A CELL-PENETRATING CD40-TRAF2,3 BLOCKING PEPTIDE DIMINISHES INFLAMMATION AND NEURONAL LOSS AFTER ISCHEMIA/REPERFUSION

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	Forward	Reverse
ICAM-1 (1)	5′-GCCTTGGTAGAGGTGACTGAG-3′	5′-GACCGGAGCTGAAAAGTTGTA-3′
CXCL1 (2)	5'-ACAGTCCCGCTGACCAAGAG-3'	5'-CACTGACAGCGCAGCTCATT-3'
NOS2 (1)	5'-CAGCTGGGCTGTACAAACCTT-3'	5'-CATTGGAAGTCAAGCGTTTCG-3'
COX-2 (3)	5′-CACAGCCTACCAAAACAGCCA-3′	5′-GCTCAGTTGAACGCCTTTTGA-3′
TNF-α (4)	5’-CATCTTCTCAAAATTCGAGTGACAA-3’	5’-TGGGAGTAGACAAGGTACAACCC-3’
IL-1β (5)	5’-CAACCAACAAGTGATATTCTCCAT G-3’	5’-GATCCACACTCTCCAGCTGCA-3’
IFN-γ (4)	5’-CATTGAAAGCCTAGAAAGTCTGAATAAC-3’	5’-TGGCTCTGCAGGATTTTCATG-3’
IL-12 p40 (5)	5’-GGAAGCACGGCAGCAGAATA-3’	5’-AACTTGAGGGAGAAGTAGGAATGG-3’
18S rRNA (6)	5’-ACTCAACACGGGAAACCTCACC-3’	5’-CCAGACAAATCGCTCCACCAAC-3’
B1 (7)	5'-AACGGGCGAGTAGCACCTGAGGAGA-3'	5'-TGGGTCTACGTCGATGGCATGACAAC-3'
L32 (8)	5’-TGTGCAACAAATCTTCACCGTGC-3’	5’-GGATTGGTGACTCTGATGGCC-3’

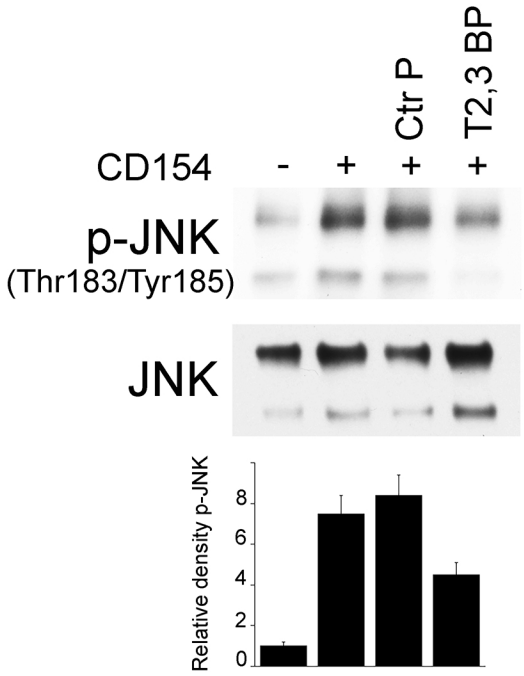
Table S1. **Oligonucleotide primer sequences for real-time PCR amplification**.

1. Park, E.-M., Cho, S., Frys, K., Racchumi, G., Zhou, P., Anrather, J., and Iadecola, C. (2004) Interaction between inducible nitric oxide synthase and poly(ADP-ribose) polymerase in focal ischemic brain injury. *Stroke* **35**, 2896-2901
2. Zhou, J. T., Pham, L., Zhang, N., He, S., Gamulescu, M.-A., Spee, C., Ryan, J., and Hinton, D. R. (2005) Neutrophils promote experimental choroidal neovascularization. *Mol. Vision* **11**, 414-424
3. Wei, X., Zhang, X., Zuscik, M. J., Drissi, M. H., Schwarz, E. M., and O'Keefe, R. J. (2005) Fibroblasts express RANKL and support osteoclastogenesis in a COX-2-dependent manner after stimulation with titanium particles. *J. Bone Min. Res.* **20**
4. Johnson, L. L., Lanthier, P., Hoffman, J., and Chen, W. (2004) Vaccination protects B cell-deficient mice against an oral challenge with mildly virulent *Toxoplasma gondii*. *Vaccine* **22**, 4054-4061
5. Overbergh, L., Valckx, D., Waer, M., and Mathieu, C. (1999) Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* **11**, 305-312
6. Subauste, C. S., Subauste, A., and Wessendarp, M. (2007) Role of CD40-dependent down-regulation of CD154 in impaired induction of CD154 in CD4+ T cells from HIV-1-infected patients. *J. Immunol.* **178**, 1645-1653
7. Bretagne, S., Costa, J. M., Vidaud, M., Tran Van Nhieu, J., and Fleury-Feith, J. (1993) Detection of *Toxoplasma gondii* by competitive DNA amplification of bronchoalveolar lavage samples. *J. Infect. Dis.* **168**, 1585-1588
8. Portillo, J.-A. C., Okenka, G., Reed, E., Subauste, A., Van Grol, J., Gentil, K., Komatsu, M., Tanaka, K., Landreth, G., Levine, B., and Subauste, C. S. (2010) The CD40-autophagy pathway is needed for host protection despite IFN-γ-dependent immunity and CD40 induces autophagy via control of p21 levels. *Plos One* **e14472**

