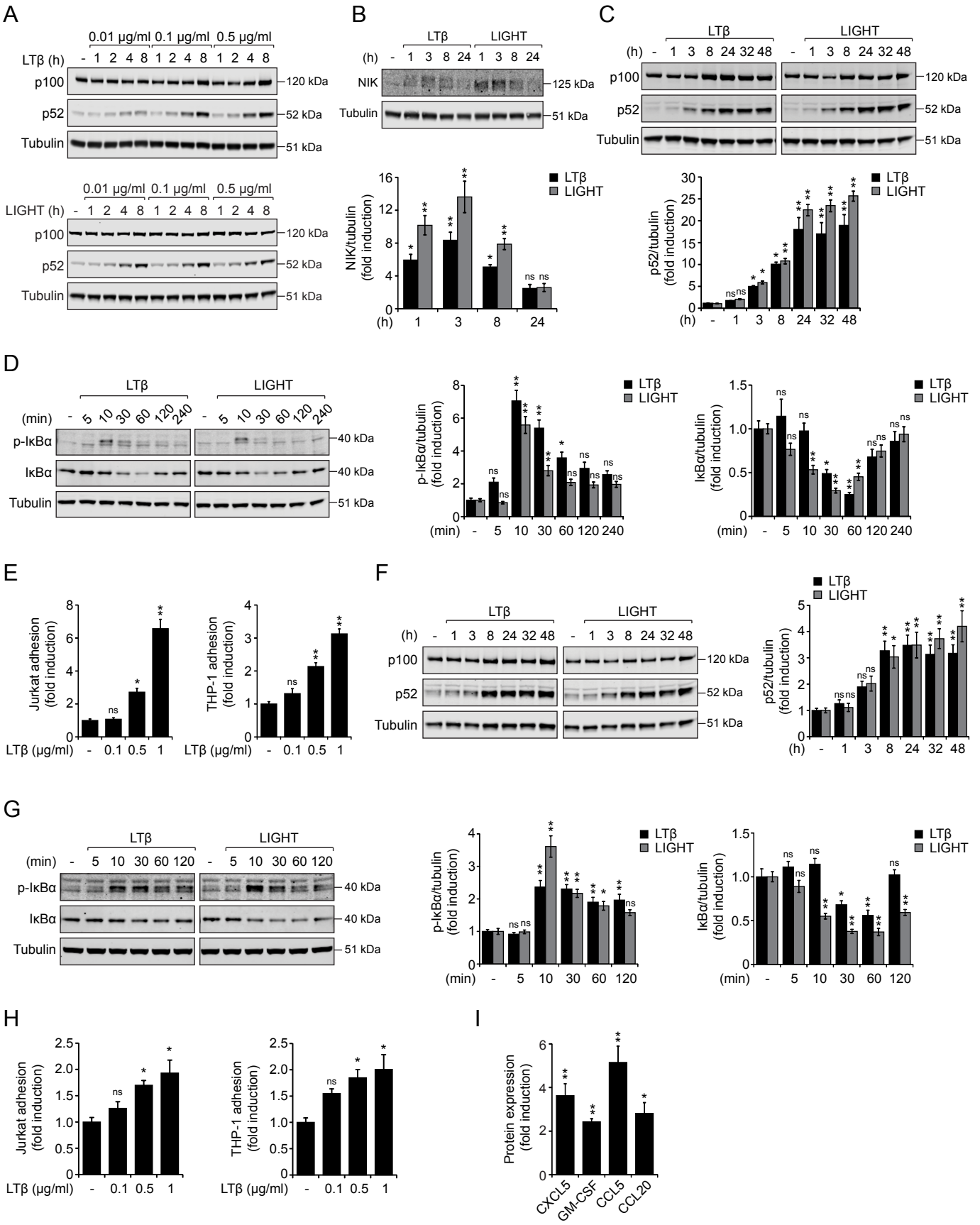

Figure S1. LTβR ligation activates NF-κB signaling and inflammation in endothelial cells. (A) Immunoblot analysis of p100/p52 and tubulin as a loading control in HUVECs treated with various concentrations of LTβ or LIGHT for the indicated time points. Data shown are representative of two independent experiments. h, hours. (B) Immunoblot analysis of NIK and tubulin as a loading control in EA.hy926 cells treated with LTβ (0.1 μg/ml) or LIGHT (0.1 μg/ml) for the indicated time points. Upper panel: immunoblotting data from a representative experiment. h, hours. Lower panel: densitometric analysis of NIK bands from three independent experiments. (C) Immunoblot analysis of p100/p52 and tubulin as a loading control in EA.hy926 cells treated with LTβ (0.1 μg/ml) or LIGHT (0.1 μg/ml) for the indicated time points. Upper panel: immunoblotting data from a representative experiment. h, hours. Lower panel: densitometric analysis of p52 bands from three independent experiments. (D) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα), IκBα and tubulin as a loading control in EA.hy926 cells treated with LTβ (0.1 μg/ml) or LIGHT (0.1 μg/ml) for the indicated time points. Left panel: immunoblotting data from a representative experiment. min, minutes. Middle and right panels: densitometric analysis of p-IκBα and IκBα bands from three independent analysis. (E) Adhesion of Jurkat cells (left panel) or THP-1 cells (right panel) to EA.hy926 cells treated