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Supplementary Information for:

Cultured macrophages transfer surplus cholesterol into adjacent cells in the absence of serum or high-density lipoproteins

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This PDF file includes: Figures S1–S11, Table S1

SI Appendix Figures S1–S10

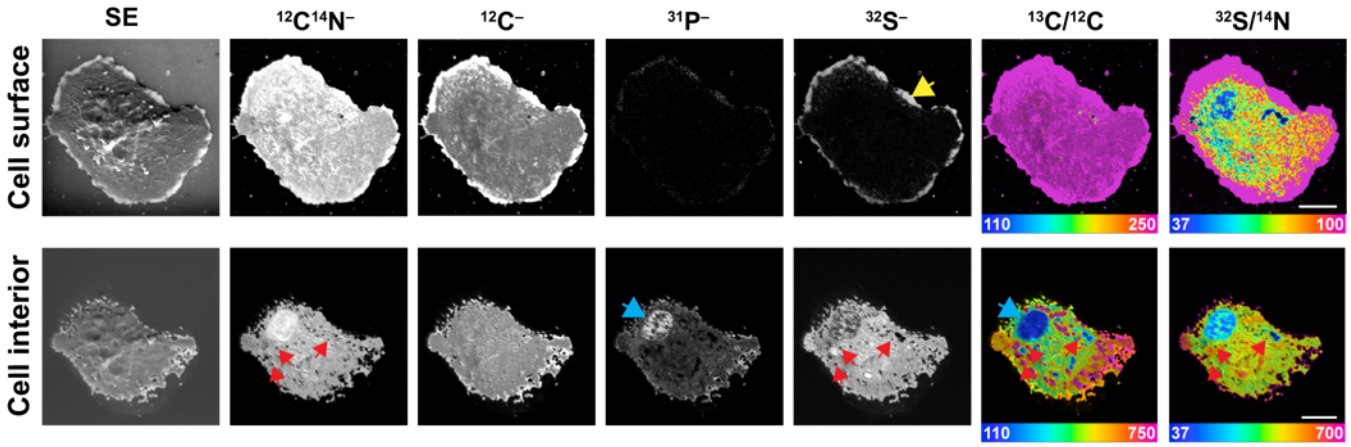

Fig. S1. NanoSIMS images of a [^{13}C]cholesterol-loaded mouse peritoneal macrophage. Macrophages loaded with [^{13}C]cholesterol were plated on a poly-D-lysine–coated silicon wafer and incubated for 24 h in serum-free medium. Secondary electron and secondary ion NanoSIMS images from the cell surface (top row) and cell interior (bottom row) are shown for a representative macrophage. The ^{32}S-rich region at the edge of the cell surface reflects extracellular heparan-sulfate proteoglycans (yellow arrow). Note the high ^{31}P$^-$ signal and low ^{13}C/^{12}C ratio in the nucleus (blue arrows). Red arrows point to examples of cytosolic LDs (identified in the ^{12}C^{14}N$^-$ and ^{32}S$^-$ images) enriched in ^{13}C. Ratio scales were multiplied by 10,000. Scale bar, 10 μm.

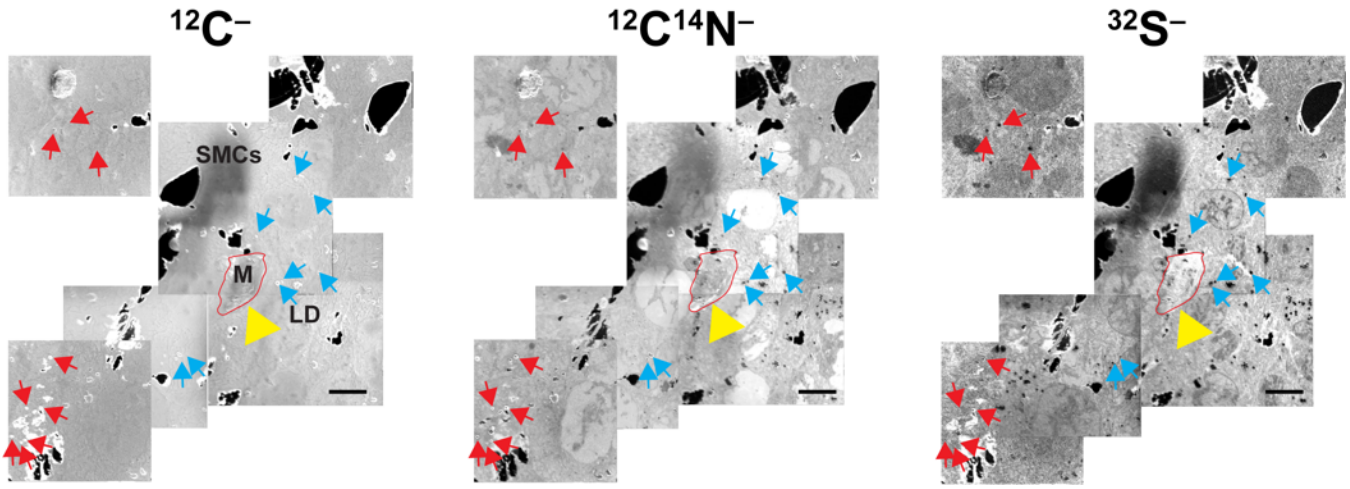

Fig. S2. Macrophages (M), smooth muscle cells (SMCs), and cytosolic lipid droplets (LDs) can be identified in $^{12}C^-$, $^{12}C^{14}N^-$, and $^{32}S^-$ NanoSIMS images. The images shown here correspond to the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ images in **Fig. 1**. A single [^{13}C]cholesterol-loaded macrophage, outlined in red and noted with a yellow arrowhead, is located on a monolayer of [^{15}N]choline-enriched SMCs. The large black holes represent regions of the silicon wafer not covered by cells. Small black holes in the $^{12}C^{14}N^-$ and $^{32}S^-$ images represent cytosolic LDs, which contain negligible amounts of ^{14}N or ^{32}S. The LDs appear white in the $^{12}C^-$ image. Blue arrows depict cytosolic LDs in SMCs adjacent to a macrophage; red arrows depict cytosolic LDs in more distant SMCs. Note the high ^{32}S content of the macrophage (also apparent in **Fig. 1C**). Scale bar, 10 μm.

