Supplemental Data

Supplementary Figure 1. iE-DAP is a specific ligand for NOD1.

(A) HT-29 cells ($1.5X10^5$/ml in 96 well plate) were transfected with a construct expressing the promoter for the gene encoding NF-κB linked to a luciferase reporter gene together with a construct expressing TLR2, TLR3, TLR4, MD2, TLR5, NOD1, or NOD2. The cells were then stimulated with ligands specific for the transfected recognition molecule as positive control (PGN 10 μg/ml, Pam_3CSK4 1 μg/m, dsRNA 25 μg/ml, LPS 1 μg/ml, flagellin 1 μg/ml, MDP 10 μg/ml) or with iE-DAP (10 μg/ml). Results of NF-κB luciferse activity normalized to galactosidase activity are presented as means ± SD. *$p<0.05$, **$p<0.01$, compared with unstimulated cells. **(B)** Bone marrow-derived dendritic cells ($1x10^6$/ml in 48 well plate) from NOD1-intact and NOD1-deficient mice were stimulated with MDP (10 μg/ml) or iE-DAP (10 μg/ml) for 24 hrs. Culture supernatant were harvested and assayed or IL-6 by ELISA. Results are expressed as means ± SD. **$p<0.01$, compared with cells from NOD1-intact mice. **(C)** HT-29 cells ($1x10^6$/ml) were stimulated with iE-DAP (synthesized), iE-DAP (commercially available), or FK156 at the indicated doses for 24 hours and then culture supernatants were harvested for IP-10 assay. Results are expressed as means ± SD.

Supplementary Figure 2. Production of chemokines by AGS cells stimulated with MDP or iE-DAP.

(A) IP-10, I-TAC, and IL-8 production by iE-DAP-stimulated AGS cells without IFN-γ pre-treatment. AGS cells ($1x10^6$/ml in 6 well plate) were stimulated with NOD1 ligand (iE-DAP, 20 or 200 μg/ml) or NOD2 ligand (MDP, 20 or 200 μg/ml) for 24 hrs. Culture supernatants were harvested and assayed. **(B)** AGS cells ($1x10^6$/ml in 6 well plate) were pre-cultured with IFN-γ or TNF (10 or 100 ng/ml) for 24 hrs and then cultured with NOD1 ligand (1 or 10 μg/ml) or NOD2 ligand (1 or 10 μg/ml) for another 24 hrs. Culture supernatants were harvested and assayed for the presence of IP-10 (top) and I-TAC (bottom). Results shown are representative of two similar experiments. Results are expressed as means ± SD. * $p<0.05$, **$p<0.01$, compared with cells stimulated with medium.

Supplementary Figure 3. Nuclear translocation of NF-κB subunits in HT-29 cells.

Translocation of NF-κB subunits (RelB, p52) in nuclear extracts was determined by Transfactor assay. Nuclear extracts were isolated from HT-29 cells stimulated with iE-DAP at the indicated time points. Nuclear extracts from Raji cells were used as positive controls. HT-29 cells were pre-incubated with (right) or without (left) IFN-γ. Results shown are representative of two studies. Results are expressed as means ± SD.

Supplementary Figure 4. Activation of NF-κB in AGS cells stimulated with iE-DAP.

Nuclear extracts were prepared from AGS cells stimulated with NOD1 ligand (200 μg/ml) at the indicated time points. Translocation of NF-κB subunits (p65, p50, p52) in nuclear extracts was determined by Transfactor assay. Nuclear extracts from AGS cells treated with 50 ng/ml of TNF for 1 hr were used as positive controls for p65 and p50. Nuclear extracts from Raji cells were used as positive controls for p52. Results shown are representative of two studies. Results are expressed as means ± SD.

Supplementary Figure 5. RICK-dependent IP-10 production by HT-29 cells stimulated with iE-DAP.