with indicated concentrations of LTβ for 24 hours. (F) Immunoblot analysis of p100/p52 and tubulin as a loading control in HMVECs treated with LTβ (0.1 μg/ml) or LIGHT (0.1 μg/ml) for the indicated time points. Left panel: immunoblotting data from a representative experiment. h, hours. Right panel: densitometric analysis of p52 bands from three independent experiments. (G) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα), IκBα and tubulin as a loading control in HMVECs treated with LTβ (0.1 μg/ml) or LIGHT (0.1 μg/ml) for the indicated time points. Left panel: immunoblotting data from a representative experiment. min, minutes. Middle and right panels: densitometric analysis of p-IκBα and IκBα bands from three independent analysis. (H) Adhesion of Jurkat cells (left panel) or THP-1 cells (right panel) to HMVECs treated with indicated concentrations of LTβ for 24 hours. (I) Quantification of CXCL5, GM-CSF, CCL5 and CCL20 protein levels by Proteome Profiler Human XL Cytokine Array Kit in conditioned medium from HMVECs treated with LTβ (0.25 μg/ml) for 24 hours. (B-I) Data represent mean ± s.e.m. of three independent experiments. ns, not significant; *$P \leq 0.05$, **$P \leq 0.01$ versus control (one-way ANOVA and Tukey's HSD post hoc test).

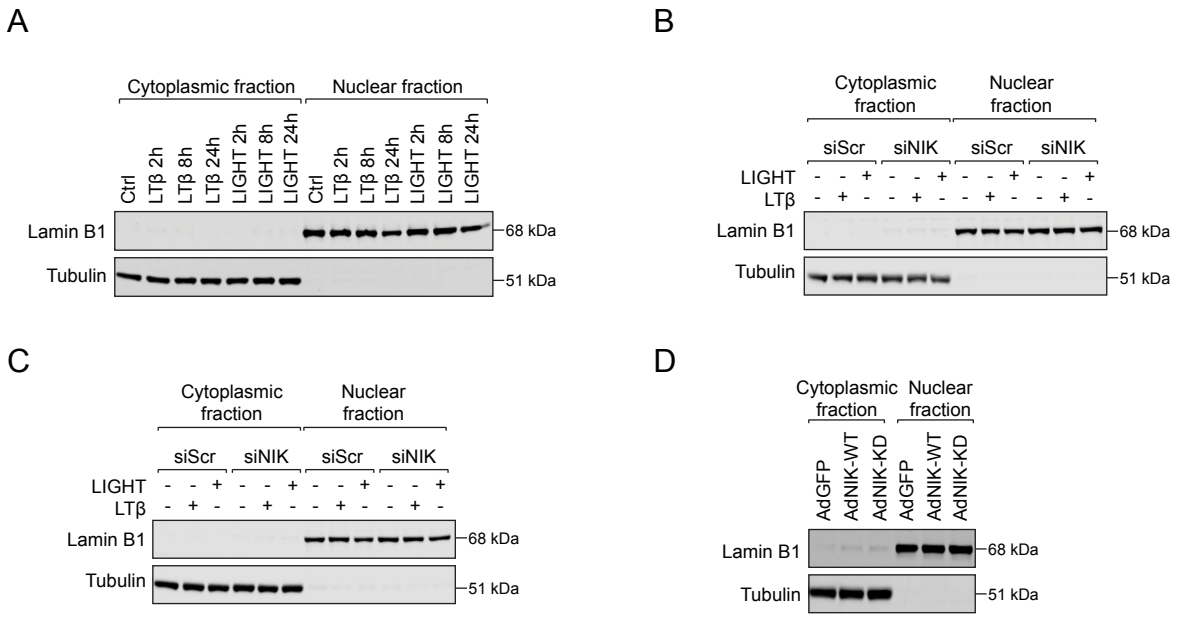

Figure S2. Fractionation of nuclear and cytoplasmic proteins. (A-D) Fractionation of cell lysates of HUVECs treated as described in Fig. 1C (A), Fig. 2B (B), Fig. S5F (C) and Fig. 3B (D).