Reagent	Clone	Source
ICAM-PE	YN1/1.7.4	BioLegend
CD19-FITC	1D3/CD19	BioLegend
CD80-BV650	16-10A1	BioLegend
CD86-AF700	GL-1	BioLegend
MHC II-BUV395	2G9	BD Bioscience
CD3-PE/Cy7	17A2	BioLegend
CD11b-PE	M1/70	BioLegend
CD11c-BV421	N418	BioLegend
Live Dead kit Aqua	-	BioLegend

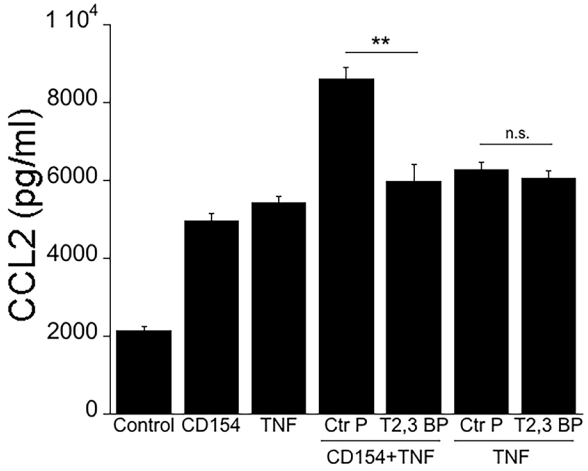
Table S2. **Reagents for flow cytometry of spleen cells**.

Figure S1



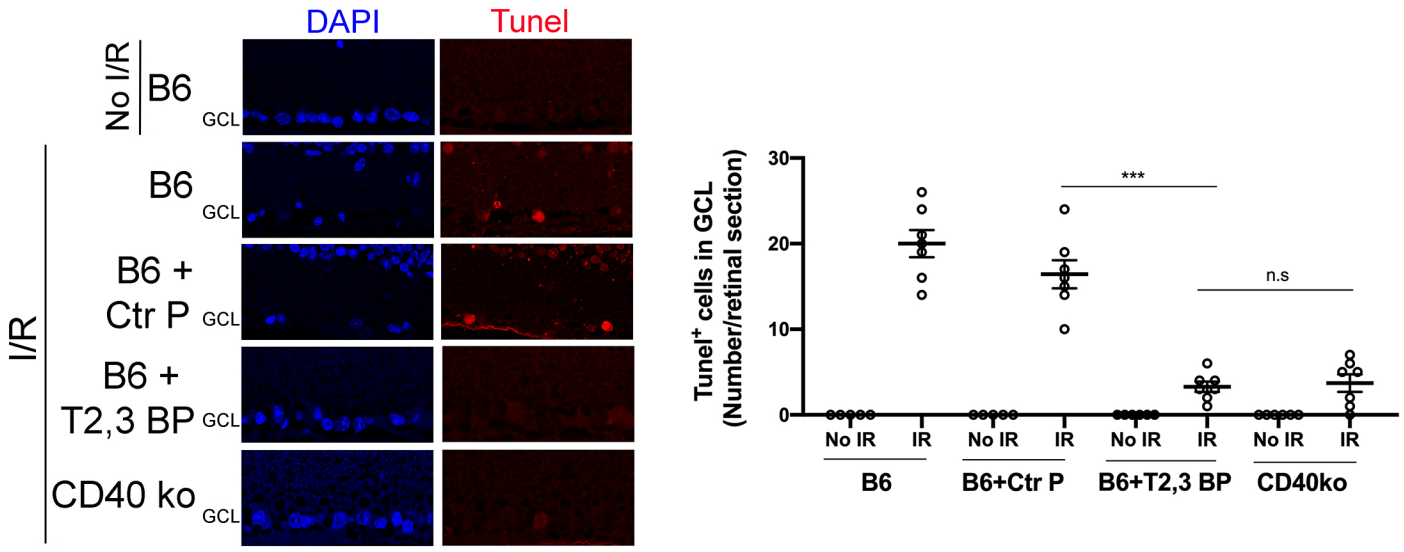
ri CD40-TRAF2,3 blocking peptide inhibits CD40-driven JNK phosphorylation. Human retinal Müller cells were treated with ri control peptide (Ctr P) or ri CD40-TRAF2,3 blocking peptide (T2,3 BP; both at 1 μM) followed by stimulation with CD154 for 15 min. Total JNK and phospho-Thr183/Tyr185 JNK were assessed by immunoblot. Relative density of phospho-JNK was obtained by normalization to total JNK. Relative density of phospho-JNK for the unstimulated sample was given a value of 1. Densitometry data represent means +/- SD of 3 independent experiments.