(A) Production of IP-10 by HT-29 cells non-treated (left) or treated (right) with IFN-γ. HT29 cells were pre-incubated with PD98059 (ERK inhibitor, PD), SB203580 (p38 and RICK inhibitor, SB), SP600125 (JNK inhibitor, SP), or BAY11-7082 (NF-κB inhibitor, BAY) for 1 hr and then stimulated with NOD1 ligand (iE-DAP, 10 or 100 μg/ml) for 24 hrs. Culture supernatants were then harvested and assayed for chemokine concentrations by ELISA. **(B)** Production of IP-10 by HT-29 cells pre-treated with 20 μM of each inhibitor for 1 hr and then stimulated with TNF (50 ng/ml) for 24 hrs. Culture supernatants were then harvested and assayed for chemokine concentrations by ELISA. Results are expressed as means ± SD. * $p<0.05$, ** $p<0.01$, compared with cells treated with DMSO and NOD1 ligand. Results shown are representative of two similar experiments.

Supplementary Figure 6. Upregulation of NF-κB activation by transfection of TRAF3 siRNA.

(A) THP1 cells ($1x10^6$/ml in 6 well plates) were pre-cultured with IFN-γ or TNF (10 or 100 ng/ml) for 24 hrs and then cultured with NOD1 ligand (iE-DAP, 1 or 10 μg/ml) or NOD2 ligand (MDP, 1 or 10 μg/ml) for another 24 hrs. Culture supernatants were harvested and assayed for the presence of IP-10 by ELISA. Results shown are representative of two studies. Results are expressed as means + SD. **p<0.01, compared with cells cultured with medium alone. **(B)** HT-29 cells ($1x10^5$/96 well plate) transfected with pNF-κB-Luc (50 ng) and pSV-β-galactosidase (10 ng) were co-transfected with control siRNA or two different TRAF3 siRNAs (#1, #2, 50 nM). HT-29 cells were stimulated with iE-DAP (100 μg/ml). **p<0.01, compared with cells transfected with control siRNA (white bar).

Supplementary Figure 7. IFN-β production by HT-29 cells stimulated with iE-DAP or dsRNA.

(A) HT-29 cells ($1x10^6$/ml) were stimulated with TNF, iE-DAP, poly(I:C) (dsRNA) for 12 hours and then culture supernatants were subjected to IFN-β assay (left panel). Expression of IRF7 in nuclear extracts was determined by Transfactor assay. Nuclear extracts from HT-29 cells treated with 1000 U/ml of IFN-β for 1 hr were used as positive controls. HT-29 cells were stimulated with NOD1 ligand (iE-DAP 100 μg/ml), dsRNA (100 μg/ml), or TNF (100 ng/ml) for 4 hrs, at which point nuclear extracts were prepared (right panel). Results are expressed as means ± SD. **(B)** Expression of ISGF3 components in HT-29 cells. HT-29 cells were stimulated with dsRNA (100 μg/ml) or TNF (100 ng/ml). Whole cell extracts prepared from the stimulated cells at the indicated time points were then subjected to western blot analysis.

Supplementary Figure 8. Production of IFN-β by HT-29 cells stimulated with iE-DAP or dsRNA in the presence of NF-κB inhibitor.

(A) HT-29 cells were incubated with NF-κB inhibitor (BAY11-7082, BAY) for one hour prior to stimulation with iE-DAP (100 μg/ml) or dsRNA (100 μg/ml). Cells were cultured for 12 hours and then culture supernatants were subjected to IFN-β assay. **p<0.01, as compared with cells treated with DMSO. Results are

expressed as means ± SD. **(B)** HT-29 cells were incubated with IFN-β at the indicated doses for 24 hours and then culture supernatants were subjected to IP-10 assay. Results are expressed as means ± SD.

Supplementary Figure 9. Activation of ISGF3 in AGS cells stimulated with iE-DAP.

Expression of ISGF3 components in AGS cells. AGS cells were stimulated with 200 μg/ml of NOD1 ligand. Whole cell extracts prepared from the stimulated cells at the indicated time points were then subjected to western blot analysis.

Supplementary Figure 10. ISGF3-dependent IP-10 production by HT-29 and AGS cells.

(A) Production of IP-10 and IFN-β by HT-29 cells. HT-29 cells ($2.5X10^5$/ml in 12 well plate) were transfected with control vector, Stat1 siRNA expressing vector, or Stat2 siRNA expressing vector and then cultured with IFN-γ (100 ng/ml) followed by stimulation with NOD1 ligand (10 μg/ml). Results are expressed as means ± SD. **p<0.01, compared with cells transfected with control vector. Results shown are representative of two similar studies.

