Methods and Material

Selective EGF-Receptor deletion in myeloid cells limits atherosclerosis

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Animals.

Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC), and were approved by the Ethical Committee of INSERM and the French Ministry of Agriculture (agreement no. A75-15-32). To generate a cell-specific knockout of Egfr in myeloid cells, we crossbred mice carrying a LysM Cre allele with mice carrying a floxed Egfr allele. All animals have been backcrossed more than ten generations on C57bl/6 background. For bone marrow transplantation experiments, 10-week old male C57bl/6 Ldlr<sup>−/−</sup> mice were subjected to medullar aplasia by lethal total body irradiation (9.5 gray). The mice were repopulated with an intravenous injection of bone marrow cells isolated from femurs and tibias of sex-matched C57BL/6 LysM<sup>Cre-Egfr<sup>lox/lox</sup></sup> mice or LysM<sup>Cre<sup>+</sup>Egfr<sup>lox/lox</sup></sup> littermates. After 4 weeks of recovery, mice were fed a pro-atherogenic diet (15% fat, 1.25% cholesterol and 0% cholate) for 4, 7 and 12 weeks. C57bl/6 Cd36<sup>−/−</sup> mice were generated in Silverstein’s lab [1].

Extent and composition of atherosclerotic lesions.

Plasma cholesterol was measured using a commercial cholesterol kit (Biomerieux). Quantification of lesion size was performed as described earlier [2]. In brief, the basal half of the ventricles and the ascending aorta were perfusion-fixed in situ with 4% paraformaldehyde. Afterwards, they were removed, transferred to a PBS-30% sucrose solution, embedded in frozen OCT and stored at −70°C. Serial 10-μm sections of the aortic sinus with valves (80 per mouse) were cut on a cryostat. Of every 5 sections, one was kept for plaque size quantification after Oil red O staining. Thus, 16 sections spanning 800 μm stretch of the aortic root were used to determine mean lesion area for each mouse. Oil Red O positive lipid contents were quantified by a blinded operator using HistoLab software (Microvisions). Oil
Red O positive area was quantified by a blinded operator using HistoLab software (Microvisions). Necrotic core was quantified after Masson’s Trichrome staining. The presence of macrophages was studied using specific antibodies, as previously described [3]. At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used. Morphometric studies were performed using Histolab software (Microvisions)[3]. For immunostaining on mouse atherosclerotic plaques, we used antibodies against MOMA-2 (MAB1852, Merck Millipore®), anti-CD36 (MABF956, Merck Millipore®) and anti-EGF-R (D38B1, Cell Signaling®).

**Splenocyte culture.**

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mM β-mercaptoethanol and antibiotics. For cytokine measurements, splenocytes were stimulated with LPS (10 µg/ml) and IFN-γ (100 UI/ml) for 24 hours. Il-10, Il-12p70 and Tnf-α production in the supernatants was measured using specific ELISAs (R&D Systems).

**Macrophage experiments**

Primary macrophages were derived from mouse bone marrow cells (BDMDs). Tibias and femurs of C57BL/6 LysMCre- Egfr\textsuperscript{lox/lox} or LysMCre+ Egfr\textsuperscript{lox/lox} littermates male mice were dissected, their marrow flushed out. Cells were grown for 7 days at 37°C in RPMI 1640 medium, 20% neonatal calf serum, and 20% Macrophage–Colony-Stimulating Factor (M-CSF)-rich L929-conditioned medium. To analyze oxidized LDL uptake, BMDMs were exposed to human oXLDL (25 µg/ml) during 24 hours. Cells were washed, fixed and stained using Oil Red O. Foam cells were quantified blindly on 6-8 fields and the mean was recorded. To analyze macrophage phenotype, BMDMs were stimulated with Lps (10 µg/ml) and Ifn-γ (100 UI/ml) for 24 hours. Il-10, Il-12p70 and Tnf-α production in the supernatant was measured using specific ELISAs (R&D Systems).
Flow cytometry

Blood samples were collected at the sacrifice for analysis of leukocyte subsets. Myeloid cells were identified as CD11b+. Monocytes were identified as CD11b+Ly6G-. Among them, classical monocytes were Gr1high (or Ly6Chigh) and non-classical monocytes were Gr1low (or Ly6Clow). Neutrophils were identified as CD11b+Ly6G+. CD4+ and CD8+ T lymphocyte subsets were also analyzed. Stainings included the following antibodies: PerCP-conjugated anti-CD45 (30-F11, Biolegend), FITC-conjugated anti-CD11b (M1/70, BD Biosciences), Anti-Gr1 (Ly6C and G)-PERCP-Cy5 (RB6-8C5, BD Biosciences), anti-F4/80-PE (BM8, eBioscience) anti-CD4-V-450 (RM4-5, eBioscience) anti-CD3ε-APC (145-2C11, eBioscience) anti-B220-V-500 (RA3-6B2, BD Biosciences) anti-CD8a-AF-700 (53-6.7,BD Biosciences) anti-MHCII-FITC (M5/114.15.2,eBioscience) anti-CD19 AF-700 (6D5, BD Biosciences) and anti-CD36 (MABF956, Merck Millipore®).

Forward scatter (FSC) and side scatter (SSC) were used to gate live cell excluding red blood cells, debris, and cell aggregates in total blood cells and splenocytes. Cells were acquired using a BD LSRII Fortessa flow cytometer (BD Biosciences, San Jose California, USA) and analyzed with FlowJo™ (TreeStar, Inc.).

Quantitative real-time PCR.

Quantitative real-time PCR was performed, after Trizol RNA extraction and RT-PCR, on an ABI Prism 7700 (Applied Biosystems™) in duplicate for each sample. Expression of all genes has been normalised using Gapdh gene. The following primer sequences were used: *Il-10* (F:5’ –AAG TGA TGC CCC AGG CA- 3’; R:5’ –TCT CAC CCA GGG AAT TCA AA-3’), *Tnf-α* (F:5’- GAT GGG GGG CTT CCA GAA CT-3’; R:5’ –CGT GGG CTA CAG GCT TGT CAC-3’), *Il-12a* (F:5’- TCA CAC GGG ACC AAA CCA GCA CAT -T-3’; R:5’ –TGT GGG GGC AGG CAG CTC CCT CT -3’), *Msr1* (F:5’- TGG ATG CAA TCT CCA
AGT CCT-3'; R: (5'- ACG TGC CTT GTT CTT CTT T-3'), \textit{Scarbl} (F: 5'- GCT CCG GAA CAA GGC AAA TA – 3'; R: 5'- GGC CAG ATC CAC GAC AGT 6 – 3'), \textit{Cd36} (F: 5'– TGG CCA AGC TAT TGC GAC ATG ATT A-3'; R: 5' – CGG GGA TTC CTT TAA GGT CGA TTT-C-3'),

\textbf{Statistical analysis.} Values are expressed as mean ± s.e.m. Differences between values were examined using the non-parametric Mann-Whitney test and were considered significant at P<0.05. For multiple group comparisons, we used a Kruskal-Wallis test and post-hoc analysis (Dunn). All these analysis were performed using GraphPad Prism version 5.0b for Mac (GraphPad Software).

\textbf{References}