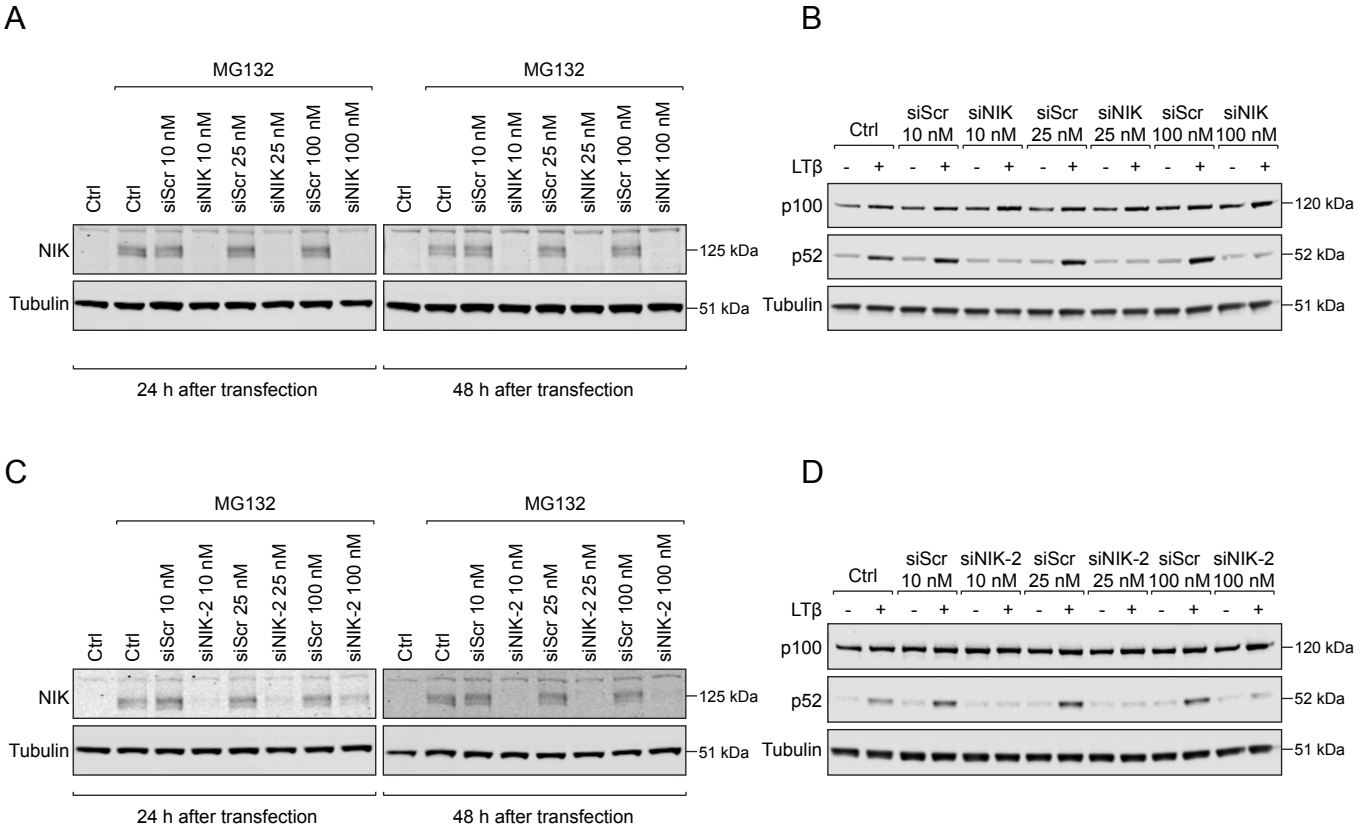

Figure S3. RNAi-mediated knockdown of NIK impairs activation of the non-canonical NF-κB pathway in HUVECs. (A,C) Immunoblot analysis of NIK in HUVECs left untreated (Ctrl) or transfected with scrambled siRNA (siScr) or siRNA targeting NIK [siNIK (A) or siNIK-2 (C)] at indicated concentrations and grown for 24 or 48 hours. Cells were incubated with the proteasome inhibitor MG132 (50 µM) for the last 3 hours to prevent NIK degradation. (B,D) Immunoblot analysis of p100/p52 in HUVECs left untreated (Ctrl) or transfected with siScr or siRNA targeting NIK [siNIK (B) or siNIK-2 (D)] at indicated concentrations for 24 hours, followed by stimulation with LTβ (0.2 µg/ml) for 16 hours. (A-D) Tubulin was used as a loading control. Data shown are representative of two independent experiments.

