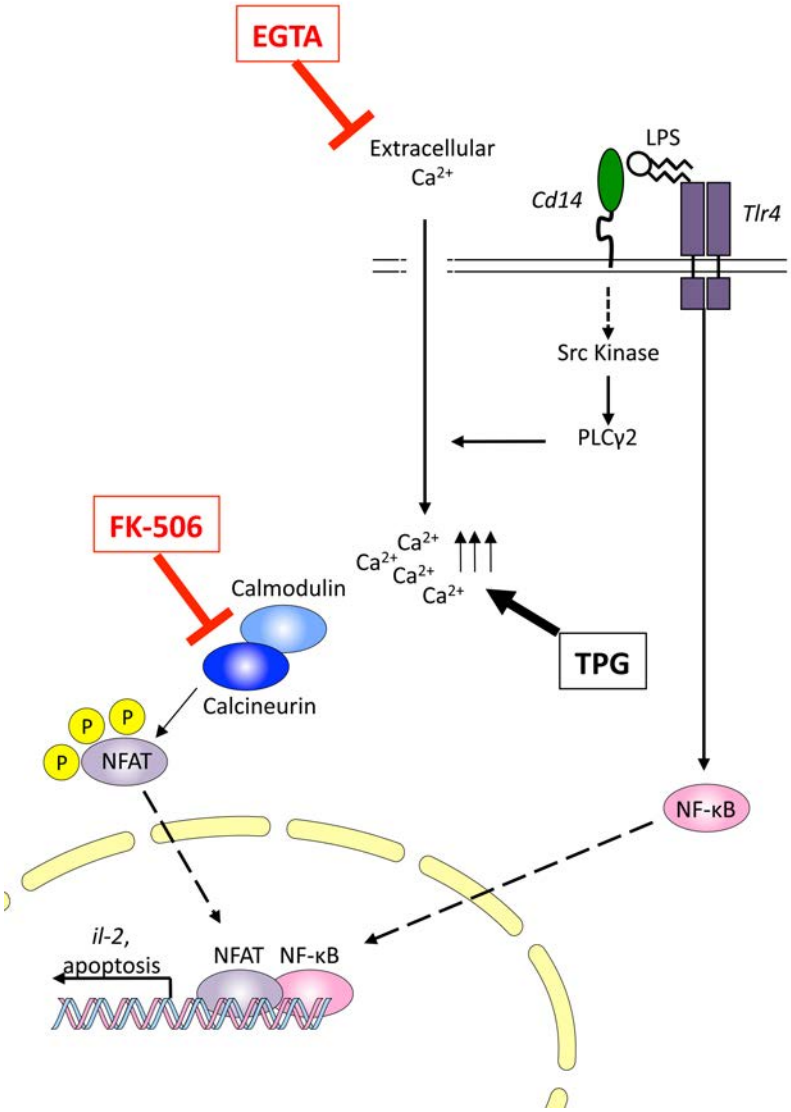
Supplementary information to: "Mechanism of lipopolysaccharide-induced skin edema formation in the mouse"

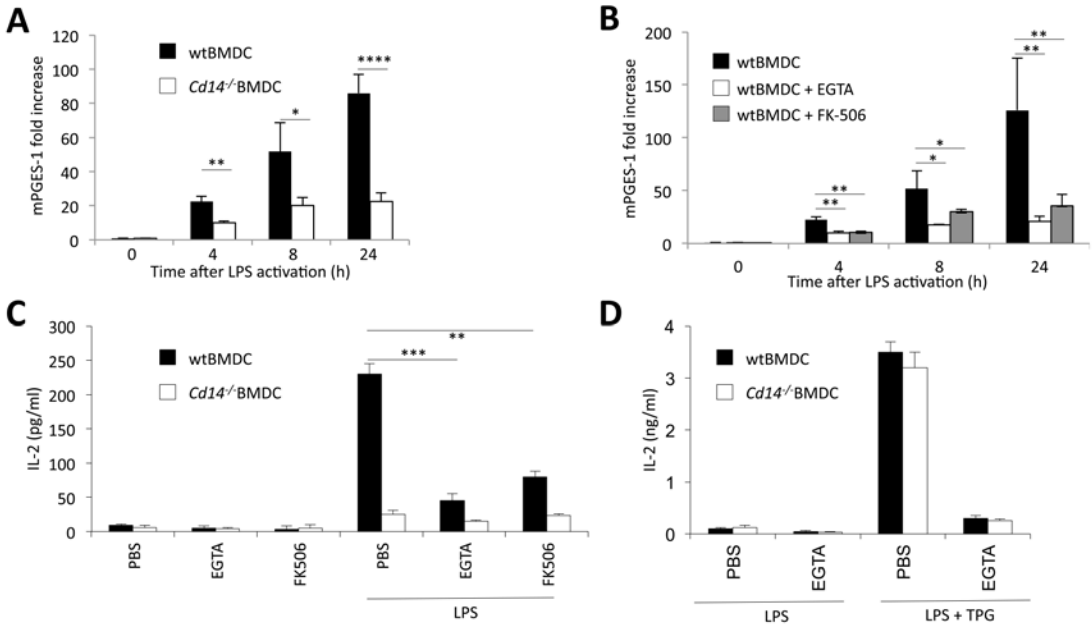
Ivan Zanoni*, Renato Ostuni*, Simona Barresi, Marco Di Gioia, Achille Broggi, Barbara Costa, Roberta Marzi and Francesca Granucci

Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy

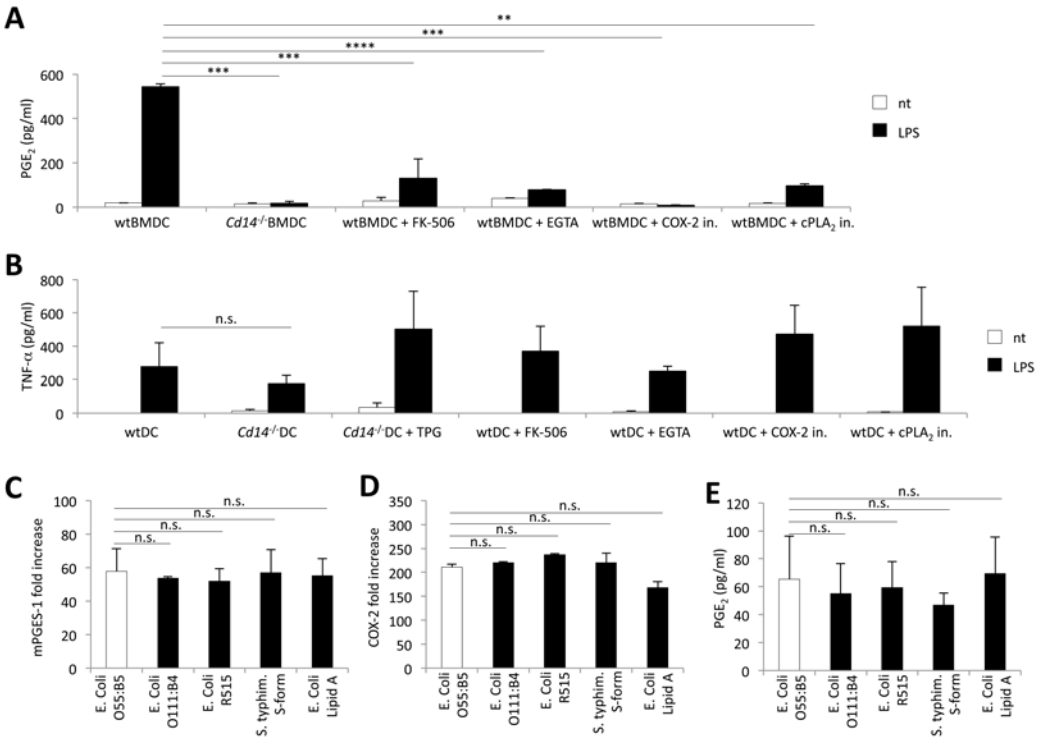
* These authors contributed equally to the work