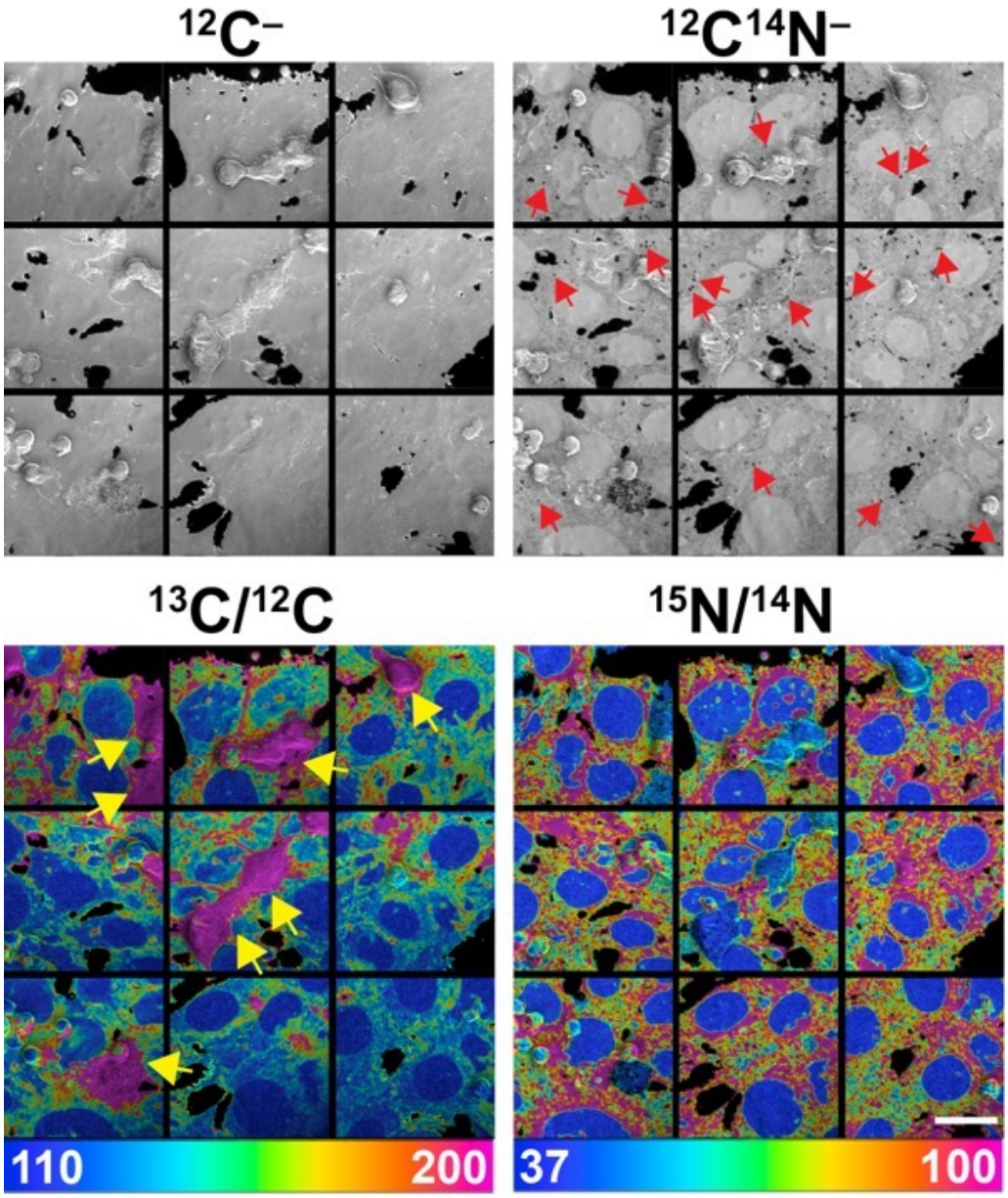

Fig. S3. NanoSIMS mosaics of [^{13}C]cholesterol-loaded macrophages that had been plated on a monolayer of [^{15}N]choline-enriched smooth muscle cells (SMCs). The $^{12}C^-$, $^{12}C^{14}N^-$, $^{13}C/^{12}C$, and $^{15}N/^{14}N$ NanoSIMS mosaic images show a ~150 μm × 150 μm region containing multiple macrophages (yellow arrows). The large black holes represent regions without cells. Small black regions in the $^{12}C^{14}N^-$ images represent cytosolic lipid droplets (red arrows), which contain negligible amounts of ^{14}N. Ratio scales were multiplied by 10,000. In this study, the number of macrophages was large; hence, ^{13}C enrichment was observed in all SMCs in the field. Scale bar, 20 μm.