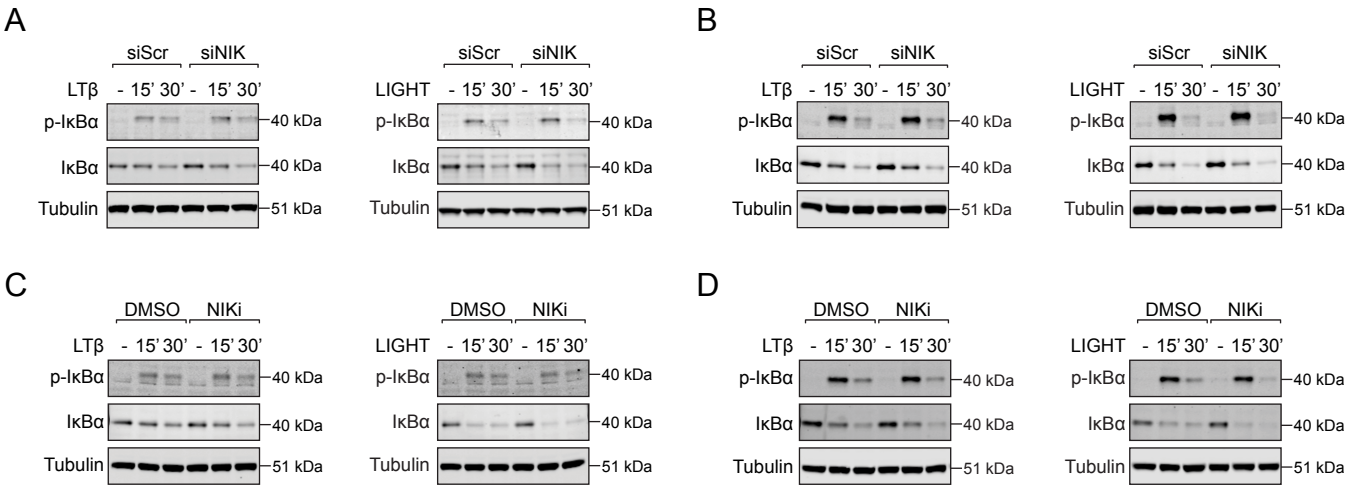

Figure S4. Immediate activation of canonical NF-κB signaling in response to LTβR ligation is not dependent on NIK activity. (A,B) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα), IκBα and tubulin as a loading control in HUVECs (A) and EA.hy926 cells (B) transfected with scrambled siRNA (siScr; 10 nM) or siRNA targeting NIK (siNIK; 10 nM) and stimulated with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for the indicated time points. (C,D) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα), IκBα and tubulin as a loading control in HUVECs (C) and EA.hy926 cells (D) treated with DMSO or NIK inhibitor (NIKi; 2.5 μM) for 30 minutes, followed by stimulation with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for the indicated time points. (A-D) Data shown are representative of two independent experiments.

