Purpose: We investigated the spatiotemporal changes in signal in draining lymph nodes of mice to ascertain the size-dependent effects of variously sized particles of iron oxide used to enhance magnetic resonance (MR) lymphography.

Materials and Methods: We injected iron oxide particles of 50-, 100-, 200-, or 1,000-nm diameter into the footpads of individual mice and obtained sequential MR images of the popliteal and inguinal lymph nodes with 11.7 tesla up to 6 weeks after particle administration.

Results: Up to 30 min after administration of particles smaller than 100 nm, we observed a marked reduction in signal in the popliteal node that spread from the periphery at first observation toward the center of the node in subsequent measurements and persisted up to 6 weeks. In contrast, 1,000-nm particles caused dot-like areas of hypointensity in the popliteal lymph node, primarily in the inner portion, that appeared after 2 days. In the inguinal lymph nodes, signal changes occurred after 2 days for 50- and 100-nm particles and after one week for 1,000-nm particles. For 1,000-nm particles, areas of hypointensity were visible in the inner portion and not the periphery of the inguinal lymph node up to 6 weeks. In this study, we demonstrate the strong dependence of MR imaging contrast in draining lymph nodes on the size of the particle-shaped contrast agents injected subcutaneously. Particle size represented passive and active targeting effects, so micron-sized particles produced delayed enhancement.

Conclusion: Choosing the size of iron oxide particles for MR imaging contrast depends on the objective of observation, such as identifying the morphology or migration of immune cells in the lymph node.

Keywords: immune cell, iron oxide particle, lymph node, magnetic resonance lymphography, mouse
Iron oxide particles may also be used for noninvasive tracking of the migration and biodistribution of immune cells. Cellular tracking typically involves labeling the cells ex vivo and subsequently implanting or systemically administering particles into the living body. These labeling methods are useful for observing the migration of immune cells toward a target, such as a tumor, and their subsequent accumulation. However, it is difficult to observe how the phagocytes that play a role in innate immune response locomote in lymphatics toward draining lymph nodes in vivo. Establishment of an in vivo phagocyte-labeling method that involves the direct administration of iron oxide particles would contribute to clarifying this mechanism.

The size of particles is known to influence phagocytosis and the permeability of the lymphatic wall as well as to affect image contrast as T2- and T2*-shortening effects. Few studies have reported the different contrast effects in lymph nodes following direct administration of various sizes of iron oxide particles. Our study aimed to test the spatiotemporal signal changes in draining lymph nodes following subcutaneous injection of variously sized particles into the footpads of mice.

Materials and Methods

Particles

We commercially purchased carboxylate-modified iron oxide particles of 4 different diameters, 50 and 100 nm (Nanomag®-D-spio, 5 mg/mL, micromod Partikeltechnologie GmbH, Rostock, Germany), 200 nm (Magnetic Beads, 3 mg/mL, ADEMTECH SA, Pessac, France), and 1,000 nm (BcMag, 20 mg/mL, Bioclon Inc., San Diego, CA, USA).

Animals

We divided 32 normal 6- to 8-week-old male C57BL/6J mice weighing 18 to 22 g (mean weight, 21 g) (Japan SLC, Shizuoka, Japan) into 4 groups according to the size of the particle they were administered. Of the 32, we examined 16 (4 animals each for each particle size) by both sequential MR imaging and histological study for 6 weeks after particle administration. We examined the other 16 mice by histology only prior to administration and at 1 hour, 2 days, and 3 weeks after particle administration, we reinduced anesthesia and performed MR imaging measurements.

MR imaging measurement

All MR imaging was performed on an 11.7-tesla vertical Bruker Avance wide bore system (Bruker BioSpin MRI GmbH, Ettlingen, Germany). Gradients could supply up to 1.5 T/m. A transmit/receive volume RF coil of 25-mm inner diameter was used for excitation and reception of signals. We imaged mouse popliteal and inguinal lymph nodes in 12 coronal slices of 300-μm thickness, with 25.6 mm × 25.6 mm field of view, 100-μm in-plane resolution. The slices substantially covered the mouse lymph nodes. To compare contrast effects, by visual inspection, we selected a representative slice that displayed the center of each lymph node. MR imaging data were obtained using a 2-dimensional (2D) fast low angle shot (FLASH) sequence (repetition time [TR]/echo time [TE], 230 ms/1.6 ms; flip angle = 20°; 8 acquisitions; and image matrix = 256 × 256).

Histology

We retrieved each popliteal and inguinal lymph node after MR imaging confirmation of the signal changes at different time points, fixed tissues in 4% formalin solution, embedded them in optimal cutting temperature (OCT) compound, and cut 10-μm sections using the cryostat. We performed Prussian blue staining and nuclear fast red counterstaining on each section, cover slipped the tissue sections, and examined them by light microscopy.