Figure S2



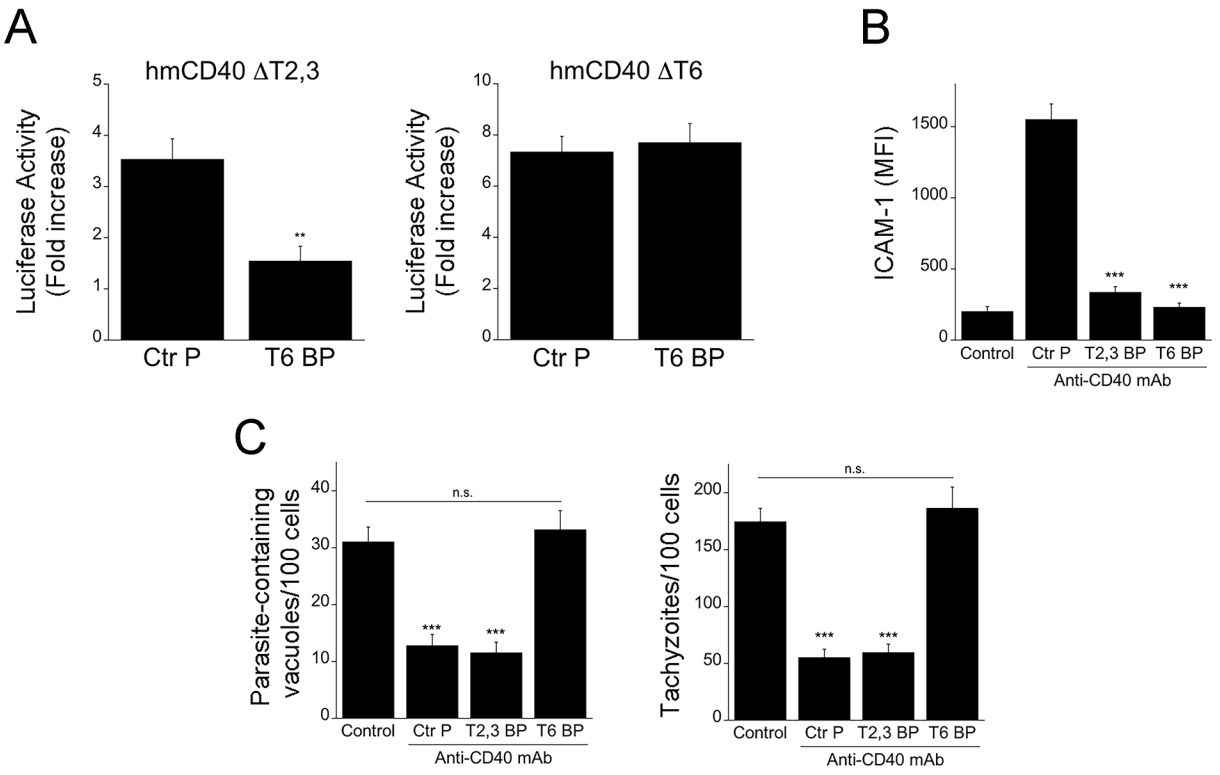
ri CD40-TRAF2,3 blocking peptide inhibits CD40-driven chemokine upregulation. Human retinal endothelial cells were treated with ri control peptide (Ctr P) or ri CD40-TRAF2,3 blocking peptide (T2,3 BP; both at 1 μM) followed by stimulation with CD154 with or without TNF-α (30 pg/ml) for 24 h. Secretion of CCL2 was examined at 24 h by ELISA. Data shown represent Mean +/- SD of triplicate samples. Results are representative of 3 independent experiments. **P<0.01 by ANOVA.

