Supporting Information

Fluorescent labeled IL-10 : SDS-PAGE and western blot analysis

The labeling of IL-10 with the fluorophore Atto655 was performed as recommended by the supplier (www.atto-tec.com). The dye was applied in a molar ratio of 2 : 1 corresponding to the molecular weight of the IL-10 monomer (~19 kD).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques were used for the detection of Atto655 labeled IL-10. All additional reagents were purchased from Bio-Rad Laboratories, München, Germany. Labeled and native IL-10 were diluted with double-distilled water and mixed in a ratio of 1:1 with Laemmli sample buffer. The protein was then applied on a 15% Tris-HCl precast gel. A broad range pre-stained SDS-PAGE standard was used for the size mapping of IL-10. After fluorescence imaging of the labeled protein excited at λ_{flu}= 750 nm (Maestro In Vivo Imaging System™, CRi, Woburn, MA), the gel was control stained with Bio-Safe™Coomassie G250 stain.

Western blotting was used for the examination of possible steric changes in IL-10 due to the fluorescence-labeling process. Samples from unstained precast gels were blotted to polyvinylidenedifluoride membranes. Immunoblots were incubated with the primary monoclonal rabbit anti-human IL-10 antibody (Epitomics, U.S.A.), detected using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Thermo Scientific, Germany) and visualized by the chemoluminescence reaction with the SuperSignal® West Femto maximum sensitivity substrate (all from Pierce Protein Research Products, Rockford, IL).

The results are summarized in Figure S1. Although the protein-dye complex tended to precipitate during dialysis, the labeling process was overall successful. During the reaction a minor amount of the IL-10 monomer formed dimers (the *native* state of IL-10) and higher molecular weight fragments in traces, which also bound the fluorescence marker. *ß*-mercaptoethanol and heat denaturation reduced the higher fragments and the dimer into the monomeric form.

Calculation of the number of lipid and IL-10 molecules per liposome

By knowing the average diameter size of extruded unilamellar IL-10-liposomes and by applying the general equation of the surface area of a sphere (1), we calculated the number of

lipid molecules per liposome (2), and consecutively the number of IL-10 molecules attached to each liposome, as follows:

$$A = 4\pi\left(\frac{d}{2}\right)^2 \qquad (1)$$

where A corresponds to the surface area of the liposome and d to the diameter

$$N_{tot} = \frac{4\pi\left(\frac{d}{2}\right)^2 + 4\pi\left(\frac{d}{2} - h\right)^2}{a} \qquad (2)$$

where N_{tot} corresponds to the total number of lipid molecules in one liposome; h to the thickness of the bilayer of about 5 nm; $d/2 - h$ to the inner radius of the liposome and a the head group area of the phospholipid molecules, which is about 0.65 nm² per molecule for phosphatidylcholines[1]. According to dynamic light scattering measurements, the average diameter size *(d)* of the extruded, IL-10-coated liposomes is about 184.5 nm ± 1.6. Knowing both, the phospholipid concentration and the final volume of the solution, and by applying the equation (3), we found that the number of liposomes/ml was equivalent to 1.75E16:

$$N_{lipo} = \frac{M_{lipid} \, \mathrm{X} \, N_A}{N_{tot} \, \mathrm{X} \, 1000} \qquad (3)$$

where N_A corresponds to the Avogadro number equal to 6.022E23; M_{lipid} to the molar concentration of lipid and N_{tot} to the number of lipids in one liposome.

Additionally, as determined by Starcher assay [2], the final concentration of IL-10 was 50.7 µg/mL. Using this value the number of molecules of IL-10 (Mw ~ 19kDa) was calculated. We first estimated the number of IL-10 moles (mole = g/MW) and multiplied them for N_A. The so obtained IL-10 molecules were then divided by the number of liposomes, resulting in a theoretical final ratio of 92 IL-10 molecules per liposome.

Intradermal skin tests

Evans blue (100 µL of 5 mg/ml NaCl 0.9%; Merck, Darmstadt, Germany) was injected into the tail vein of immunized mice on day 70. Subsequently, 30 µL of the following substances were administered intradermally into the shaved abdominal skin: IL-10-liposomes or non-

targeted liposomes (both 200 µg lipids/ml PBS); IL-10 (200 µg/ml PBS); codfish extract (50 µg/ml PBS) as irrelevant control allergen, mast cell degranulation compound 48/80 (20 µg/ml PBS; Sigma, Steinheim, Germany) as positive control, and PBS as negative control. After 20 minutes, mice were sacrificed and the color reaction examined on the inside of the abdominal skin.