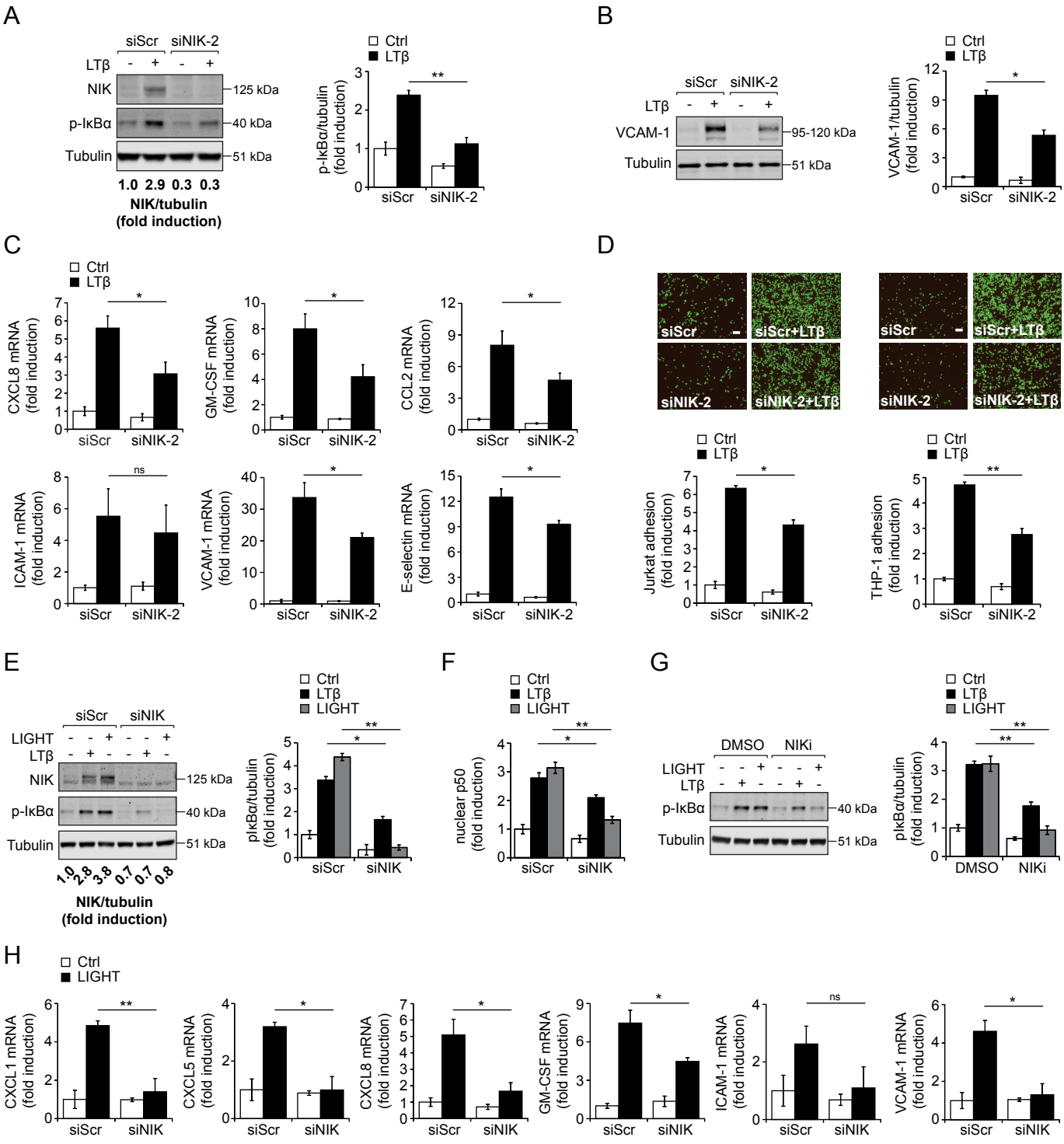

Figure S5. NIK-dependent activation of canonical NF-κB signaling pathway in endothelial cells upon LTβR ligation. (A) Immunoblot analysis of NIK, phospho-IκBα (Ser32/36) (p-IκBα) and tubulin as a loading control in HUVECs transfected with scrambled siRNA (siScr; 10 nM) or siRNA targeting NIK (siNIK-2; 10 nM) and stimulated with LTβ (0.25 μg/ml) for 3 hours. Cells were incubated with the proteasome inhibitor MG132 (50 μM) for the final hour to prevent NIK and p-IκBα degradation. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p-IκBα bands from three independent experiments. (B) Immunoblot analysis of VCAM-1 and tubulin as a loading control in HUVECs transfected with siScr or siNIK-2 as in A and stimulated with LTβ (0.25 μg/ml) for 24 hours. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of VCAM-1 bands from two independent experiments. (C) Real-time qPCR quantification of the CXCL8, GM-CSF, CCL2, ICAM-1, VCAM-1 and E-selectin transcripts expressed in HUVECs transfected with siScr or siNIK-2 as in A and stimulated with LTβ (0.25 μg/ml) for 6 hours. (D) Adhesion of Jurkat cells (left panels) and THP-1 cells (right panels) to HUVECs transfected with siScr or siNIK-2 as in A and stimulated with LTβ (0.25 μg/ml) for 24 hours. Upper panels: representative images of calcein-labeled leukocytes attached to HUVECs. Scale bar, 100 μm. Lower panels: quantification of leukocyte adhesion to HUVECs relative to siScr cells. (E) Immunoblot analysis of NIK, phospho-IκBα (Ser32/36) (p-IκBα) and tubulin as a loading control in EA.hy926 cells transfected with scrambled siRNA (siScr; 10 nM) or siRNA targeting NIK (siNIK; 10 nM) and stimulated with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for 3 hours. Cells were incubated with the proteasome inhibitor MG132 (50 μM) for the final hour to prevent NIK and p-IκBα degradation. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p-IκBα bands from three independent experiments. (F) Quantification of p50 DNA binding activity by ELISA in nuclear protein extracts from EA.hy926 cells transfected with siScr or siNIK as in E and stimulated with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for 6 hours. (G) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα) and tubulin as a loading control in EA.hy926 cells treated with DMSO or NIK inhibitor (NIKi; 2.5 μM) for 30 minutes, followed by stimulation with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for 3 hours. Cells were incubated with the proteasome inhibitor MG132 (50 μM) for the final hour to prevent p-IκBα degradation. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p-IκBα bands from five independent experiments. (H) Real-time qPCR quantification of the CXCL1, CXCL5, CXCL8, GM-CSF, ICAM-1 and VCAM-1 transcripts expressed in HMVECs transfected with scrambled siRNA (siScr; 10 nM) or siRNA targeting NIK (siNIK; 10 nM) and stimulated with LIGHT (0.25 μg/ml) for 6 hours. Data represent mean ± s.e.m. of two (B), three (A,C-F,H) or five (G) independent experiments. ns, not significant; *$P \leq 0.05$, **$P \leq 0.01$ [Student's *t* test (A-D,H) or one-way ANOVA and Tukey's HSD post hoc test (E-G)].

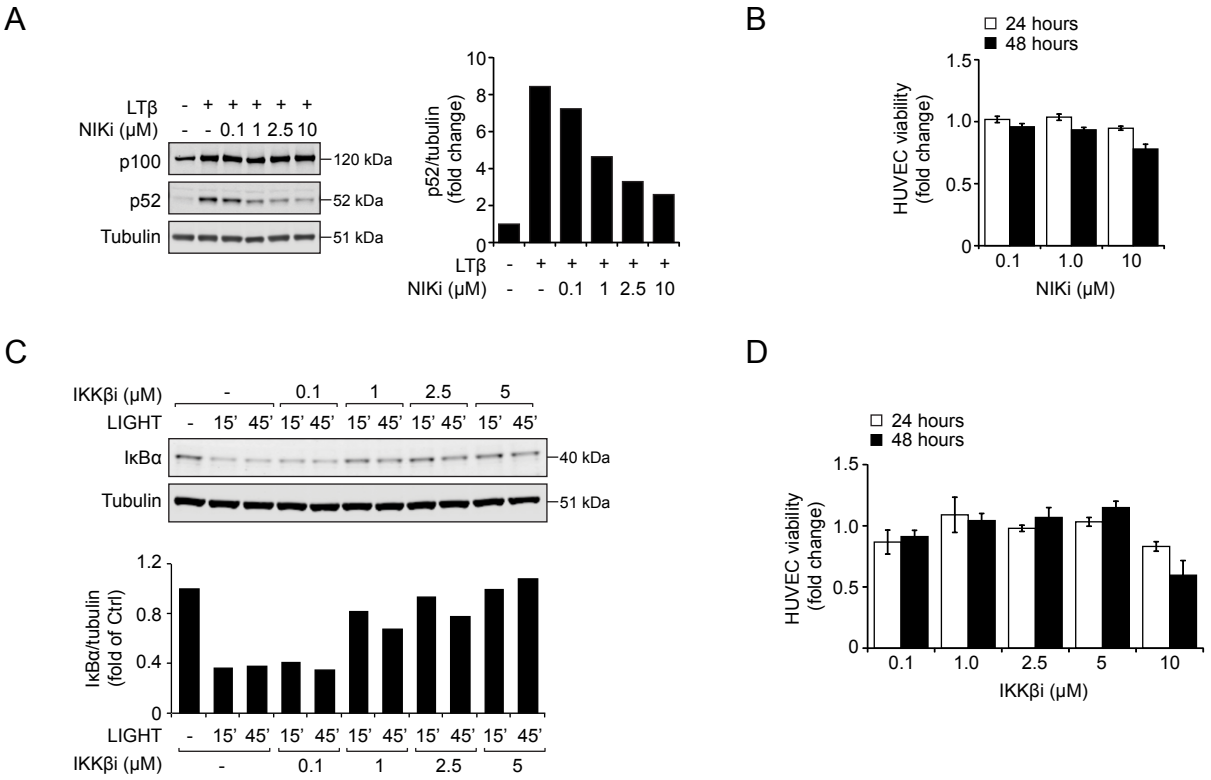

