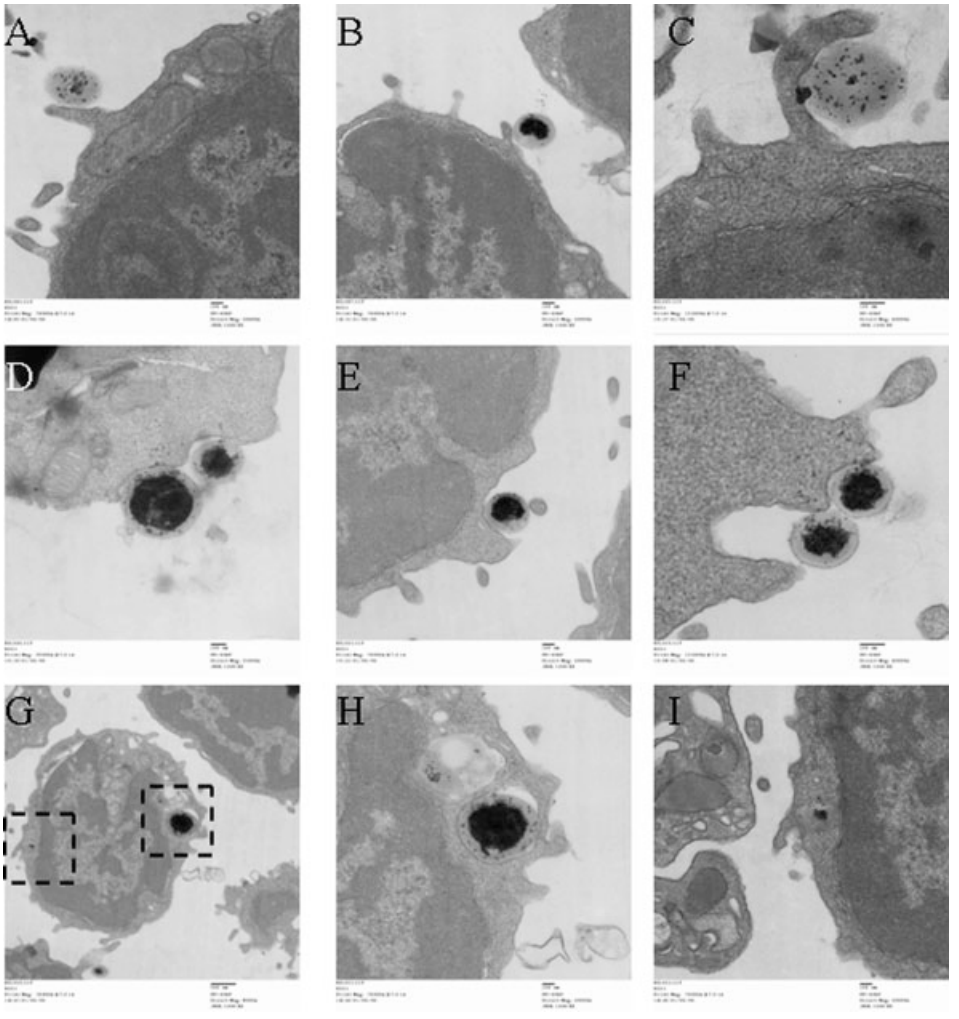

Figure 3. Electron microscopy of labeled T cells. MPIOs were found sticking to cellular processes (A–C), in concave extracellular cavities (D–F) and sometimes in intracellular compartments (G). (H and I) Expansions of two MPIOs from the cell in (G) (dashed boxes).