Figure S3



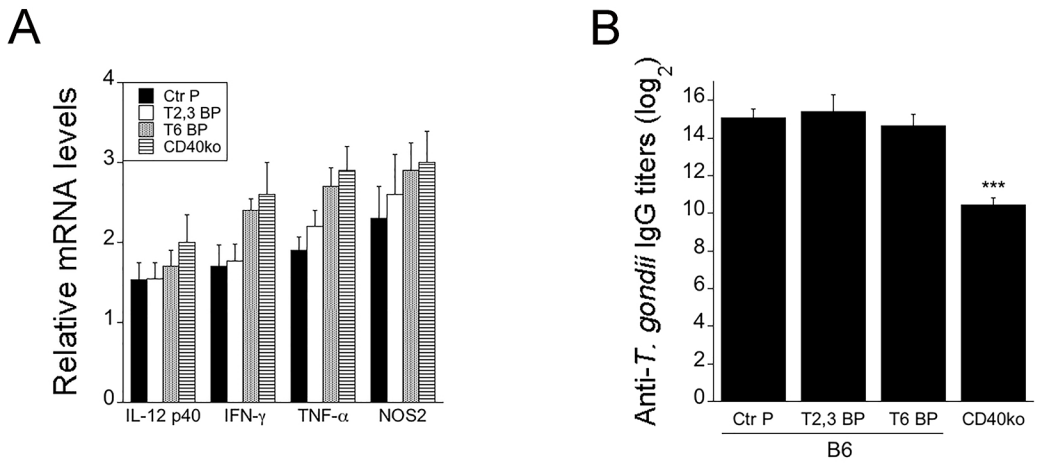
ri CD40-TRAF2,3 blocking peptide protects against programmed cell death in the GCL of retinas subjected to I/R. One eye from each B6 and Cd40-/- mouse was subjected to I/R. Contralateral non-ischemic eye was used as control. Eyes subjected to I/R in B6 mice were treated intravitreously with or without ri control peptide (Ctr P) or ri CD40-TRAF2,3 blocking peptide (T2,3 BP) 1 hr. prior to increase in IOP. Eyes were collected 2 d after I/R and were stained with ApopTag Red, In situ Apoptosis Detection kit. Original magnification X400. Scale bar, 50 μm. GCL = Ganglion cell layer). Tunel+ cells in the GCL were counted in whole retinal sections. Horizontal bars represent mean +/- SEM (7 mice per group).***P<0.001 by ANOVA.