(B) Production of IP-10 by AGS cells. AGS cells ($2.5X10^5$/ml in 12 well plate) were transfected with control vector, Stat1 siRNA expressing vector, or Stat2 siRNA expressing vector and then stimulated with NOD1 ligand (200 μg/ml) for 24 hrs in the absence of IFN-γ. AGS cells transfected with control vector, Stat1 siRNA expressing vector, or Stat2 siRNA expressing vector were cultured for 48 hrs. Cells were then lysed and the whole lysates obtained were subjected to western blot analysis (top). Culture supernatants were harvested and assayed for chemokine content by ELISA (bottom). Results are expressed as means ± SD. **p<0.01, compared with cells transfected with control vector.

Supplementary Figure 11. IFN-β production induced by NOD1 activation depends on TBK1 and IKKε. Wild type ($IKK\beta^{+/+}$) or IKKβ deficient ($IKK\beta^{-/-}$) MEFs ($1x10^6$/ml) were stimulated with iE-DAP (100 μg/ml) for 24 hours (top panel). Wild type ($TBK1^{+/+}IKK\varepsilon^{+/+}$) or TBK1/IKKε double deficient ($TBK1^{-/-}IKK\varepsilon^{-/-}$) MEFs were stimulated with iE-DAP (100 μg/ml) for 24 hours (bottom panel). Culture supernatants were subjected to IFN-β assay. Nuclear translocation of

Stat1 and p65 was also analyzed. Cells were stimulated with NOD1 ligand (iE-DAP 100 μg/ml) and then nuclear extracts were prepared. Cells were stimulated with NOD1 ligand for one hour for p65 assay and for 4 hours for Stat1 assay. Nuclear extracts isolated from cells stimulated with IFN-β (1000 U/ml) or TNF (100 ng/ml) were used as positive controls. Results are expressed as means ± SD. **p<0.01, as compared with wild type cells.

Supplementary Figure 12. Induction of IFN-β production by AGS cells infected with *H. pylori*.

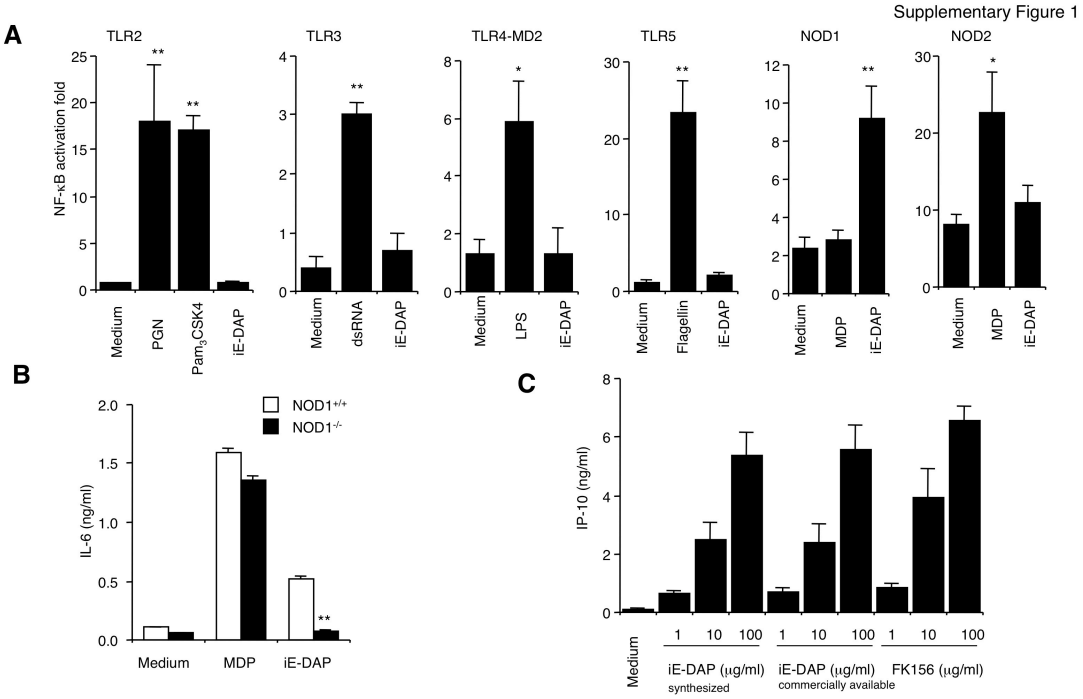
(A) AGS cells ($5x10^5$/ml) were infected with *H. pylori* for 24 hours and then culture supernatants were assayed for the presence of IP-10, IL-8, and IFN-β by ELISA. **(B)** Translocation of NF-κB subunits (p65, p50) and Stat1 in nuclear extracts was determined by Transfactor assay. Nuclear extracts from AGS cells stimulated with *H. pylori* for an hour and for 4 hours were used for p65/p50 and Stat1 assays, respectively. **(C)** Expression of ISGF3 components and IκBα in AGS cells stimulated with *H. pylori*. Whole cell extracts prepared from the stimulated cells at the indicated time points were then subjected to western blot analysis. Results are expressed as means ± SD. ** p<0.01, as compared with cells without infection.