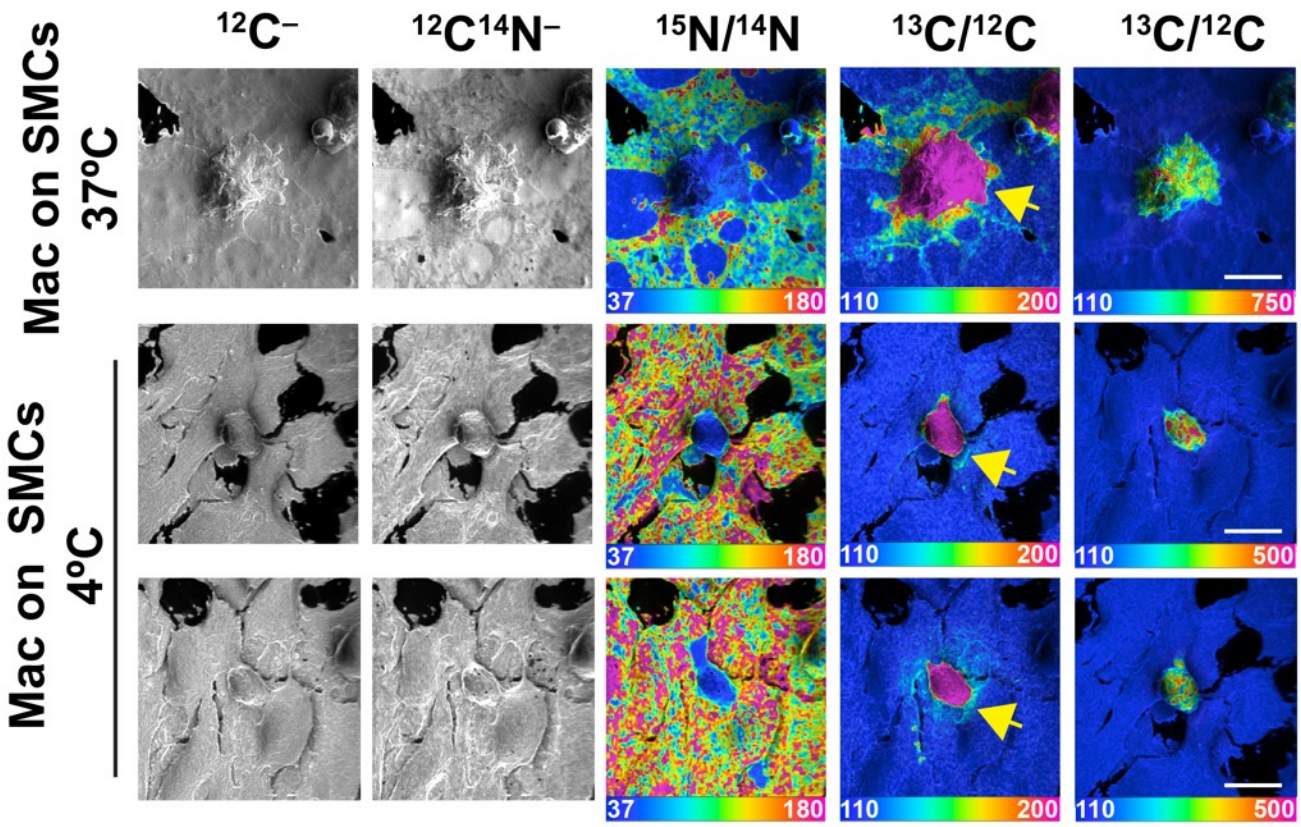

Fig. S4. NanoSIMS images of [^{13}C]cholesterol-loaded macrophages that had been plated onto a monolayer of [^{15}N]choline-labeled SMCs and then incubated at either 37°C or 4°C. Macrophages are noted with large yellow arrows. NanoSIMS images were recorded from the cell interior. The large black holes represent regions of the silicon wafer not covered by cells. At 4°C, ^{15}N enrichment of macrophages was very difficult to see but was ~13% above natural abundance. ^{13}C enrichment in the surrounding SMCs was visible, but was not seen in abutting SMC plasma membranes or in cytosolic lipid droplets. Scale bar, 10 μm. Ratio scales were multiplied by 10,000.

