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 $\lambda_{\text{flu}} = 750$ nm (Maestro In Vivo Imaging System™, CRi, Woburn, MA), the gel was control stained with Bio-Safe™ Coommassie 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. $\beta$-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:

\[
A = 4\pi \left( \frac{d}{2} \right)^2
\]

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

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

where \(N_{\text{tot}}\) 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. 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:

\[
N_{\text{lipo}} = \frac{M_{\text{lipid}} \times N_A}{N_{\text{tot}} \times 1000}
\]

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

Additionally, as determined by Starcher assay, 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. 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 $\lambda_{\text{flu}} = 750$ 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 + β-mercaptoethanol at 96°C for 10 min; lane 3, 2 µg IL-10-Atto655; lane 4, 2 µg IL-10-Atto655 + β-mercaptoethanol at 96°C for 10 min; lane 5, 4 µg IL-10-Atto655; lane 6, 4 µg IL-10-Atto655+ β-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, native IL-10 resolved precipitate + β-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 + β-mercaptoethanol at 96°C for 10 min.
**Figure S2. Preparation of PEGylated stealth liposomes.**

- **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-succinimidoyloxylutaryl) aminopropyl, polyethylene glycol-200 carbamyl/distearoylphosphatidyl-ethanolamine

**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

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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.