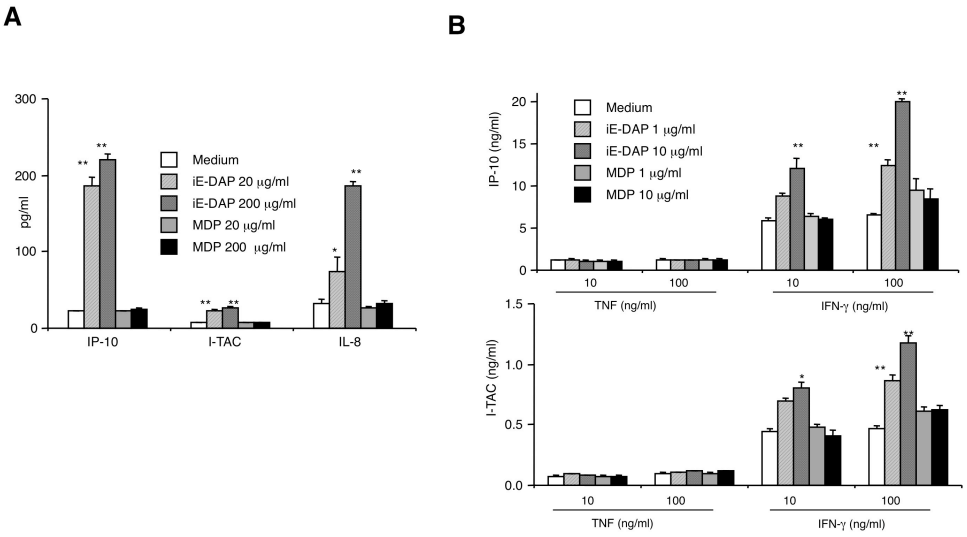
Supplementary Figure 13. NOD1-independent nuclear translocation of p65 in AGS cells infected with *H. pylori*.

AGS cells ($2.5X10^5$/ml in 12 well plate) were transfected with NOD1 siRNA, p65 siRNA, Stat1 siRNA or control siRNA (20 nM each) and after 48 hours cells were cultured for a further 24 hours with or without *H. pylori* as described in supplementary Fig. 12. After culture supernatant was harvested, cells were lysed and the whole cell lysates obtained were subjected to western blot analysis. **(A)** Expression of NOD1, p65, and Stat1 in AGS cells transfected with siRNAs. **(B)** Translocation of p65 and Stat1 in nuclear extracts was determined by Transfactor assay. Nuclear extracts from AGS cells stimulated with *H. pylori* for an hour and for 4 hours were used for p65 and Stat1 assays, respectively. **(C)** Production of IP-10, IL-8, and IFN-β by siRNA-transfected cells stimulated with *H. pylori* for 24 hours. Results are expressed as means ± SD. **p<0.01, compared with cells transfected with control siRNA.

Supplementary Figure 14. Induction of IFN-β production and activation of the ISGF3 signaling pathway by NOD1. NOD1 activation induces recruitment of RICK followed by an interaction between RICK and TRAF3 that leads to the production of IFN-β through activation of TBK1, IKKε and IRF7. IFN-β production induces production of IP-10 through transactivation of ISGF3 (Stat1-Stat2-IRF9 complex).