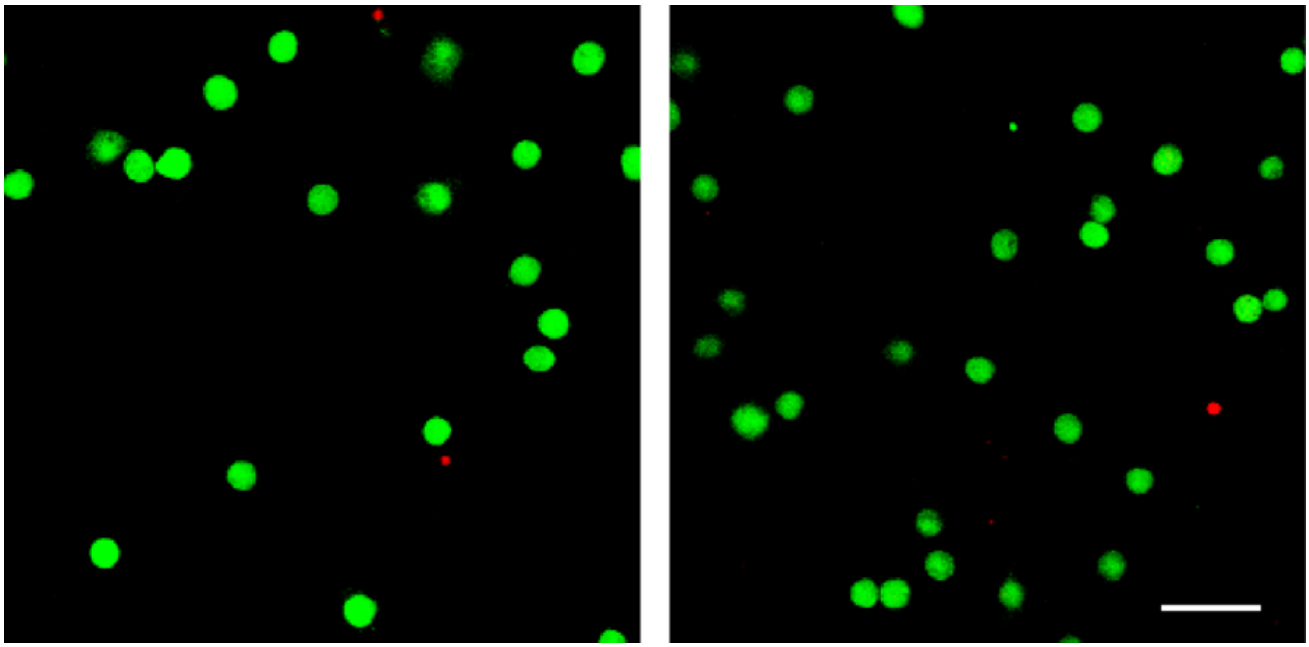

Fig. S5. Cell viability assay on [^{13}C]cholesterol-loaded macrophages after a 30-min incubation with methyl-β-cyclodextrin (MβCD). Cell viability was assessed with the fluorescence-based LIVE/DEAD Cell Imaging Kit (ThermoFisher). Two fields are shown. Live cells fluoresce green; DNA from dead cells fluoresces red. Scale bar, 50 μm.

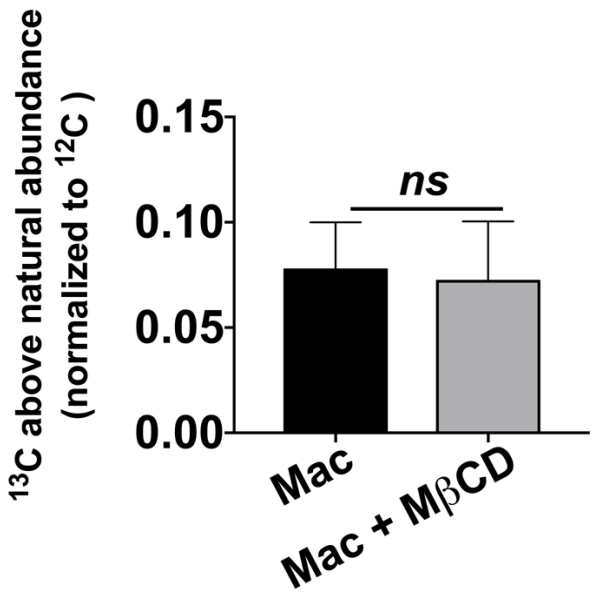

Fig. S6. Pre-incubating [^{13}C]cholesterol-loaded macrophages for 30 min with methyl-β-cyclodextrin (MβCD) did not result in a significant depletion of [^{13}C]cholesterol from the interior of macrophages. The *y*-axis shows ^{13}C secondary ions (above natural abundance and normalized to ^{12}C) in the interior of macrophages. This graph shows ^{13}C enrichment (mean ± standard deviation) in macrophages (Mac) that were not pre-incubated with MβCD (n = 9) and macrophages that had been pre-incubated for 30 min with MβCD (Mac + MβCD) (n = 10).