Results

Size-dependent effects of administration of iron oxide particles in the popliteal lymph node

Experimental procedure

Following induction of 2.5% isoflurane inhalational anesthesia, each mouse was placed within a volume radiofrequency (RF) coil to allow simultaneous observation of the popliteal and inguinal lymph nodes. To study effects of each size of iron oxide particle, we performed MR imaging before subcutaneously injecting a 20- to 30-μL suspension of a given-sized particle into the right footpad of each mouse using a 30-G needle (Fig. 1a). Mice were aroused immediately after particle administration and allowed to move freely in the cage. At 30 min, 2 days, and one, 3, and 6 weeks after particle administration, we reinduced anesthesia and performed MR imaging measurements.
Figure 1. Negative contrast effects of iron oxide particles in lymph nodes of mice. (a) Graphic depiction of lymphatic tissues in mice. Dots indicate lymph nodes, and lines indicate major lymphatic vessels that connect nodes to each other. We injected iron oxide particles into the footpad of the right hind leg in mice. (b) Coronal image of draining lymph nodes with injection of 50-nm particles. A fast low angle shot (FLASH) sequence with fat saturation clearly depicts oval-shaped lymph nodes (arrows). On the other hand, accumulation of particles in regional lymph nodes on the same side as injection causes signal reductions on T2*-weighted magnetic resonance imaging scans, such as the FLASH sequence (arrowheads).

Figure 2 shows FLASH images of representative popliteal lymph nodes from each group. The sequential contrast effects in the lymph nodes for individual animals within each experimental group were generally similar. Thirty min after administration of 50-nm particles, we observed marked signal reductions throughout the lymph node (Fig. 2a-2). Thirty min after administration of 100-nm particles, we observed areas of hypointensity that were extend in the periphery of the nodes (Fig. 2b-2). After 2 days, the areas of hypointensity had spread throughout the lymph nodes (Fig. 2b-3). Following 200-nm particle administration, we observed 2 distinct spatiotemporal patterns of signal change; half the mice showed peripheral areas of hypointensity after 30 min (200 nm-A; Fig. 2c-2), and half showed areas of hypointensity within the inner portion of the node at 2 days in the absence of any remarkable change at 30 min (200 nm-B; Fig. 2d-3). After administration of 1,000-nm particles, we observed no signal changes at 30 min (Fig. 2e-2), but after 2 days, we noted a few dots of hypointensity of approximately 100-μm diameter in the inner portion of the popliteal lymph node (Fig. 2e-3; arrows) and none in the periphery (Fig. 2e-3). In all cases, the areas of hypointensity spread throughout the lymph nodes during subsequent measurements and remained up to 6 weeks after administration of each particle size.

Size-dependent effects of iron oxide particle administration in the inguinal lymph node

Figure 3 shows FLASH images of representative inguinal lymph nodes in each group. The sequential contrast effects in the lymph nodes for individual animals within each experimental group were generally similar. Thirty min after administration of 50-nm particles, we observed areas of hypointensity throughout the lymph node that were more extensive in the periphery (Fig. 3a-2). Thirty min after administration of 100-nm particles, we observed areas of hypointensity at the center of the lymph node and strong reductions at the periphery (Fig. 3b-3). After 50- and 100-nm particle administration, these areas of hypointensity remained during subsequent measurements up to 6 weeks. We also observed 2 distinct spatiotemporal patterns of signal changes after 200-nm particle administration. In the first group (50% of subjects; 200 nm-A), areas of hypointensity appeared at 2 days (Fig. 3c-3), spread into the center of the ingui-
Fig. 2. Representative examples of size-dependent changes in spatiotemporal signal in the popliteal lymph nodes. Magnetic resonance (MR) imaging was performed prior to and 30 min, 2 days, and one, 3, and 6 weeks after administration of variously sized particles into the footpad of the right hind leg. Sequential images in the same representative mouse within each experimental group were arranged in each line. Thirty min following administration of 50- and 100-nm particles, we observed areas of hypointensity in the popliteal lymph node (a-2, b-2, arrows). Similar effects were observed in half the mice administered 200-nm particles (200 nm-A, c-2, arrow); in the other half, the signal remained up to 30 min (d-2), and signal reductions in the inner portion of the node were observed at 2 days (200 nm-B, d-3, arrow). Furthermore, dot-like hypointense areas were obtained 2 days after administration of 1,000-nm particles (e-3, arrows). In all cases, these areas of hypointensity spread throughout the lymph node during subsequent measurements.