Supplementary Figure 1

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IP-10
I-TAC
IL-8
IP-10 (ng/ml)
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15
10
5
0
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iE-DAP 10 μg/ml
MDP 1 μg/ml
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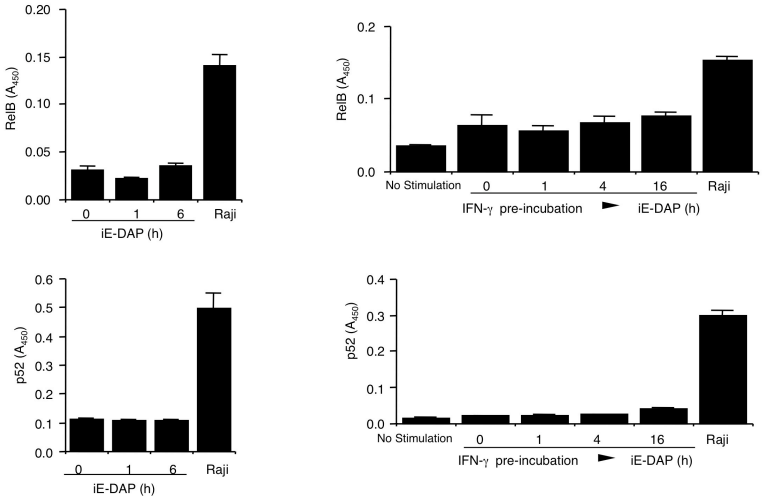
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0
1
6
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0.1
0.0
No Stimulation
0
1
4
16
Raji
IFN-γ pre-incubation
iE-DAP (h)
p52 (A450)
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0.5
0.4
0.3
0.2
0.1
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0
1
6
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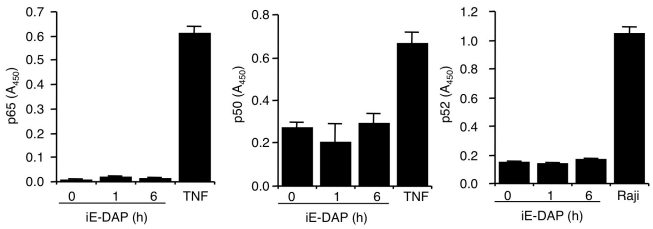
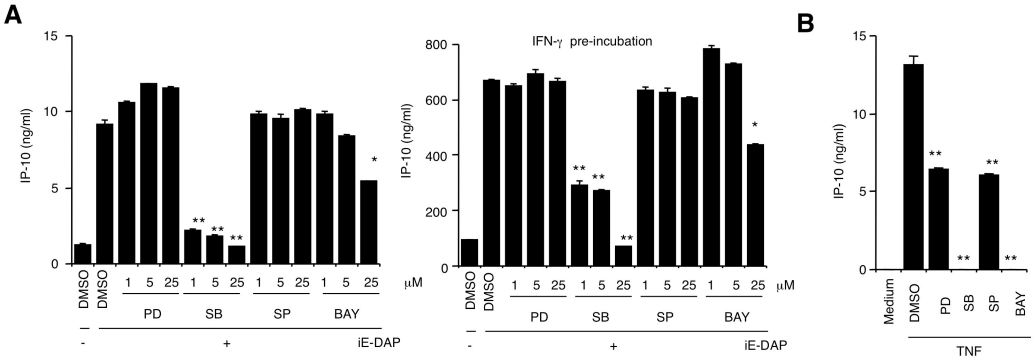
Supplementary Figure 4