Isolation of splenocytes and evaluation of cytokines in stimulated spleen cells

Preparation of spleen cell suspensions and stimulation was performed as described previously [3]. For stimulation, medium as negative control, Con A (Sigma, Steinheim, Germany; 5 µg/ml) as positive control, non-targeted liposomes (40 µg/ml lipids) or IL-10-liposomes (40 µg/ml) were added for 72 hours.

Mouse IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, TNF-α and GM-CSF were measured in pooled supernatants of stimulated splenocytes by a multiplex immunoassay and analyzed on a flow cytometer (eBioscience, Vienna, Austria), according to manufacturer's instructions. Additionally, measurement of IL-13 was performed by ELISA with anti-mouse cytokine antibodies and standards (Bender MedSystems, Vienna, Austria), according to manufacturer's instructions.

Supplement Figures

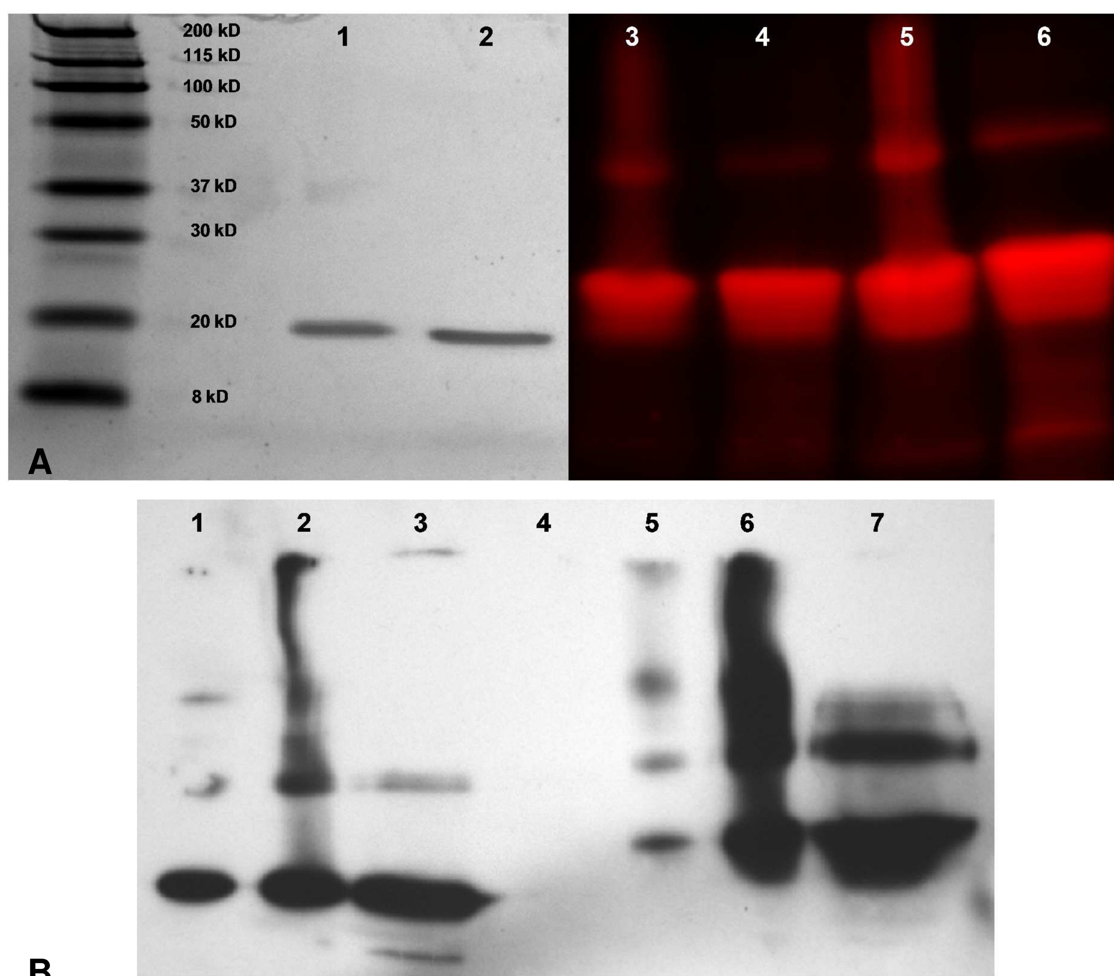
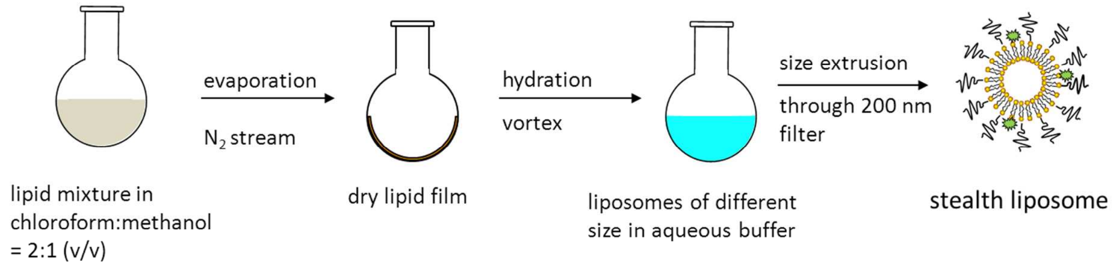