Figure S6. Activity of NIK and IKKβ inhibitors in HUVECs. (A) Immunoblot analysis of p100/p52 and tubulin as a loading control in HUVECs treated with DMSO or indicated concentrations of NIK inhibitor (NIKi) for 30 minutes, followed by stimulation with LTβ (0.1 μg/ml) for 16 hours. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p52 bands from a representative experiment. (B) Quantification of LDH viability assay performed on HUVECs treated with indicated concentrations of NIKi for 24 or 48 hours. Graph represents the fold change in cell survival relative to untreated cells ± SD (n = 3). Data shown are representative of two independent experiments. (C) Immunoblot analysis of IκBα and tubulin as a loading control in HUVECs treated with DMSO or IKKβ inhibitor (IKKβi) at indicated concentrations for 30 minutes, followed by stimulation with LIGHT (0.1 μg/ml) for 15 and 45 minutes. Upper panel: immunoblotting data from a representative experiment. Lower panel: densitometric analysis of IκBα bands from a representative experiment. (D) Quantification of MTT viability assay performed on HUVECs treated with indicated concentrations of IKKβi for 24 or 48 hours. Graph represents the fold change in cell survival relative to untreated cells ± SD (n = 3). Data shown are representative of two independent experiments.

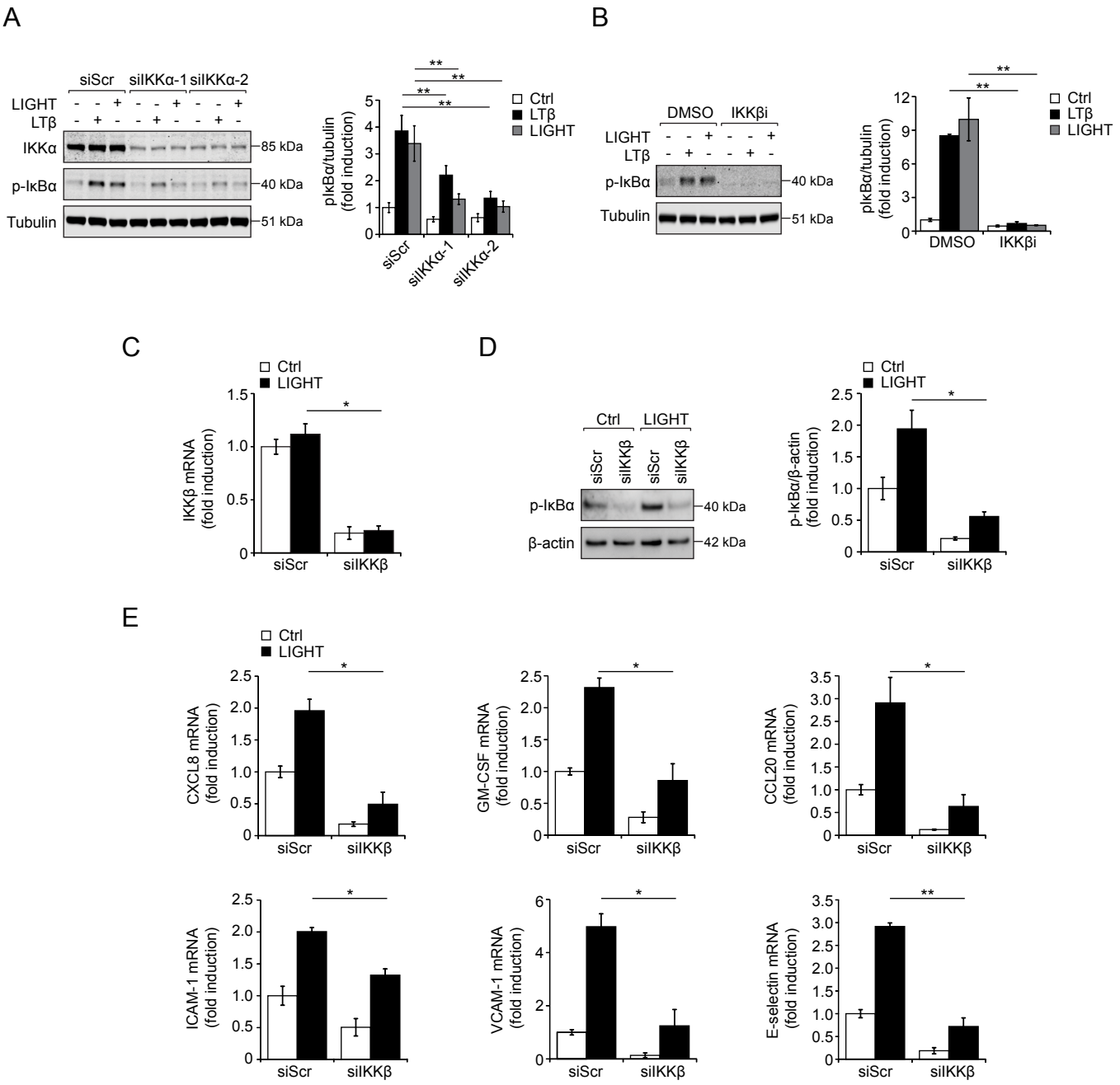

Figure S7. IKKα- and IKKβ-dependent activation of canonical NF-κB pathway in EA.hy926 cells and HUVECs upon LTβR ligation. (A) Immunoblot analysis of IKKα, phospho-IκBα (Ser32/36) (p-IκBα) and tubulin as a loading control in EA.hy926 cells transfected with scrambled siRNA (siScr; 10 nM) or siRNAs targeting IKKα (siIKKα-1 or IKKα-2; 10 nM) and stimulated with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for 3 hours. Cells were incubated with the proteasome inhibitor MG132 (50 μM) for the final hour to prevent p-IκBα degradation. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p-IκBα bands from four independent experiments. (B) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα) and tubulin as a loading control in EA.hy926 cells treated with DMSO or IKKβ inhibitor (IKKβi; 2.5 μM) for 30 minutes, followed by stimulation with LTβ (0.25 μg/ml) or LIGHT (0.25 μg/ml) for 3 hours. Cells were incubated with the proteasome inhibitor MG132 (50 μM) for the final hour to prevent p-IκBα degradation. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p-IκBα bands from two independent experiments. (C) Real time qPCR quantification of the IKKβ transcript expressed in HUVECs transfected with scrambled siRNA (siScr; 50 nM) or siRNA targeting IKKβ (siIKKβ; 50 nM) and stimulated with LIGHT (0.25 μg/ml) for 6 hours. (D) Immunoblot analysis of phospho-IκBα (Ser32/36) (p-IκBα) and β-actin as a loading control in HUVECs transfected with siScr or siIKKβ as in C and stimulated with LIGHT (0.25 μg/ml) for 3 hours. Cells were incubated with the proteasome inhibitor MG132 (50 μM) for the final hour to prevent p-IκBα degradation. Left panel: immunoblotting data from a representative experiment. Right panel: densitometric analysis of p-IκBα bands from two independent experiments. (E) Real-time qPCR quantification of the CXCL8, GM-CSF, CCL20, ICAM-1, VCAM-1 and E-selectin transcripts expressed in HUVECs transfected with siScr or siIKKβ as in C and stimulated with LIGHT (0.25 μg/ml) for 6 hours. Data represent mean ± s.e.m. of two (B-E) or four (A) independent experiments. *$P \leq 0.05$, **$P \leq 0.01$ [one-way ANOVA and Tukey's HSD post hoc test (A,B) or Student's *t* test (C-E)].