Supplementary Information

Pleiotropic action of CpG-ODN on endothelium and macrophages attenuates angiogenesis through distinct pathways

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Supplementary Tables

Table S1. List of CpG-ODNs

	Class	Backbone	Sequence (5'-3')
PS-CpG-ODN2216	A	PS	ggGGGACGA:TCGTCgggggg
PS-GpC-ODN2216	control	PS	ggGGGA**GC**A:T**GC**TGgggggg
PS-CpG-ODN1826	B	PS	TCCATgACgTTCCTgACgTT
PD-CpG-ODN1826	B	PD	TCCATgACgTTCCTgACgTT
PS-ApG-ODN1826	control	PS	TCCATgA**A**gTTCCTgA**A**gTT
PS-CpG-ODN2395	C	PS	TCgTCgTTTTCggCgCgCgCCg
PD-CpG-ODN2395	C	PD	TCgTCgTTTTCggCgCgCgCCg
PS-ApG-ODN2395	control	PS	T**A**gT**A**gTTTT**A**gg**A**g**A**g**A**g**AA**g

Table S2. List of sequences

Gene name	Sequences
mVegf	F 5'-AGCAGAAGTCCCATGAAGTGA-3'
	Rv 5'-ATGTCCACCAGGGTCTCAAT-3'
msFlt1	F 5'-GCCGGGCCTTCAATAAAATA-3'
	Rv 5'-CTTTTTGCCGCAGTGCTC-3'
mGapdh	F 5'-TTCACCACCATGGAGAAGGC-3'
	Rv 5'-GGCATGGACTGTGGTCATGA-3'
hVegf	F 5'-GAAGTGGTGAAGTTCATGGATGT-3'
	Rv 5'-TGGAAGATGTCCACCAGGGTC-3'
hsFlt1	F 5'-GGCTGTTTTCTCTCGGATCTC-3'
	Rv 5'-CATCTCCTCCGAGCCTGA AAG-3'
hDll4	F 5'-GCGGGGTACCTTCTCGCTCATCAT C-3'
	Rv 5'-GCCTCCCCAGCCCTCATCACAAGTA-3'
hNotch1	F 5'-CAGGCAATCCGAGGACTATG-3'
	Rv 5'-CAGGCGTGTTGTTCTCACAG-3'
hTie2	F 5'-CACAAGTACCCTACTGCGGGATGACTTGTG-3'
	Rv 5'-TTCTCCCGCCAGCATTGTCT-3'
hGapdh	F 5'-GGTGTGAACCATGAGAAGTATGA-3'
	Rv 5'-GAGTCCTTCCACGATACCAAAG-3'

Table S3. Corneal neovascularisation scoring

Score	Vessel Length
0	No Vessel
1	0-0.25 mm
2	0.25-0.5 mm
3	0.5-0.75 mm
4	0.75-1.0 mm