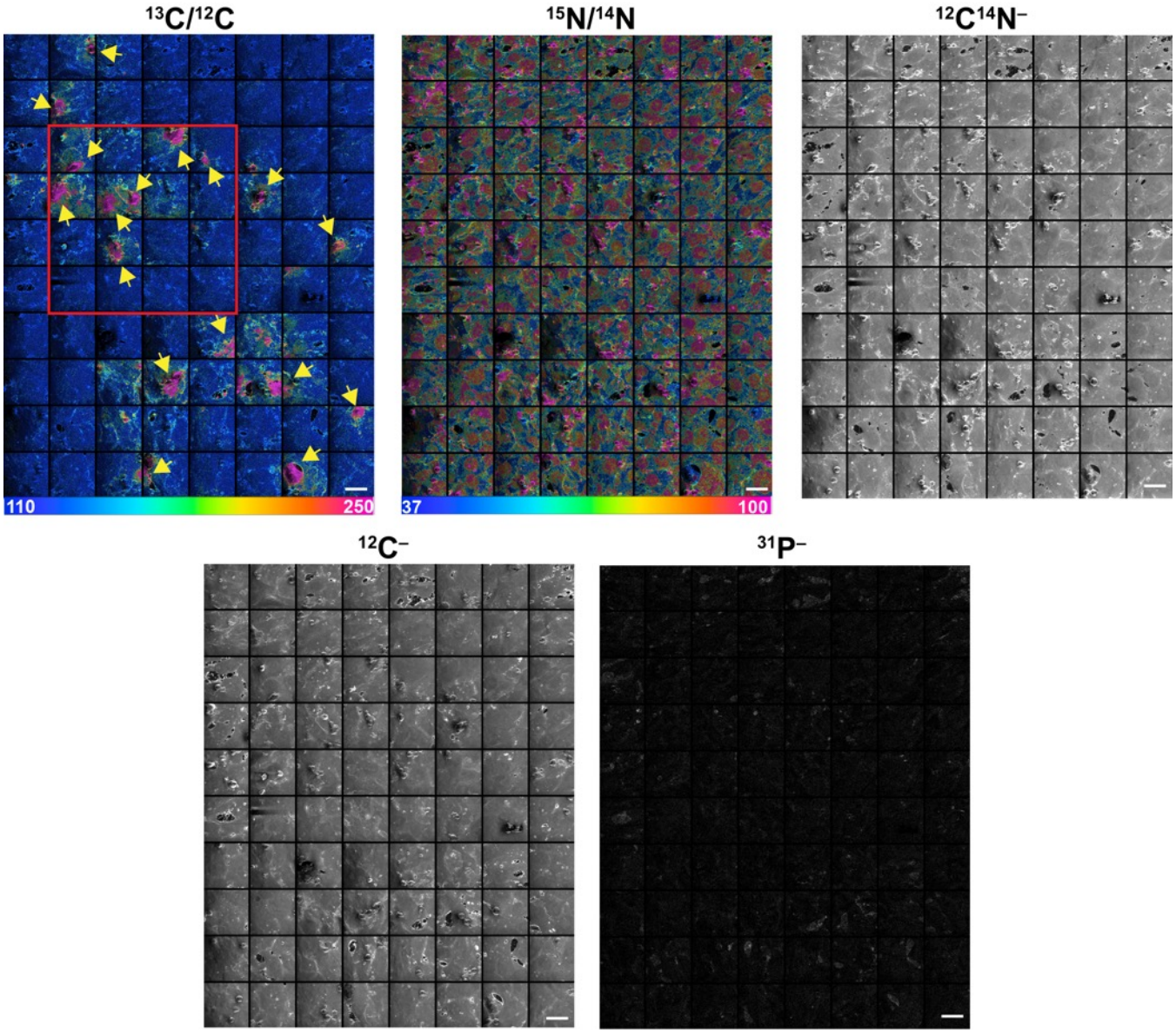

Fig. S7. Transfer of [^{13}C]cholesterol from [^{13}C]cholesterol-loaded macrophages to adjacent smooth muscle cells (SMCs). In this experiment, "accessible cholesterol" was removed from the plasma membrane of [^{13}C]cholesterol-loaded macrophages by incubating the cells with methyl-β-cyclodextrin (MβCD) for 30 min. The macrophages were then washed extensively and plated onto SMC (~90–95% confluent) that had been grown for 21 days in medium containing [^{15}N]choline. [^{13}C]cholesterol-loaded macrophages are noted with yellow arrows. A portion of this mosaic is shown in **Fig. 4** (boxed region). In this figure, the imaged area is ~400 µm × 500 µm. Shown here are $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{12}C^{14}N^-$, $^{12}C^-$, and $^{31}P^-$ NanoSIMS images from the surface of cells. A slightly smaller NanoSIMS mosaic from the cell interior is shown in the *SI Appendix*, Fig. S8. Ratio scales were multiplied by 10,000. Scale bar, 20 µm.