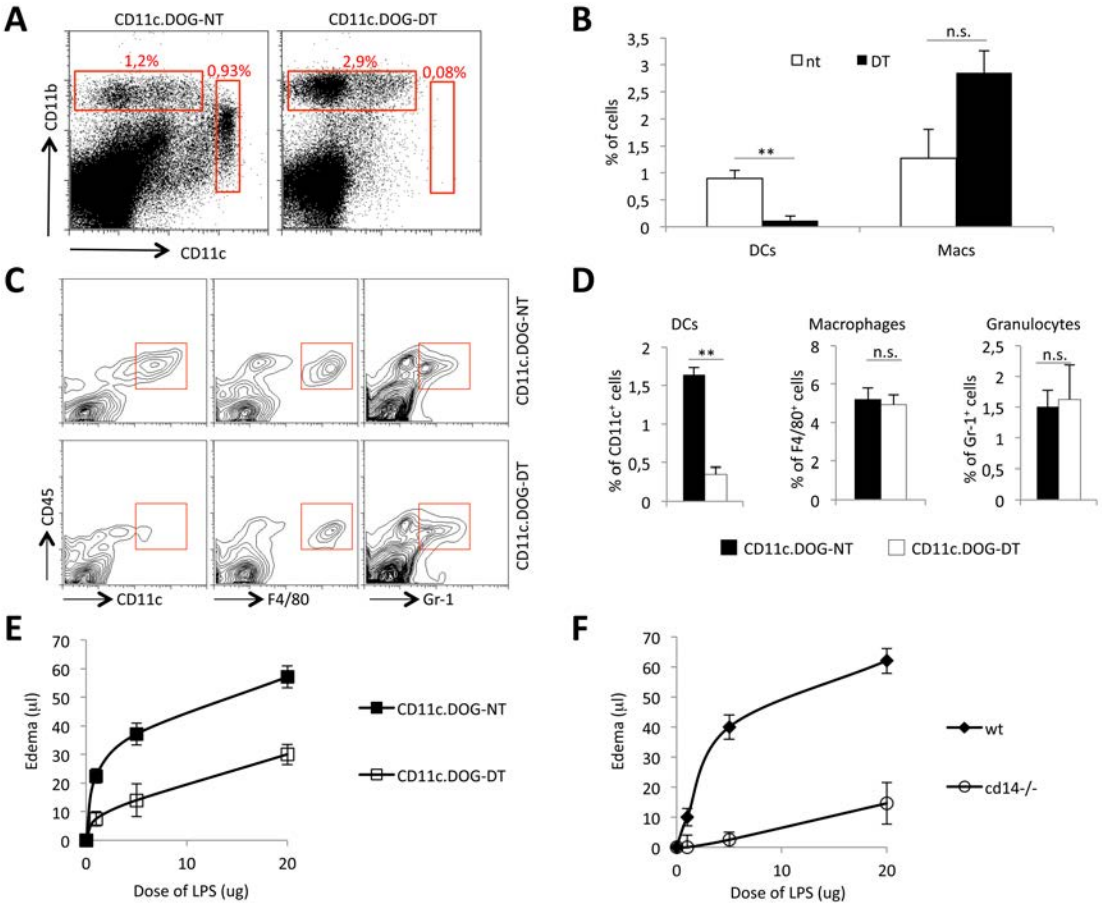
Supplementary Figure 1. CD14-dependent and TLR4-independent NFAT activation in DCs. CD14 has autonomous signaling functions. Upon LPS engagement, CD14 transiently recruits and activates a Src family kinase (SKF) member. Active SFK then phosphorylates PLCγ2, which in turn catalyzes the hydrolysis of PI(4,5)P2 into the second messengers diacylglycerol (DAG) and IP3. IP3 directly triggers Ca^{2+} influx. The increased intracellular Ca^{2+} concentration stimulates activation of calcineurin, which dephosphorylates NFAT and promotes its nuclear translocation. EGTA and FK-506 are two inhibitors of the NFAT pathway. EGTA blocks extracellular Ca^{2+} influxes and FK-506 inhibits calcineurin activation. Diversely, thapsigargin (TPG) is an activator of the NFAT pathway. By blocking the SERCA pumps induces an increase of intracellular Ca^{2+} concentration and therefore NFAT activation.