Comparison of particle size in spatiotemporal signal change

We made 4 important observations regarding the signal changes in MR imaging. (1) The use of 50-nm particles induced marked signal reductions throughout the popliteal lymph node and in the periphery of the inguinal lymph node immediately after administration. (2) The use of 100-nm particles also induced areas of hypointensity, mainly in the peripheries of the popliteal lymph nodes at 30 min and the inguinal nodes at 2 days after administration. In addition, over time, the areas of hypointensity spread toward the center of the nodes during measurements. (3) The use of 1,000-nm particles led to visualization of hypointense dots in the inner portions of the popliteal lymph nodes at 2 days and of the inguinal lymph nodes at one week, and the size of these dots in the inguinal lymph nodes remained up to 6 weeks. (4) The use of intermediate-sized 200-nm particles yielded patterns resembling those noted after the administration of 100-nm and 1,000-nm particles.

Histological examination

Figure 4 shows representative histological images
that confirm the presence and distribution of particles in the popliteal and inguinal lymph nodes. The 100-nm particles accumulated in the periphery, called the marginal sinus, of the popliteal lymph nodes within one hour (Fig. 4A) and the periphery of the inguinal nodes at 2 days after administration (Fig. 4J), spread around the marginal sinus, and persisted there for 6 weeks (Fig. 4D, 4L). Some particles were incorporated into cells and deposited in the inguinal lymph nodes during the experiments (Fig. 4J, arrows). In contrast, the number of 1,000-nm particles in the marginal sinus was much lower than that of 100-nm particles, and at 2 days, we also observed 1,000-nm particles in the inner portion of the popliteal lymph nodes (Fig. 4F). Many of the observed particles were clearly incorporated into the cells. Particles were also dispersed throughout the popliteal lymph node, including in the marginal zone, at 6 weeks (Fig. 4H). In the inguinal lymph node, particles were absent at 2 days (Fig. 4N), but we observed a few particles in the inner portion of the inguinal lymph node at one and 6 weeks (Fig. 4O, 4P). Most 1,000-nm particles remained, mainly intracellularly, throughout the measurements (white arrows).

**Discussion**

Our results are consistent with those obtained in other studies carried out using microscopy\textsuperscript{15,19} and scintigraphy.\textsuperscript{20} A study using colloidal particles assumed 2 pathways by which particles are incorporated into the lymphatics—the first involving the passive transport of particles along extracellular interstitial fluid channels directly into the initial lymphatics, and the other involving phagocytosis of particles by phagocytes, such as dendritic cells or macrophages, which then migrate into the initial lymphatics and carry the particles via the intracellular active pathways along the lymphatics into the lymph nodes.\textsuperscript{15} These transport patterns of particles into lymphatics are also known to depend upon particle size.\textsuperscript{15,21} The good agreement of our MR imaging results with results of the histological examination confirmed that iron oxide particles provided adequate contrast. MR imaging discriminat-
Fig. 4. Histological comparison of particle presence in draining lymph nodes. Higher magnified views marked by red squares are shown in the corner of each image. Highly dense blue pigmentation in Prussian blue staining, which indicates iron accumulation in the tissue, can be observed mainly in the peripheries of popliteal lymph nodes within one hour (A) and inguinal lymph nodes at 2 days (J) after 100-nm particle administration. Meanwhile, the 1,000-nm particles were localized in the inner portion of popliteal lymph node at 2 days (F, arrows) and in the inguinal lymph nodes at one week following particle administration (O, arrow). At 6 weeks, many particles were observed both in the periphery (H, black arrow) and the inner portion of the popliteal lymph nodes (H, white arrows). In contrast, only a few particles were identified in the inner portion of the inguinal lymph nodes through subsequent experiments (O-P, arrows).