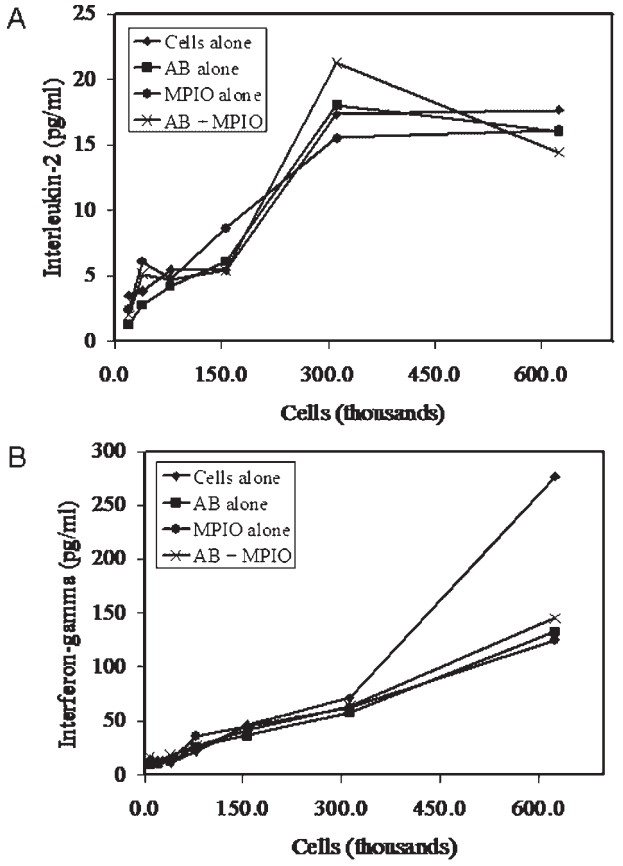

Figure 4. ELISA cytokine assays for (A) Interleukin-2 and (B) Interferon-gamma production and release. The four conditions are (1) cells alone, (2) cells labeled only with anti-CD5 antibody (AB alone), (3) cells incubated only with MPIOs (MPIO alone), and (4) cells first labeled with the antibody, then incubated with the MPIOs (AB + MPIO).

This resulted in $57.0 \pm 2.1\%$ T cell labeling and >95% viability. After two days in culture, cellular viability was $88.0 \pm 1.4\%$ and the percentage of T cells that had fluorescent MPIOs was $45.2 \pm 4.4\%$. This was 79.3% of the original labeling percentage.

Figure 4 summarizes the cytokine release assays after two day exposure to anti-CD3, an antibody against the T cell antigen receptor. Interleukin-2 production and release from stimulated, labeled T cells was nearly identical for all experimental conditions, over the entire range of cell concentrations [Fig. 4(A)]. Similarly, interferon-gamma [Fig. 4(B)] production and release were not affected by any of the experimental conditions, except for at the highest cell concentration, where control unlabeled cells exhibited greatly augmented cytokine release. However, at 625 000 cells in 100 μl, this exceeds the concentration of T cells in peripheral blood.

MRI showed multiple punctate, isolated, hypointense spots from the labeled cells [Fig. 5(A)]. Signal attenuation from single cells at 100 μms isotropic was $78 \pm 10\%$, with dimensions of the enhancement of 300 μms in all three dimensions [Fig. 5(B)]. The heterogeneity of the sizes of the spots is reflective both of the differences in the number of MPIOs incorporated on each cell, as well as partial volume effects from the large voxel sizes, with respect to the size of the cells. Unlabeled cells were undetectable, as previously demonstrated (9) (data not shown).

DISCUSSION

T cells have been implicated in a number of diseases, in maladies such as multiple sclerosis (MS), and therapeutically in immunotherapy of cancer. The ability to detect the presence of T cells by MRI could assist in the early detection of disease, or in the case of immunotherapy, allow an analysis of homing kinetics and efficiency of T cells to tumors. Measuring these processes at the single cell level facilitates quantification of these parameters, as well as studying rare or potentially novel cellular movements.