Figure S4



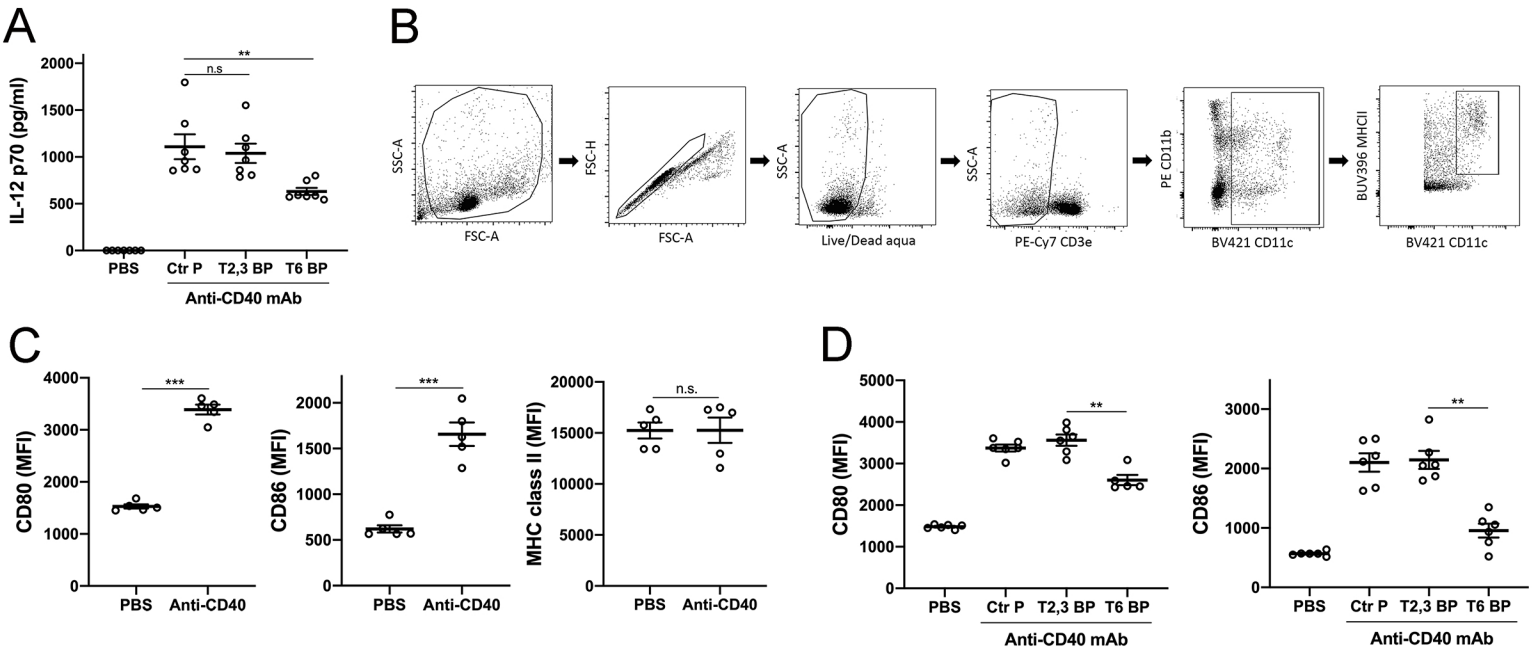
ri CD40-TRAF6 blocking peptide inhibits CD40-TRAF6 signaling, impairs CD40-driven adhesion molecule upregulation and CD40-induced toxoplasmacidal activity. *A*, Mouse endothelial cells (mHEVc) that express an NF-κB response element that drives transcription of a luciferase reporter plus either hmCD40 ΔT2,3 or hmCD40 ΔT6 were pre-incubated with ri Tat peptide (Ctr P) or ri CD40-TRAF6 blocking peptide (T6 BP; both at 1 μM) or medium alone followed by stimulation with human CD154. Data are expressed as fold-increase in normalized luciferase activity in cells stimulated with CD154 compared to cells treated with respective peptide in the absence of CD154. *B*, Mouse retinal endothelial cells were treated with ri Tat peptide (Ctr P), ri CD40-TRAF2,3 (T2,3 BP) or ri CD40-TRAF6 blocking peptide (T6 BP; all 1 μM) followed by incubation with a stimulatory anti-CD40 mAb for 24 h. Expression of ICAM-1 was assessed by flow cytometry. *C*, Mouse retinal endothelial cells were treated as above and infected with *T. gondii* tachyzoites. The numbers of vacuoles and tachyzoites per 100 cells were assessed at 24 hr. Data shown represent mean +/- SD of triplicate samples. Results are representative of 3 independent experiments. **P<0.01 by ANOVA.

Figure S5



Effect of CD40 and blocking peptides on the expression of IL-12, IFN-γ, TNF-α and NOS2 mRNA levels in the eyes of *T. gondii*-infected mice and on serum anti-*T. gondii* IgG levels. B6 and Cd40-/- mice were infected with *T. gondii* tissue cysts. B6 mice received peptides intravitreally in both eyes 4 days after infection. *A*, Eyes were collected 14 d post-infection. Levels of IL-12 p40, IFN-γ, TNF-α and NOS2 mRNA were assessed by real time PCR. One infected B6 mouse was given an arbitrary value of 1. Data are expressed as fold-increase compared to this animal. Each group contained 4-7mice. *B*, Serum anti-*T. gondii* IgG titers at 14 d post-infection. Results are shown as the mean +/- SEM.

Figure S6



The ri CD40-TRAF6 but not the ri CD40-TRAF2,3 blocking peptide impairs IL-12 p70 production and dendritic cell activation after systemic administration of stimulatory anti-CD40 mAb. B6 mice were injected i.p. with 100 μg of stimulatory anti-CD40 mAb or PBS. Mice received peptides (10 μg/kg ii.p.) 3 h prior to anti-CD40 mAb. *A*, Serum levels of IL-12 p70 were assessed by ELISA after 24 hr. *B-D*, Splenocytes were isolated 48 h after administration of anti-CD40 mAb and subjected to flow cytometric analysis. *B*, Dot plots show gating strategy for dendritic cells. *C*, Expression of CD80, CD86 and MHC class II were assessed on gated dendritic cells. *D*, Expression of CD80 and CD86 on gated dendritic cells after administration of peptides. Results show median +/- SEM of 5-7 mice per group and are representative of 3 independent experiments. *P<0.05; **P<0.01 by ANOVA.