The use of 50- and 100-nm particles induced signal reductions relatively early after administration, mainly in the peripheries of the popliteal and inguinal lymph nodes. First identified in the periphery, the areas of hypointensity tended to spread toward the center of each node. The lymphatic wall is known to have low permeability, which may bar the passage of macromolecules, such as proteins, whereas water and substances of low molecular weight retain their high permeability. A previous study reports colloid particles between 10 and 100 nm were the optimal size for lymphatic uptake. These smaller particles would be quickly transported across the initial lymphatic wall, and they could pour into the marginal sinuses of draining lymph nodes via afferent vessels and accumulate there. Consequently, we may consider that 50- and 100-nm particles can quickly, easily, and noninvasively provide MR imaging contrast of intact lymph nodes, mainly via the extracellular transport pathways. In our histological study, our observation of some particles within cells during experiments raises the possibility that some particles were transported via the intracellular pathways or phagocytosed within the lymph node in conjunction with the extracellular transport pathways. However, the overwhelming effects of the extracel-
Lymph nodes for presentation to T cells.1,2,28 After transfer the antigen to resident dendritic cells in the nodes, then migrate to the paracortical area to differentiate into mature dendritic cells during migration through the interstitium from the injection site and uptake into the lymphatics by a passive process requiring administered particles smaller than 100-nm diameter.25 Micron-sized particles are considered physically incapable of entering the lymphatics because of the size limit of the interstitium and the initial lymphatic wall, so identification of these particles in the lymph nodes requires the active process of phagocytes. Langerhans cells, one of the phagocytes in the skin, take up the particles locally and then migrate toward the draining lymph node. Langerhans cells mobilize relatively slowly following inflammatory stimuli, so they reach draining lymph nodes only after one to 4 days.26,27 Langerhans cells differentiate into mature dendritic cells during transport to the marginal sinuses of draining lymph nodes, then migrate to the paracortical area to transfer the antigen to resident dendritic cells in the lymph nodes for presentation to T cells.1,2,28 After administration of 1,000-nm particles, we observed focal hypointense dots in the inner portion of the node significantly later than observed for 50- and 100-nm particle administration, and these dots maintained their diameters until final observation. The upper size limit for passive lymphatic uptake has not been strictly defined, but the principal barrier to particle drainage from subcutaneous injection has been drainage through the interstitium into the initial lymphatics.24 A previous study reported that drainage through the interstitium from the injection site and uptake into the lymphatics by a passive process requires administered particles smaller than 100-nm diameter.25 Micron-sized particles are considered physically incapable of entering the lymphatics because of the size limit of the interstitium and the initial lymphatic wall, so identification of these particles in the lymph nodes requires the active process of phagocytes. Langerhans cells, one of the phagocytes in the skin, take up the particles locally and then migrate toward the draining lymph node. Langerhans cells mobilize relatively slowly following inflammatory stimuli, so they reach draining lymph nodes only after one to 4 days.26,27 Langerhans cells differentiate into mature dendritic cells during transport to the marginal sinuses of draining lymph nodes, then migrate to the paracortical area to transfer the antigen to resident dendritic cells in the lymph nodes for presentation to T cells.1,2,28 After administration of 1,000-nm particles, we observed focal hypointense dots in the inner portion of the node significantly later than observed for 50- and 100-nm particle administration, probably because the micron-sized particles reflected the active transport by phagocytic cells that had migrated into the paracortical area or the T-cell zone of lymph nodes.

In accordance with our histological findings (Fig. 4F), MR imaging could detect the existence of a small amount of particles as dot-like signal reductions with 100-µm diameter (Fig. 2e-3). Previous ex vivo studies have stated that a single cell containing labeled micron-sized iron oxide particles can provide adequate contrast effect for gradient-echo MR imaging because of the $T_2^*$ effect, and only a single particle is required for detection.29 Phagocytosed particles were observed as dots in MR imaging, so further improvements in the spatiotemporal resolution of MR imaging are expected to enable noninvasive observation of cell movement in the lymph nodes. One week after 1,000-nm particle administration onward, the hypointense areas spread throughout the popliteal lymph node. We may consider that phagocytes that incorporate particles and are then activated at the periphery migrate toward the draining lymph node. Further dose-response experiments are necessary to establish the protocol for obtaining the best contrast for observing single cells in lymphatic tissue. There are relatively few reports of active targeting of iron oxide particles achieved with in vivo labeling. Our results demonstrate that direct injection of micron-sized particles noninvasively produces active targeting effects of phagocytic cells. Such information could allow detection of mechanisms of disease and natural resistance.

3. Demarcating diameter contributes for both passive and active transport effects

The use of 200-nm particles induced passive and active targeting effects in various individuals, so we may consider this approximate size as the size limit for particles for demarcating the 2 effects for our method. A previous study reported that the optimal size of molecules for lymphatic uptake is smaller than 100 nm because of the limited gap opening at the junctions of endothelial cells.16 Particle size and other factors, such as injection pressure15,19,24 or surface characteristics,24,50 may affect the permeability of the lymphatic wall. We could achieve a much clearer contrast by determining the size limit of particles and appropriate injection pressure to permit passage of particles through the endothelial cell junctions of lymphatic vessels.

Conclusion

In this work, we elucidated the spatiotemporal characteristics of in vivo MR imaging using variously sized iron oxide particles that we injected directly into the footpads of mice. MR imaging contrast in the draining lymph nodes depended strongly on particle size and represented the different manner of particle transportation. Passive target-
ing effects of small particles, with diameters of 50 and 100 nm, enabled visualization of lymphatic structures via passive transport of large quantities of particles across lymphatic walls. Further, micron-sized particles could potentially demonstrate the migration of phagocytes with MR imaging noninvasively, without ex vivo cell labeling. The appropriate size of particles should be considered for acquiring contrast-enhanced MR lymphography with direct injection of contrast agents. Our findings would be important for future research involving the immune system and for the development of contrast agents intended specifically for lymphatic tissues.

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