T cells were labeled with MPIOs by way of an antibody-mediated, biotin–streptavidin recognition strategy,

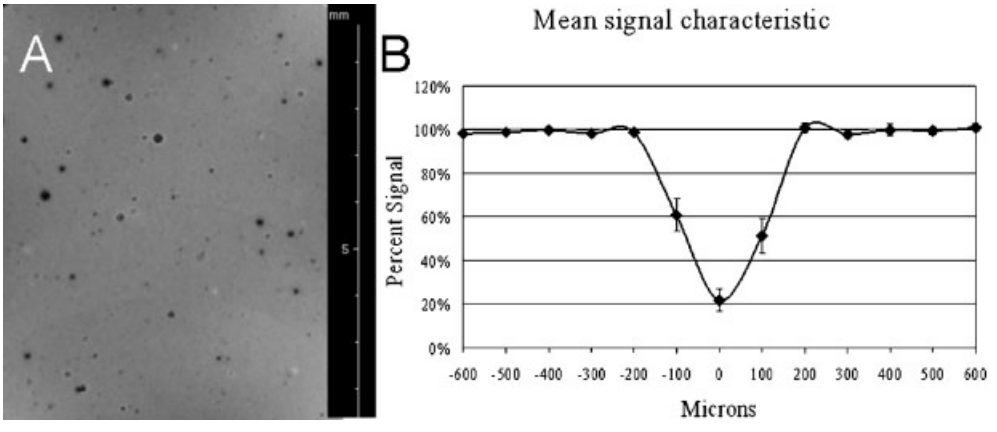

Figure 5. (A) MRI slice from the labeled lymphocyte phantom showing isolated, punctate, dark contrast spots. The barscale is in millimeters. (B) A mean plot profile through 12 spots, measuring the linear size and percentage contrast generated by labeled lymphocytes in the MRI experiments performed here.

achieving cellular iron loads previously demonstrated to be sufficient for *in vivo* single cell detection (15–17). The large iron load delivered by MPIOs to T cells in this study ranged from a few picograms for cells with one or two MPIOs in the less granular, lightly labeled population, to as much 60 pg/cell in the highly granular, well labeled population. The iron content in the highest labeled cells was at least 30 times larger than that currently achievable with (U)SPIOs (4). The reason for the large disparity in cell labeling from cell to cell is not known. Indeed, in an effort to ensure the dispersity of the MPIOs and antibody by shaking the samples, heterogeneous cell labeling was still observed. This may reflect a true heterogeneous expression of CD5.

In most cell labeling techniques, the iron oxide particles are endocytosed and distributed within endosomes or lysosomes. In the present work, antibody-mediated coupling of MPIOs to T cells led to a predominantly extracellular label. It remains to be tested whether the presence of the MPIOs on the cell surface will interfere with migration properties of the lymphocytes. A few of the lymphocytes had taken the MPIOs up into intracellular spaces and it may be that incubating longer or adding agents to stimulate endocytosis, such as TAT peptide (3) or polyamine groups (19) may increase the number of MPIOs that can be endocytosed.

The high percentage of live labeled cells following two days of cell culture, 88%, suggests that the label does not significantly affect cellular viability. The decrease in the percentage of cells with an incorporated label during the cell culture period, 79% of the original labeling percentage, may be due to either the small percentage of cell death measured by trypan value exclusion test, through cell division of labeled cells, or simply the label falling off the cell.

A major issue with using antibodies to label lymphocytes is that the antibody may activate the cells or alter production and release of cytokines. Activation of T cells through the T cell antigen receptor complex (CD3) results in the triggering of several signaling pathways. This leads to the induction of a number of genes, including those encoding cytokines such as interleukin-2 and interferon-gamma which have important immunoregulatory effects. ELISA assays revealed no changes in production and release by labeled cells of these two cytokines. That the labeling scheme used here had no detrimental effect on T cell behavior may be due to the number of attached MPIOs to cells. This is an advantage of using MPIOs to label lymphocytes, in that only one or a few MPIOs are necessary for single cell detection, requiring only a few productive antibody-receptor complexes. Lastly, antibody-mediated magnetic cell labeling with MPIOs should be particularly useful for labeling other cells in suspension, such as hematopoietic stem cells, or cells where low receptor copies are expressed.

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