Figure S1. SDS-PAGE and Western Blot. Recombinant mouse IL-10 was Atto655 fluorescence labeled, applied on a SDS-PAGE and transferred to a WB Membran. **A** SDS-Page visualized by fluorescence imaging excited at λ_{flu}= 750 nm (right panel) and stained with Coomassie blue for the visualization of the *native* IL-10 (left panel). Lane **1**, 2 µg *native* IL-10; lane **2**, 2 µg *native* IL-10 + *ß*-mercaptoethanol at 96°C for 10 min; lane **3**, 2 µg IL-10-Atto655; lane **4**, 2 µg IL-10-Atto655 + *ß*-mercaptoethanol at 96°C for 10 min; lane **5**, 4 µg IL-10-Atto655; lane **6**, 4 µg IL-10-Atto655+ *ß*-mercaptoethanol at 96°C for 10 min. **B** WB of the membrane after the incubation with a primary anti-IL-10 Ab, a secondary HRP-conjugated Ab and the substrate reaction visualized by luminescence. Lane **1**, *native* IL-10 supernatant; lane **2**, *native* IL-10 resolved precipitate; lane **3**, *nativ*e IL-10 resolved precipitate + *ß*-mercaptoethanol at 96°C for 10 min; lane **4**, -; lane **5**, IL-10-Atto655 supernatant; lane **6**, IL-10-Atto655 resolved precipitate; lane **7**, IL-10-Atto655 resolved precipitate + *ß*-mercaptoethanol at 96°C for 10 min.



Lipid mixture :

POPC : 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine)

DSPE-PEG2000 : 1,2-distearoyl-sn -glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]

CH : cholesterol

DOPE-CF : 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (ammonium salt)

DSPE-PEG2000-NHS: 3-(N-succinimidyloxyglutaryl) aminopropyl, polyethylene glycol-200 carbamyldistearoylphosphatidyl-ethanolamine

Figure S2. Preparation of PEGylated stealth liposomes.

