SUPPORTING INFORMATION

MODULATING LIPOPROTEIN TRANSCELLULAR TRANSPORT AND
ATHEROSCLEROTIC PLAQUE FORMATION IN APOE\textsuperscript{-/-} MICE
VIA NANOFORMULATED LIPID-METHOTREXATE CONJUGATES

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**SUPPLEMENTARY RESULTS**

**DSPE-MTX prodrug synthesis.** Considering the poor solubility of the Methotrexate (MTX), a prodrug with amphiphilic properties was generated by binding MTX to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-amino (DSPE-NH2). In order to generate the prodrug, MTX was pre-activated *in loco* with a mixture of DCC and NHS, then conjugated with DSPE-NH2. Compound purification was achieved by precipitation in cold diethyl ether.

![Synthesis of prodrug](image)

**Figure S1.** Synthesis of prodrug.
MTX-loaded nanoparticles stability under slightly acidic conditions

**Figure S2.** Particle stability at pH=6.5.
**MTX-SPN optimization. Optimization of SPN.** To find the optimal formulation, MTX-SPNs were produced using different amounts of PLGA and DPPC and using PLGA with different molecular weights (25-35KDa or 38-53KDa) as reported in Table S1, Figure S3. To find the optimal formulation, MTX-SPNs were produced using different amounts of PLGA and DPPC and using PLGA with different molecular weights (25-35KDa or 38-53KDa). All the formulations presented average sizes ranging from 200 to 225 nm and showed a negative $\zeta$-potential ranging from -41 to -54 mV. With the increase of PLGA amount a relative increase on the nanoparticles size was retrieved. The negative charge is related to DSPE-PEG-COOH carboxylic groups exposed on SPNs surface. As shown in Figure S3A and B the amount of PLGA also affected DSPE-MTX encapsulation efficiency (EE%) which increases proportionally to PLGA amount, passing from about 0.2% to 0.5 % for the lowest molecular weight PLGA (25-35KDa) and from 0.4% to 1.5% for the highest (38-53kDa). EE% was also affected by the presence of DPPC, by removing DPPC the amount of internalized prodrug increased. This finding is probably related to a competition between DSPE-MTX and DPPC on the stabilization of the PLGA during the addition to the aqueous phase on the preparation of the nanoparticles. Basing on these considerations, SPNs 12 formulation was selected (Table S1, Figure S3). Starting from now on, we will refer to SPNs12 as MTX-SPNs.
Table S1: Optimization of SPNs. The SPNs were made in triplicate (n=3) for each single experiment.

| Sample | PLGA (23-35 kDa) (mg) | PLGA (38-53 kDa) (mg) | DPPC (mg) | DSPE-MTX (mg) | DSPE-PEG (mg) | Size (nm) | Pdl | Z Pot (mV) | Mass of DSPE-MTX (µg) | EE(%) |
|--------|-----------------------|-----------------------|-----------|---------------|---------------|-----------|-----|------------|------------------------|-------|
| SPN 1  | 0.5                   | -                     | 0.09      | 0.2           | 0.11          | 290±12    | 0.12±0.02 | -52±2.3     | 0.25±0.025             | 0.13±0.91 |
| SPN 2  | 0.5                   | -                     | -         | 0.2           | 0.11          | 230±13    | 0.11±0.02 | -49±15      | 0.20±0.06              | 0.18±0.93 |
| SPN 3  | 1                     | -                     | 0.09      | 0.2           | 0.11          | 291±9     | 0.13±0.03 | -54±11      | 0.36±0.06              | 0.18±0.94 |
| SPN 4  | 1                     | -                     | -         | 0.2           | 0.11          | 210±12    | 0.18±0.02 | -44±15      | 0.52±0.02              | 0.26±0.91 |
| SPN 5  | 2                     | -                     | 0.09      | 0.2           | 0.11          | 223±14    | 0.18±0.03 | -51±12      | 0.58±0.16              | 0.25±0.38 |
| SPN 6  | 2                     | -                     | -         | 0.2           | 0.11          | 215±6     | 0.18±0.02 | -48±13.6    | 0.70±0.09              | 0.38±0.05 |
| SPN 7  | -                     | 0.5                   | 0.09      | 0.2           | 0.11          | 200±9     | 0.11±0.02 | -46.5±0.5   | 0.26±0.06              | 0.13±0.93 |
| SPN 8  | -                     | 0.5                   | -         | 0.2           | 0.11          | 202±10    | 0.13±0.03 | -41±1.5     | 0.41±0.04              | 0.21±0.02 |
| SPN 9  | -                     | 1                     | 0.09      | 0.2           | 0.11          | 215±6     | 0.18±0.02 | -44±1.5     | 0.52±0.02              | 0.26±0.91 |
| SPN 10 | -                     | 1                     | -         | 0.2           | 0.11          | 200±7     | 0.16±0.03 | -46±1.2     | 0.87±0.2               | 0.43±0.1  |
| SPN 11 | -                     | 2                     | 0.09      | 0.2           | 0.11          | 218±11    | 0.19±0.02 | -48±1.6     | 0.70±0.09              | 0.38±0.05 |
| SPN 12 | -                     | 2                     | -         | 0.2           | 0.11          | 208±12    | 0.15±0.02 | -45±0.02    | 3±0.34                 | 1.5±0.17  |

Figure S3: A. and B. Different formulations with different amounts of PLGA and DPPC: EE% of DSPE-MTX.
Oxidized Low Density Lipoproteins (oxLDL)

**Figure S4.** TEM images of LDL molecules (left), oxLDL molecules (center), Dil-oxLDL (right) (scale bar = 50 μm).

**Figure S5.** Incubation of oxLDL with macrophages (RAW 264.7). A. 0 µg/ml oxLDL; B. 20 µg/ml oxLDL; C. 50 µg/ml oxLDL; D. 80 µg/ml oxLDL (scale bar = 25 μm).