Supplementary Figure S1

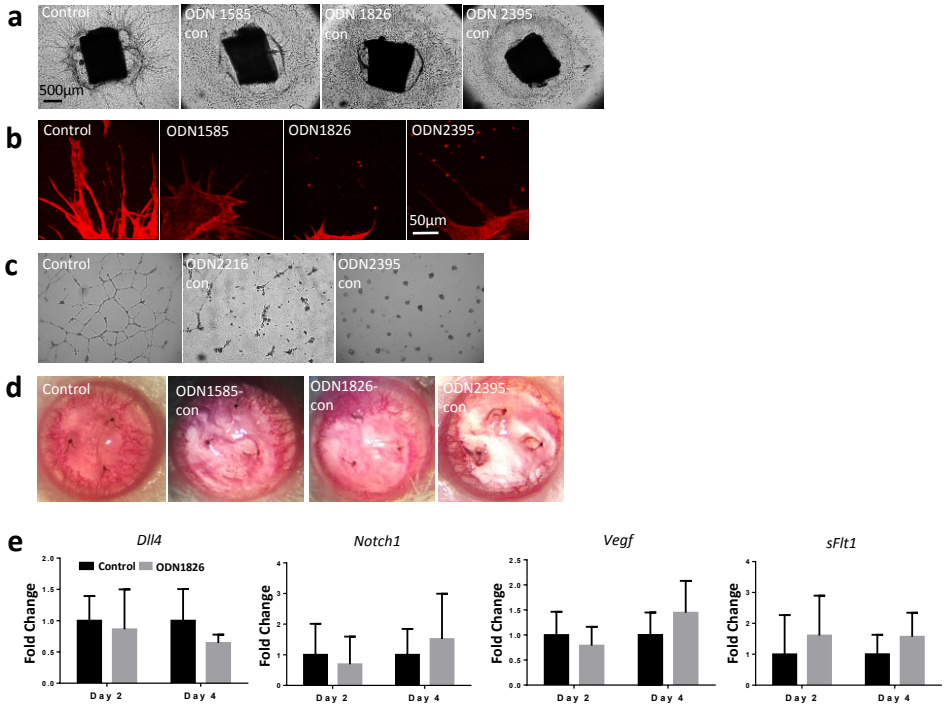

Figure S1: (a) Aortic rings were seeded in collagen gel with stimulation of non-CpG controls of three classes of CpG-ODNs at the dose of 5 μM. All non-CpG-ODN controls suppressed the angiogenic sprouts of aortic rings. (b) Aortic rings were stained for anti-CD31-APC (red) and imaged under ×20 magnification for counting of explants. (c) Both of the CpG-ODN controls (non-CpG-ODN2216 or non-CpG-ODN2395) at concentration of 5 μM inhibited HUVECs tube formation after 24 h. (d) None of the non-CpG-ODNs inhibited corneal angiogenesis. (e) Sutured corneas were dissected from either day 2 or day 4 post sub-conjunctival administration of CpG-ODN1826 or water (n = 12-14 per condition). There was no significant change in the expression of *Dll4, Notch1, Vegf* and *sFlt1* in corneas between ODN1826 and water control. Data represents means ± SD of relative values vs control from 3 independent experiments. Statistical analysis was performed with unpaired Student's *t* test and Mann-Whitney test.

Supplementary Figure S2

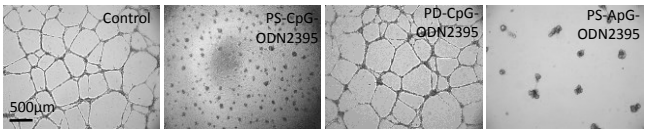

Figure S2: P4 HUVECs were seeded on Matrigel with stimulation of CpG-ODN2395 or its customized controls. Phase contrast photos were taken after 24h incubation. Both PS-CpG-ODN and PS-ApG-ODN but not PD-CpG-ODN suppressed HUVEC tube formation. Scale bar: 500 μm.