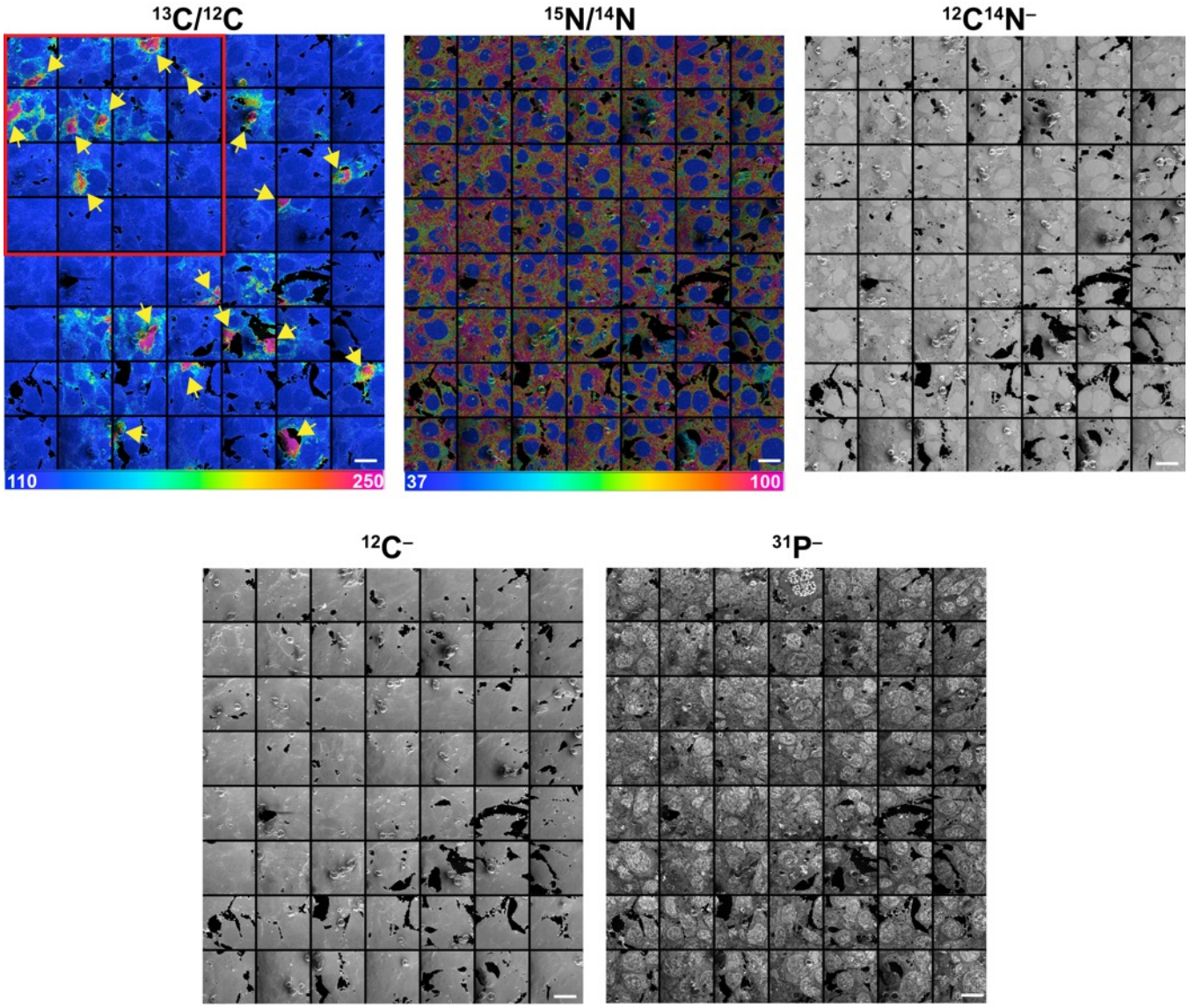

Fig. S8. Transfer of [^{13}C]cholesterol from [^{13}C]cholesterol-loaded macrophages to adjacent smooth muscle cells (SMCs). Cells were treated as described in **Fig. S7**. [^{13}C]cholesterol-loaded macrophages are noted with yellow arrows. A portion of this mosaic is shown in **Fig. 4** (boxed region). In this figure, the imaged area is ~350 μm × 400 μm. Shown here are $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{12}C^{14}N^-$, $^{12}C^-$, and $^{31}P^-$ NanoSIMS images from the cell interior. A slightly larger NanoSIMS mosaic from the cell surface is shown in the *SI Appendix*, Fig. S7. Ratio scales were multiplied by 10,000. Scale bar, 20 μm.

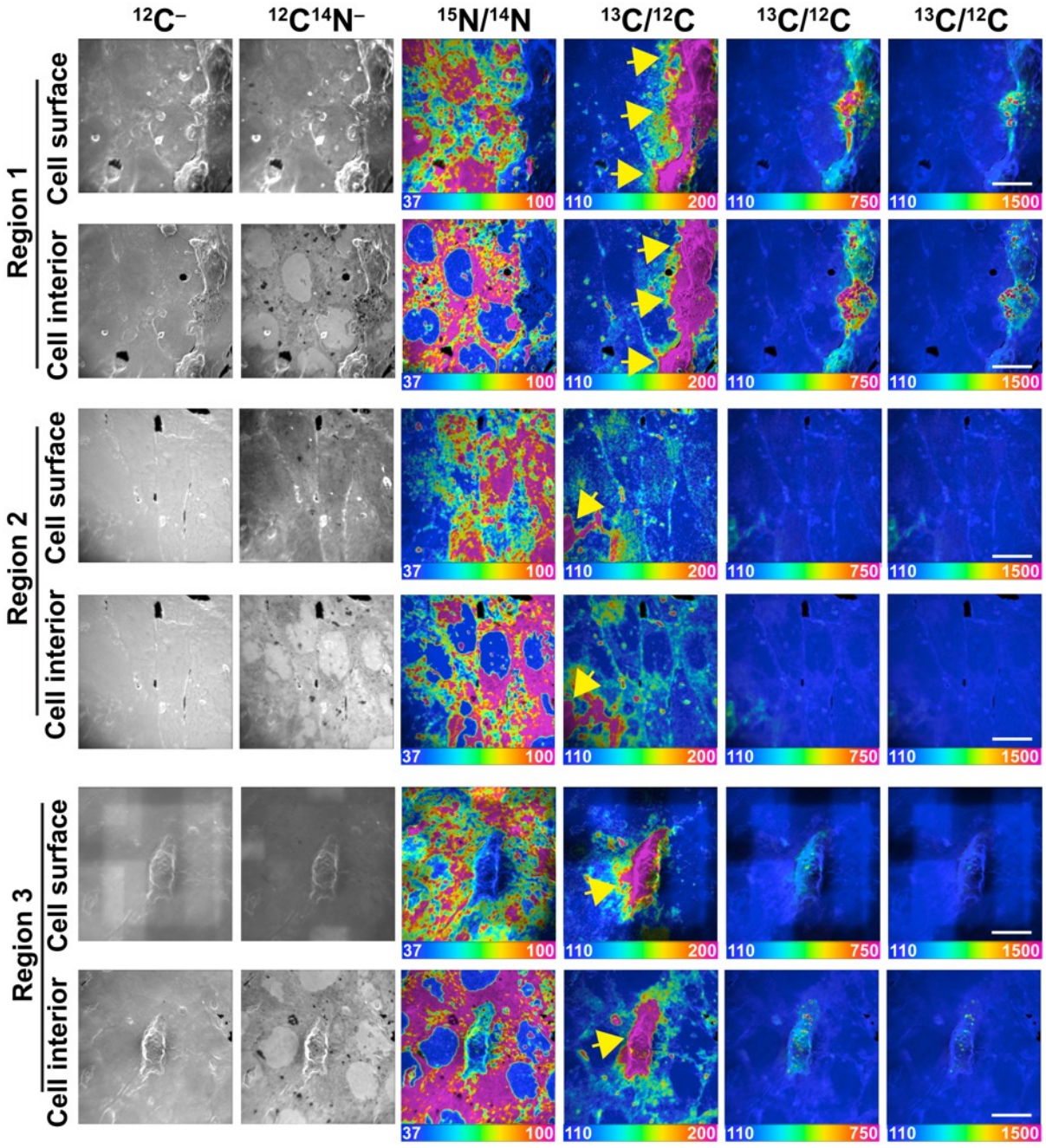

Fig. S9. Transfer of [^{13}C]cholesterol from [^{13}C]cholesterol-loaded macrophages to adjacent smooth muscle cells (SMCs). [^{13}C]cholesterol-loaded macrophages were plated onto a monolayer of SMCs (~90–95% confluency) that had been grown in medium containing [^{15}N]choline for 21 days. Shown here are NanoSIMS images of three fields of [^{13}C]cholesterol-loaded macrophages that had not been pre-incubated with methyl-β-cyclodextrin. Images of both the cell surface and cell interior are shown. $^{12}C^-$, $^{12}C^{14}N^-$, $^{13}C/^{12}C$ (three different scales), and $^{15}N/^{14}N$ NanoSIMS images depict the transfer of [^{13}C]cholesterol from macrophages (yellow arrows) to SMCs. Ratio scales were multiplied by 10,000. Scale bar, 10 μm.