**Intracellular localization of oxLDL.** To observe oxLDL intracellular distribution at confocal microscopy oxLDL were stained using DiI; for this experiment living RAW 264.7 were treated with 488-lysotracker to highlight lysosomes and with a concentration of Dil-oxLDL equivalent to 50 µg/ml of oxLDL. As it is possible to appreciate the green signal from lysosomes and the red signal from oxLDL co-localize almost completely, proving oxLDL accumulation mainly occurs into lysosomes (**Figure S6A**). Control cells were treated only with DiI (instead of DiI-oxLDL) following the same conditions, as it is possible to observe in **Figure S6B**. No co-localization at lysosomes was observed. Red signal coming from DiI was confined to plasma membrane. It is also important to underline that in cells treated with Dil-oxLDL no fluorescent signal was retrieved at plasma membrane thus proving the absence of any leakage of DiI from fluorescent oxLDL.
Figure S6. Raw 264.7 cells treated with A. Dil-oxLDL and B. Free Dil. In red Dil and Dil-oxLDL, in green Lysotracker and in blue DAPI.

Figure S7 TEM images of macrophages. A. RAW 264.7 cells not exposed to oxLDL show dark lysosomal structures. B. RAW 264.7 cells exposed to oxLDL (overnight, 50 μg/ml) show light lysosomal structures due to the high lipid content.
Stable association of Dil and oxLDL molecules. To furtherly demonstrate the stability of oxLDL and Dil a time-lapse microscopy experiment was performed (Movie S1 and S2). Following the same condition living BMDM were incubated overnight with Dil-oxLDL or with Dil. 14 hours after treatment cells were observed at the microscope and movies (Movie S1) were recorded. In Dil-oxLDL treated cells it is possible to note particulate movement inside the cell referring to Dil-oxLDL trafficking; in free Dil treated cells, Movie S2, instead the fluorescent signal is revealed from firm spots most likely located in plasma membrane.

Figure S8 A. DLS analysis of Cy5-LIP and Cy5-SPN. B. Release study of Cy5 from LIP and SPN.
Expression levels for the genes regulating the cholesterol transport

Figure S9 A-D. Foam cells markers expression level (ABCA1, CD36, and SR-A1) in foam cells (treated for 8 and 24 hours with the free and empty/loaded drug in LIP and SPN). Results are expressed as average ± SD (n = 5). ***p < 0.0001; **p < 0.001; *p < 0.01
Expression levels for the genes associated with the anti-inflammatory cytokines

Figure S10 A-D. Pro-inflammatory cytokines expression levels (TNF-α, IL-1β, and IL-6) in foam cells (treated for 8 and 24 hours with the free and empty/loaded drug in LIP and SPN). Results are expressed as average ± SD (n = 5). ***p < 0.0001; **p < 0.001; *p < 0.01
Figure S11 A-E. Viability of BMDM incubated with MTX, DSPE-MTX, empty SPN, MTX-SPN and empty LIP at 3 different time points.
**Table S2** Luminex results.

| Cytokine (pg/ml) | LIP     | MTX-LIP  |
|------------------|---------|----------|
| IL-1α            | 7.7±1   | 11.1±1.2 |
| IL-1β            | -       | -        |
| IL-2             | -       | -        |
| IL-3             | 7.3±0.67| 8.13±0.67|
| IL-4             | 2.6±0.2 | 2.4±0.3  |
| IL-5             | 7±0.8   | 10.1±1.1 |
| IL-6             | 11.7±3  | 13.6±3.75|
| IL-9             | 41.3±6.3| 40.6±3.3 |
| IL-12p40         | 970.8±68| 930.5±72 |
| IL-12p70         | 2263±98 | 2541.9±  |
| IL-13            | 82.2±13 | 89±8.2   |
| IL-17A           | 189.9±28| 227.4±18.5|
| Eotaxin          | 2084.3±210| 1866.3±160|
| G-CSF            | 308.6±85| 419.6±129.5|
| GM-CSF           | -       | -        |
| IFN-Y            | 43.8±3.4| 43.8±4.9 |
| KC               | 58±8.9  | 53.3±10  |
| MCP-1            | 257.8±31.6| 265.5±26.6|
| MIP-1α           | 6.2±0.2 | 6.29±0.4 |
| MIP-1β           | 25.8±1.7| 25.2±1.36|
| RANTES           | 68.6±8.44| 51.84±6.6 |
| TNF-α            | 1386.2±60.7| 1493±108 |
**Figure S12** Representative Bio-distribution confocal imaging after injection of Cy5-liposomes (red signal) in a one-year-old female apoE<sup>−/−</sup> mouse. Liver (A), and kidney (B), aortic arch: initial portion (C), middle portion (D) and aortic sinus (C). In C and D α-smooth muscle actin (α-SMA) was stained by immunofluorescence (green signal), in all of the images cell nuclei were stained using DAPI (blue signal). Insets: M= Media; P= Plaque.

**Figure S13** Bio-distribution study. A. DLS analysis of $^{64}$Cu-LIP. B. Release study of $^{64}$Cu from LIP. C. Quantitative assessment of radioactivity distribution in selected tissues 24 h after injection (n = 5).

**SUPPLEMENTARY MOVIES**

Movie S1: Trafficking of Dil-oxLDL.

Foam: BMDM + Dil-oxLDL for 14h
Movie S2: Cellular membrane staining.

BMDM + Dil for 14h