Supplementary Figure S3

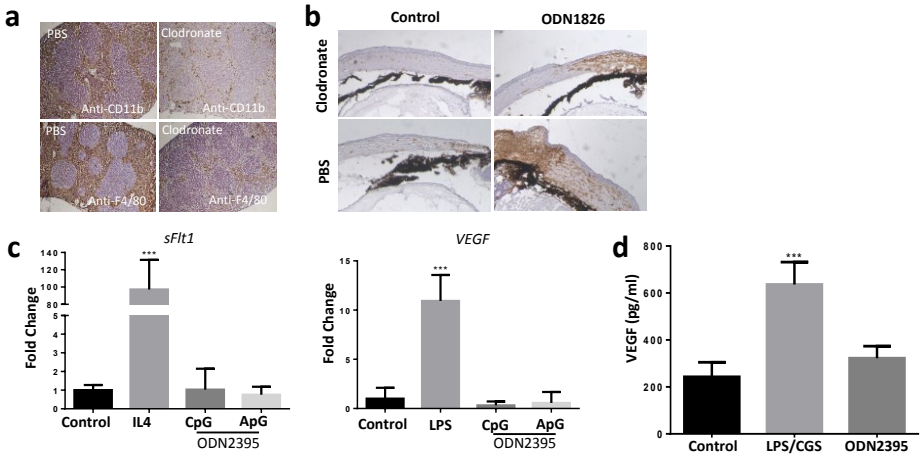

Figure S3: (a) The spleen sections from clodronate or PBS control treated mice were stained for anti-CD11b (1:100) and anti-F4/80 (1:100) for examining the efficiency of systemically macrophages depletion by clodronate. (b) The sutured eyes were collected from clodronate or its PBS control treated mice after 7 days post sub-conjunctival administration of either water or CpG-ODN1826. The sections of eyes were stained for anti-CD11b for efficiency of local macrophages depletion. (c) BMDMs were cultured with stimulation of 5 μM CpG-ODN2395 or ApG-ODN2395 for 6 h or 24 h. IL4 (20 U/mL) and LPS (1 μg/mL) were used as the positive control for sFlt1 and VEGF expression respectively. The expression of *sFlt1* and *Vegf* were not significantly regulated by either CpG-ODN or ApG-ODN. (d) BMDM were cultured for 48 h with stimulation of medium alone, LPS/CGS (1 μg/mL/10 nM) or CpG-ODN2395 (5 μM). The VEGF level in supernatant was increased by LPS/CGS but not CpG-ODN2395 compared to medium alone (n = 12 per condition). Data represents means ± SD of relative values vs control from 3 independent experiments. ***$p < 0.0005$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Supplementary Figure S4

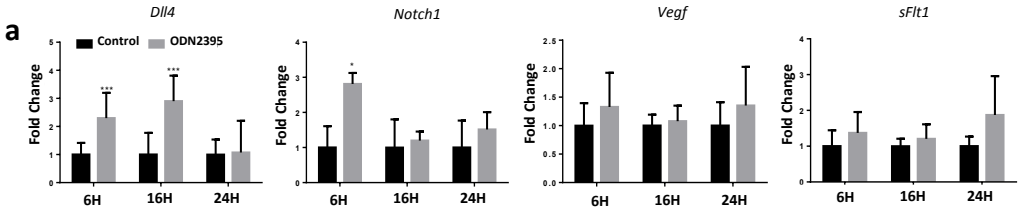

Figure S4: HUVECs were cultured for 6 h, 16 h or 24 h with CpG-ODN2395 (5 µM) or medium alone as control. CpG-ODN2395 significantly up-regulated expression of *Dll4* at 6 h and 16 h and *Notch1* at 6 h compared to control (n = 12 per condition). The expression of *Vegf* and *sFlt1* was not significantly regulated at any time point. Data represents means ± SD of relative values vs control from 3 independent experiments. **p* < 0.05; ****p* < 0.0005, statistical analysis was performed with unpaired Student's *t* test and Mann-Whitney test for two individual comparisons.

Supplementary Figure S5

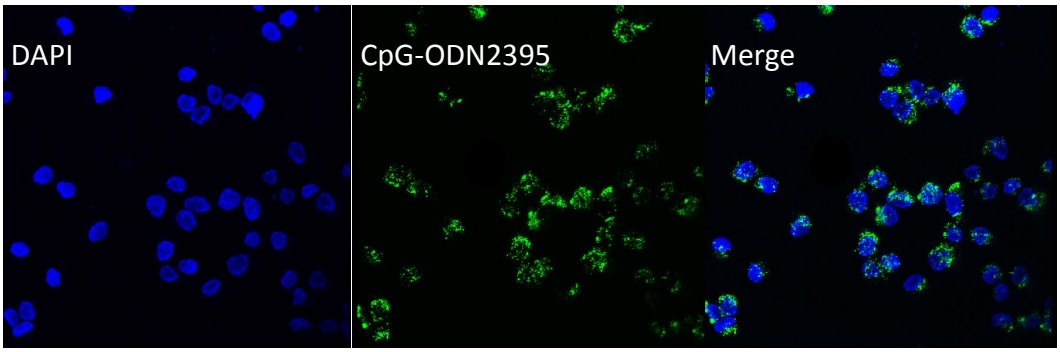

Figure S5: HUVECs were cultured for 6 h with CpG-ODN2395 conjugated to FITC (5 μM). Cells were fixed and followed by DAPI staining before Confocal scanning (n = 6 per condition). Photos represent that CpG-ODN2395 has entered cytoplasm after 6 hours stimulation.