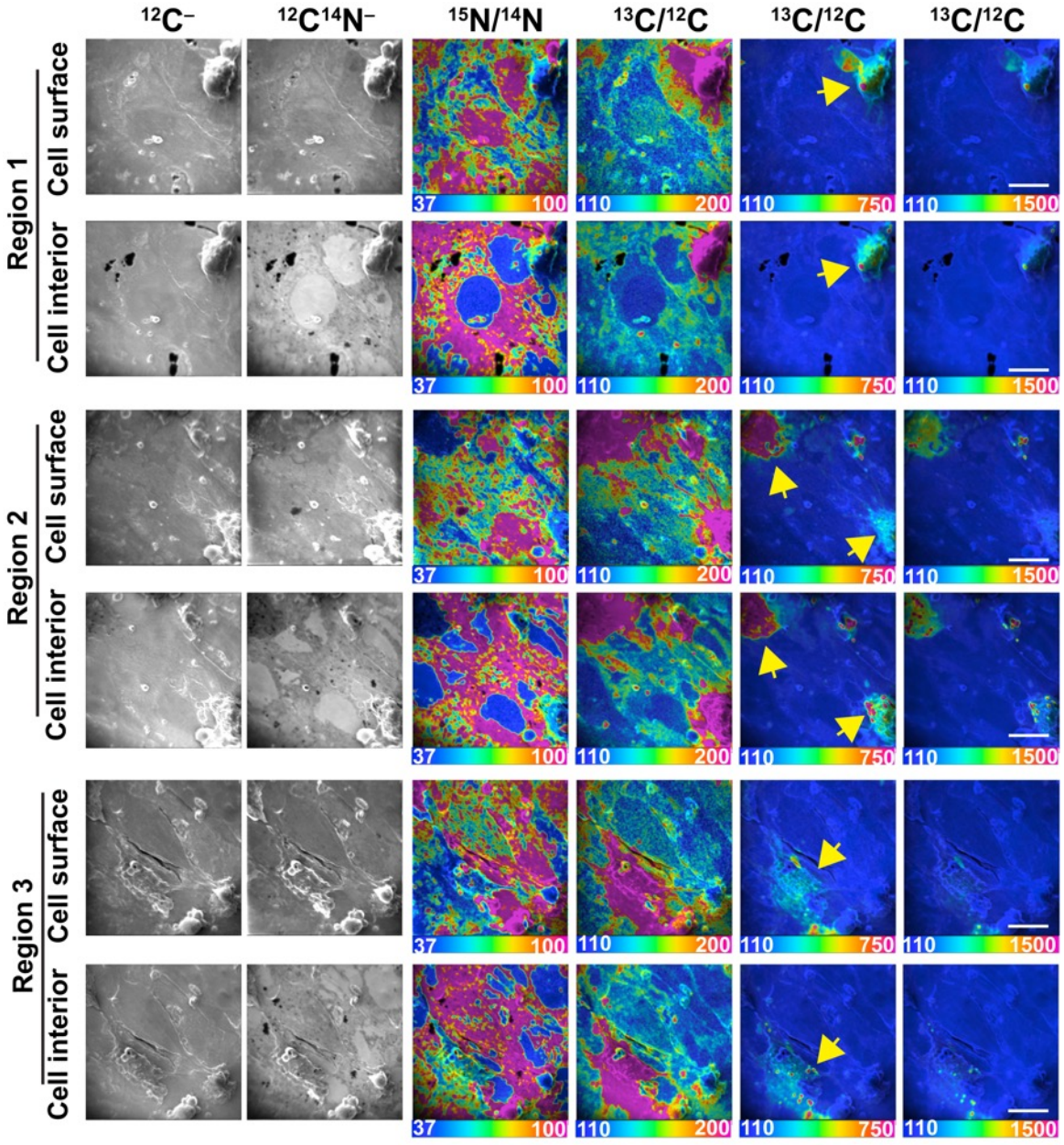

Fig. S10. Transfer of [^{13}C]cholesterol from methyl-β-cyclodextrin (MβCD)–treated [^{13}C]cholesterol-loaded macrophages to adjacent smooth muscle cells (SMCs). [^{13}C]cholesterol-loaded macrophages were plated onto a monolayer SMCs (~90–95% confluent) that had been grown in medium containing [^{15}N]choline for 21 days. Shown here are NanoSIMS images of three fields of [^{13}C]cholesterol-loaded macrophages that had been pre-treated with MβCD for 30 min before they were plated onto the SMC monolayer. Images of the cell surface and cell interior are shown. $^{12}C^-$, $^{12}C^{14}N^-$, $^{13}C/^{12}C$ (three different scales), and $^{15}N/^{14}N$ NanoSIMS images depict the transfer of [^{13}C]cholesterol from macrophages (yellow arrows) to SMCs. Ratio scales were multiplied by 10,000. Scale bar, 10 μm.