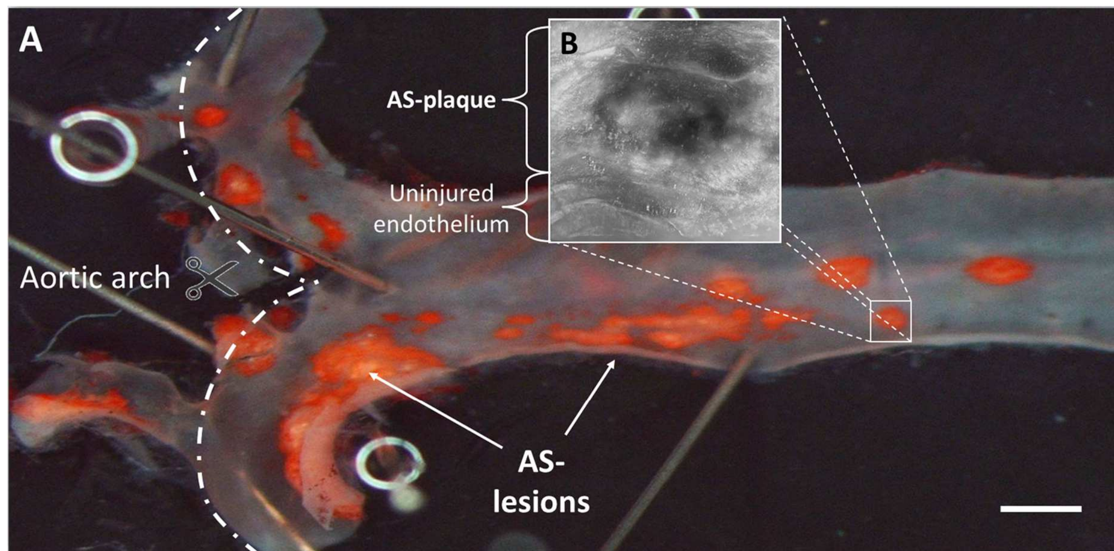


Figure S3. Orientation of the aortic specimens for CLSM imaging. A Dissected aorta and arch cut open and incubated with oilred-o solution to stain the lipid content of AS-lesions. **B** Optical sections from the inner aortic surface projected into one plane. A series of 25 transmitted light images in Z (1 µm consecutive intervals) were projected in a single image showing one single plaque and the surrounding area. Bar corresponds to 1 mm.

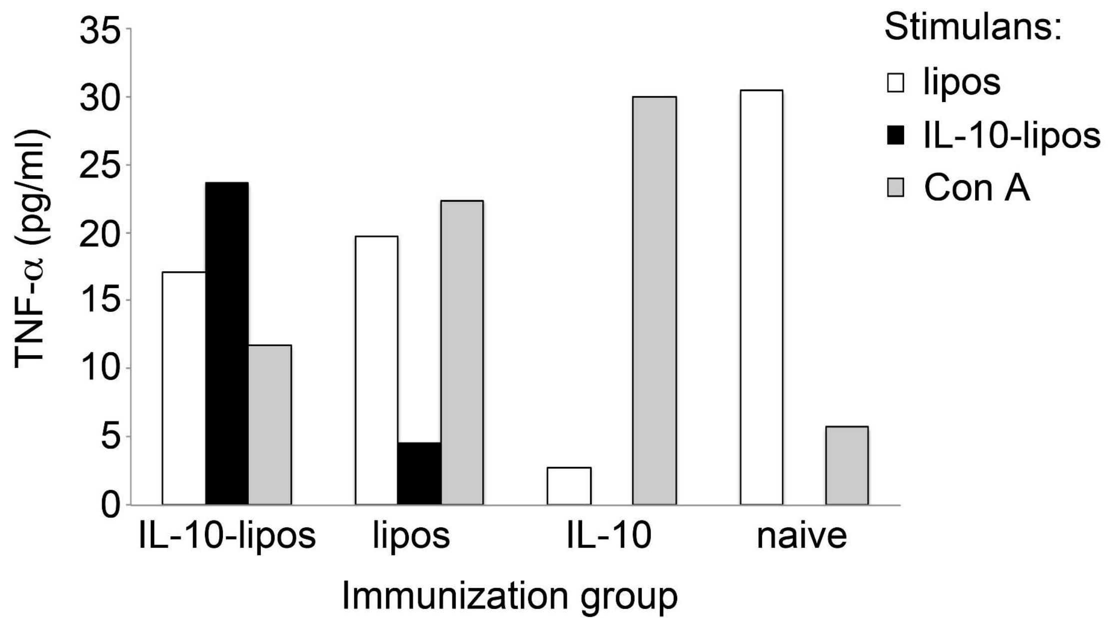


Figure S4. Cytokine levels in supernatants of stimulated splenocytes. Spleen cells of Balb/c mice immunized with IL-10-liposomes (IL-10-lipos) i.v., non-targeted liposomes (lipos) i.v, IL-10 i.v. or of naïve animals were incubated with ConA, medium, liposomes or IL-10-liposomes. TNF-α was elevated in splenocyte supernatants of mice immunized with IL-10-liposomes and liposomes i.v. when stimulated with liposomes or IL-10-liposomes, as well as in naïve animals when stimulated with liposomes. Due to usage of pooled supernatants no statistical analysis could be performed. Values of medium stimulation are subtracted.

Supplement References

1. Lewis, B. A.; Engelman, D. M. Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles. *J Mol Biol* **1983,** *166*, (2), 211-7.
2. Starcher, B. A ninhydrin-based assay to quantitate the total protein content of tissue samples. *Anal Biochem* **2001,** *292*, (1), 125-9.
3. Pali-Scholl, I.; Herzog, R.; Wallmann, J.; Szalai, K.; Brunner, R.; Lukschal, A.; Karagiannis, P.; Diesner, S. C.; Jensen-Jarolim, E. Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **2010,** *40*, (7), 1091-8.