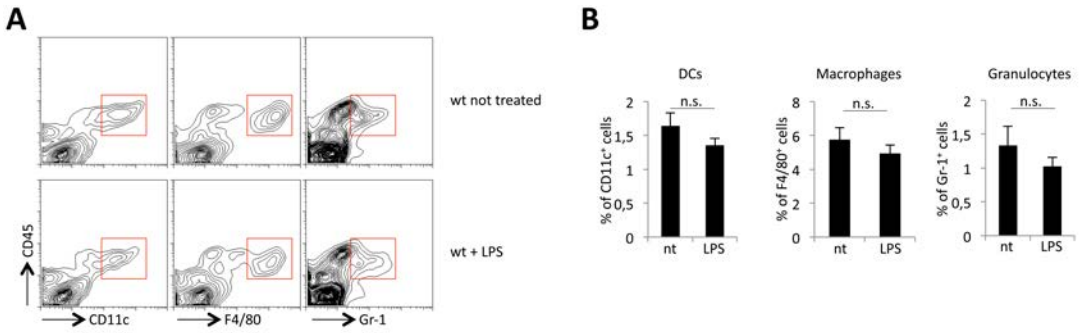
Supplementary Figure 2. mPGES-1 is a potential target of CD14/NFAT signaling in BMDCs. (A) Real-Time PCR analysis of mPGES-1 mRNA induction kinetics in wt and CD14-deficient BMDCs stimulated with LPS (1 μg/ml). (B) Real-Time PCR analysis of mPGES-1 mRNA up-regulation after LPS (1 μg/ml) administration in wt BMDCs pre-treated with PBS, FK-506 (1 μM, 90 min) or EGTA (2 mM, 30 min) at the indicated time points. (C, D) Production of IL-2 by wt and *Cd14*$^{-/-}$ BMDCs in the indicated conditions; TPG, thapsigargin (50 nM). Values represent at least three independent experiments performed in duplicate + s.e.m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.



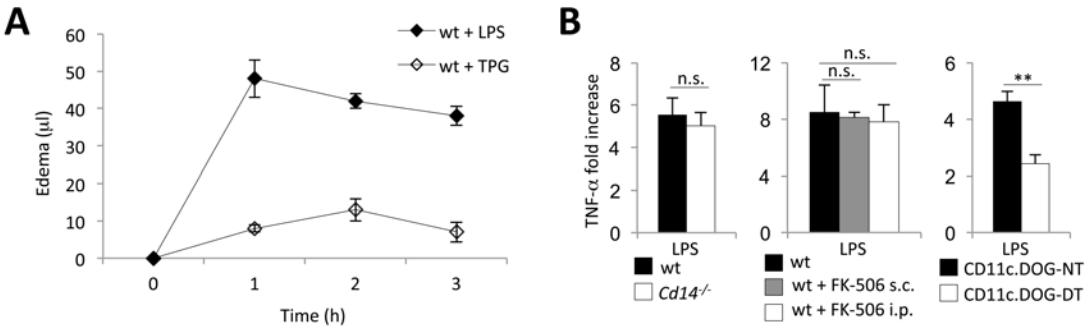
Supplementary Figure 3. PGE_2 production by BMDCs. (A) wt and *Cd14*$^{-/-}$ BMDCs were treated with LPS and PGE2 production measured in the supernatants four hours later. Where indicated wt BMDCs were pretreated with FK-506 (90 min, 1 μM), EGTA (30 min, 2 mM), COX-2 inhibitor (COX-2 in, 1 μM, 30 min) or $cPLA_2$ inhibitor ($cPLA_2$ in, 1 μM, 30 min). (B) TNF-α production by ex vivo wt or CD14-deficient DCs treated with LPS and the indicated stimuli/inhibitors; TPG, thapsigargin; COX-2 in, COX-2 inhibitor; $cPLA_2$ in, $cPLA_2$ inhibitor. Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$. (C, D, E) mPGES-1 and COX-2 mRNA upregulation and PGE_2 secretion induced by the indicated species of LPS in wt BMDCs.