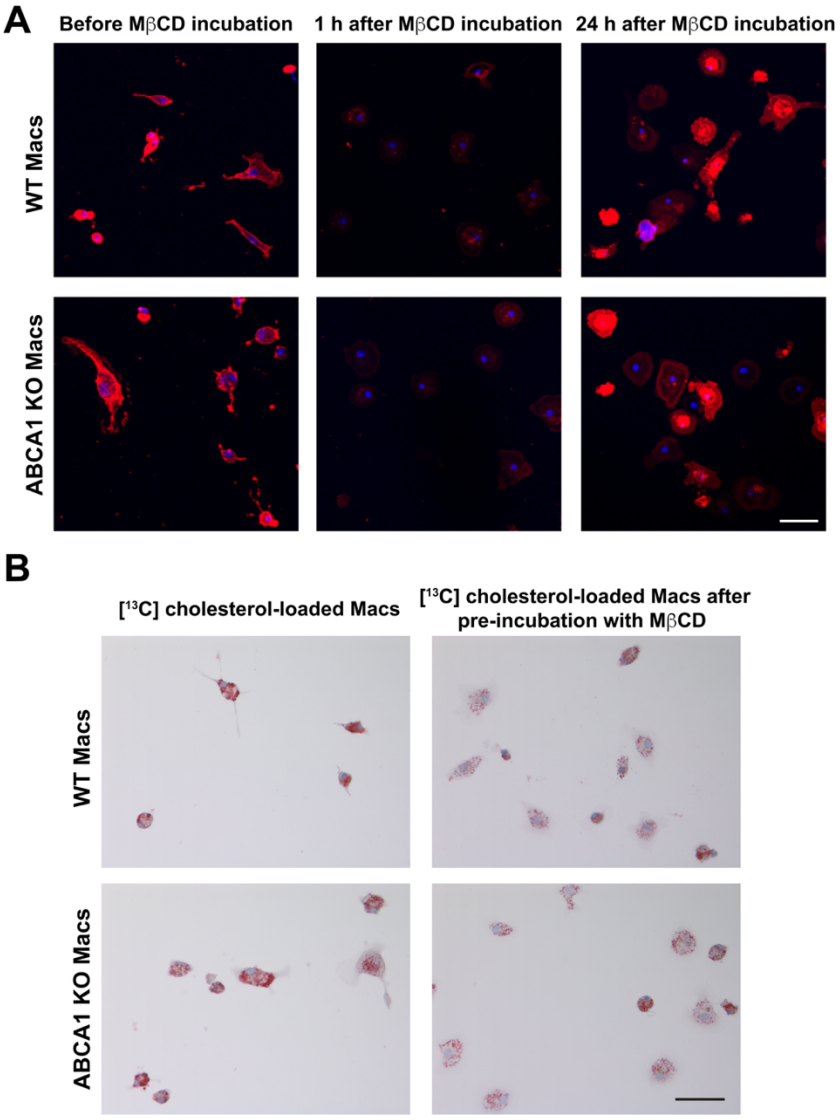

Fig. S11. Depletion of "accessible cholesterol" from the plasma membrane of [^{13}C]cholesterol-loaded wild-type or ABCA1 knockout (KO) macrophages by incubating the cells with methyl-β-cyclodextrin (MβCD). [^{13}C]Cholesterol-loaded macrophages were incubated with 10 mM MβCD for 30 min, and the binding of Alexa 594-labeled ALO-D4 (red) to the cells was assessed 1 h and 24 h later. (A) Fluorescence microscopy of ALO-D4 binding to macrophages before and after the MβCD treatment. In the absence of MβCD, the binding of ALO-D4 (red) to macrophages was robust, but there was negligible binding of ALO-D4 1 h after MβCD treatment. After 24 h, ALO-D4 binding returned to baseline levels. Cell nuclei were stained with DAPI (blue). Photomicrographs were obtained with identical settings. (B) Oil red O–stained wild-type and ABCA1 KO [^{13}C]cholesterol-loaded macrophages at baseline and 1 h after a 30-min incubation with 10 mM MβCD. Cell nuclei were lightly stained with hematoxylin (blue). Scale bars, 50 μm.

Table S1. Transfer of [^{13}C]cholesterol from [^{13}C]cholesterol-loaded mouse peritoneal macrophages to smooth muscle cells (SMCs).[1]

Figures	Genotype of Macrophages	Pretreatment with MβCD[2]	Percent ^{13}C ions in SMCs (mean ± SD)[3]	Number of Macrophages
Fig. 2A & Fig. S3	WT	–	11.7 ± 1.8	25
Fig. S8	WT	+	8.1 ± 2.2	22
Fig. S9	WT	–	8.9 ± 2.9	6
Fig. S10	WT	+	12.9 ± 3.2	11
Fig. 6	WT	+	7.2 ± 3.9	9
Fig. 6	ABCA1 KO	+	5.9 ± 2.4	8

[1] This table summarizes data from six [^{13}C]cholesterol coculture transfer experiments, showing the percentage of $^{13}C^-$ secondary ions (above natural abundance and normalized to ^{12}C ions) in SMCs relative to $^{13}C^-$ secondary ions (above natural abundance and normalized to ^{12}C secondary ions) in the [^{13}C]cholesterol-loaded macrophages. In each experiment, [^{13}C]cholesterol-loaded macrophages were plated on a SMC monolayer and incubated overnight. NanoSIMS images from each experiment are found in the indicated figures. Quantitative analyses of NanoSIMS data made it possible to quantify transfer of [^{13}C]cholesterol from [^{13}C]cholesterol-loaded mouse peritoneal macrophages to adjacent SMCs.

[2] In some experiments, [^{13}C]cholesterol-loaded macrophages were pre-treated with MβCD (to deplete "accessible cholesterol" from the plasma membrane) before they were plated onto the SMC monolayer.

[3] Number of ^{13}C secondary ions (above natural abundance and normalized to ^{12}C secondary ions) in SMCs adjacent to [^{13}C]cholesterol-loaded macrophages, expressed as a percentage of ^{13}C secondary ions (above natural abundance and normalized to ^{12}C secondary ions) in the [^{13}C]cholesterol-loaded macrophages.