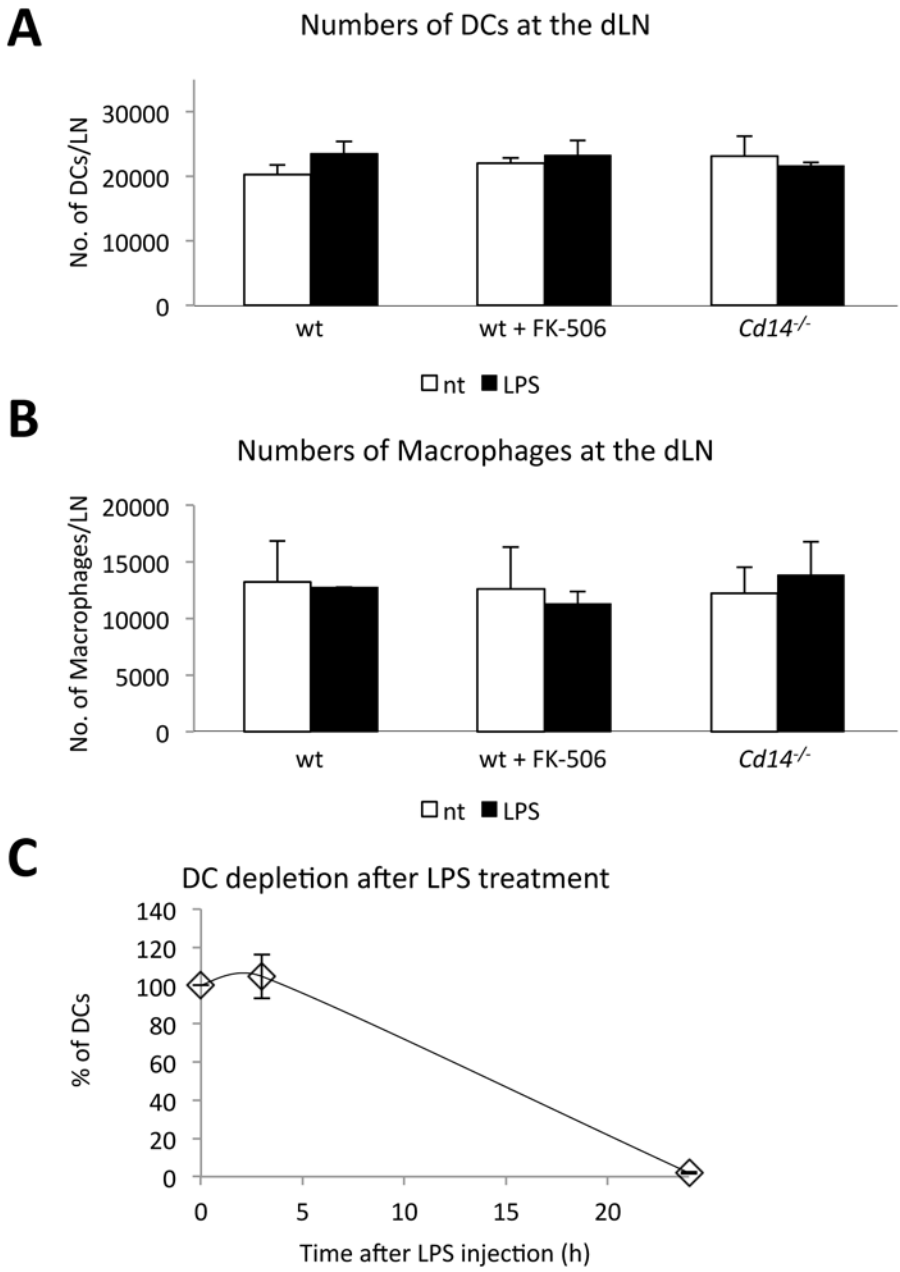
Supplementary Figure 4. DC depletion from the spleen and the skin of CD11c.DOG mice after DT treatment. (A) Representative dot plots of splenocytes from CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). $CD11c^{+}CD11b^{int}$ DC and $CD11b^{+}CD11c^{int}$ macrophages populations are shown. (B) Quantification and statistical analysis of the percent of DCs and macrophages in the spleen of CD11c.DOG mice before (nt) and after (DT) DT treatment. Data represent men and s.e.m. of 5 mice; ** $P < 0.005$. (C) Representative contour plots of $CD11c^{+}$ (DCs), $F4/80^{+}$ (macrophages) and $Gr\text{-}1^{+}$ (granulocytes) cells in the skin of CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). (D) Quantification and statistical analysis of the percent of DCs, macrophages and granulocytes in the skin of CD11c.DOG mice before (CD11c.DOG-NT) and after (CD11c.DOG-DT) DT treatment. ** $P < 0.005$. (E, F) Inflammatory footpad swelling induced by different doses of LPS three hours after treatment in (E) CD11c.DOG mice treated or not with DT and (F) wt and CD14-deficient mice. Data represent men and s.e.m. of 5 mice.



Supplementary Figure 5. LPS injection in the footpad does not induce early inflammatory cell recruitment in the skin. (A) Representative contour plots of $CD45^+CD11c^+$ (DCs), $CD45^+F4/80^+$ (macrophages) and $CD45^+Gr\text{-}1^+$ (granulocytes) skin cell populations before and 1 hour after LPS treatment. (B) Quantification and statistical analysis of the percent of DCs, macrophages and granulocytes in the skin of wt mice before (nt) and 1 hour after LPS treatment (LPS).



Supplementary Figure 6. (A) Inflammatory swelling induced by LPS or thapsigargin (TPG) alone. (B, left and middle panels) Real-Time PCR analysis of TNF-α mRNA induction in the footpad skin of wild type and *Cd14*$^{-/-}$ mice 2 hours after subcutaneous injection of LPS; where indicated wt mice were injected 18 hours before LPS administration with FK-506 sub-cute (s.c.) or intra-peritoneum (i.p.). (B, right panel) Real-Time PCR analysis of TNF-α mRNA induction by LPS in the footpad of CD11c.DOG mice treated (-DT) or not (-NT) with DT. Values represent at least two independent experiments (n=5) + s.e.m. ** $P < 0.005$, n.s. not significant.



Supplementary Figure 7. (A, B) Absolute numbers of DCs ($CD11c^{+}CD11b^{int}$) and macrophages ($F4/80^{+}$) in the draining lymph nodes of wt and CD14-deficient mice before (nt) and after LPS (three hours) treatment. Where indicated the mice were pretreated with FK-506 18 hours before LPS administration. (C) Percentage of $CD11c^{+}$ cells in draining lymph nodes after s.c LPS administration (20 μg) at the indicated time points. Data are representative of two independent experiments (four mice per group).