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SB
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BAY
–
-
+
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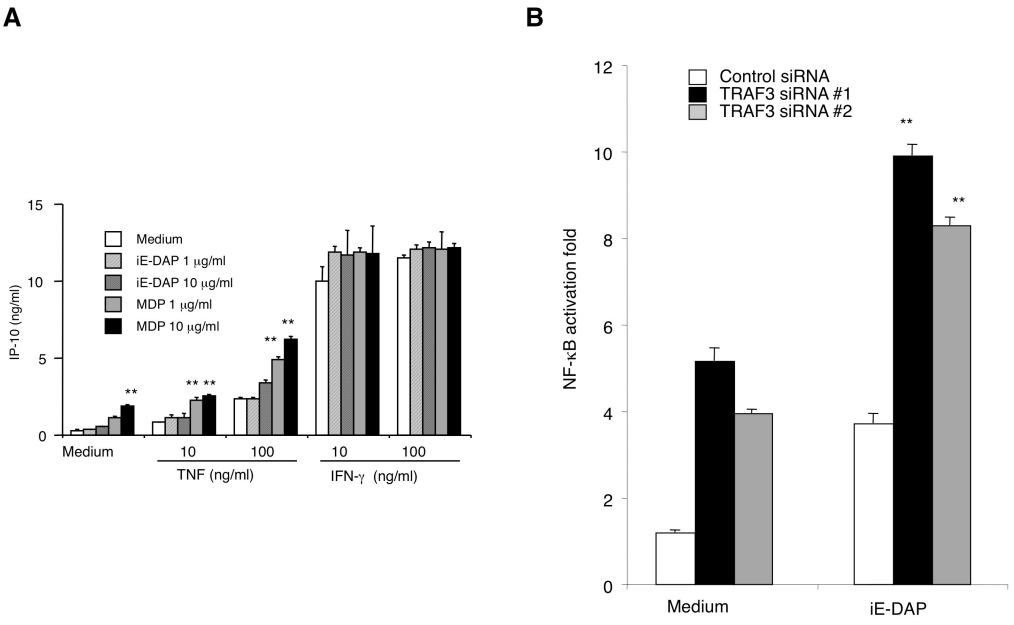
TNF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100
10
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Medium
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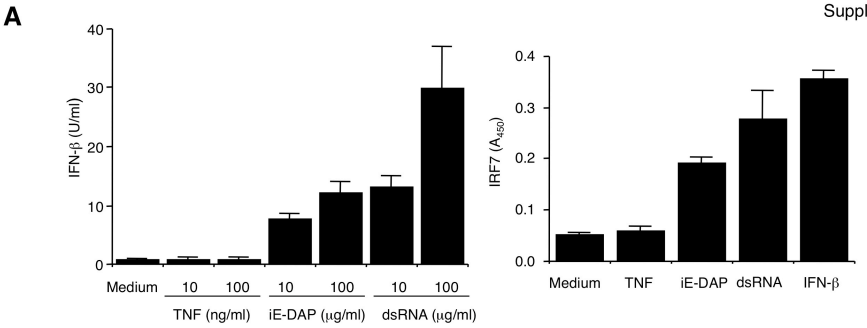
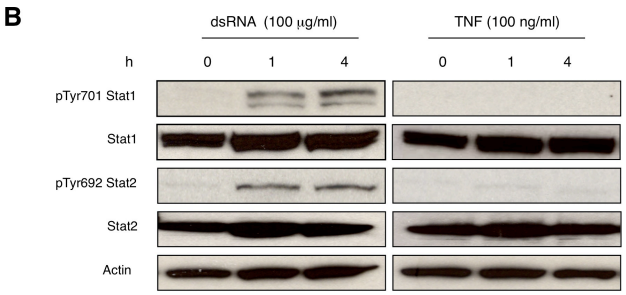
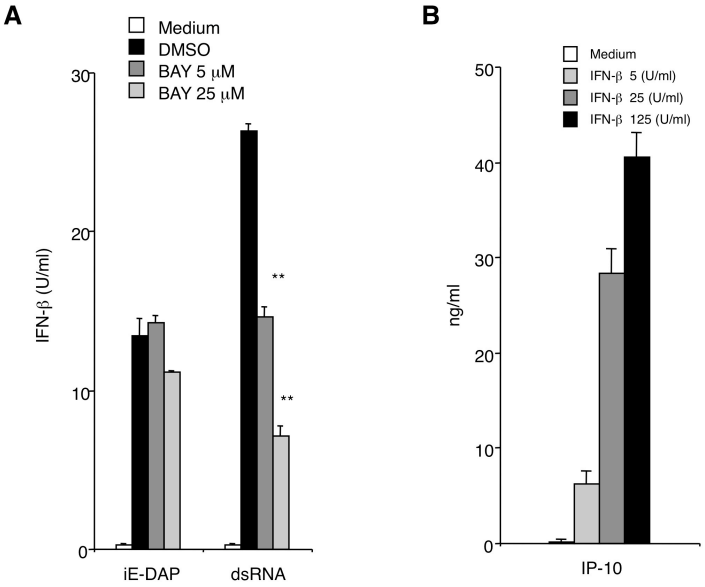
Supplementary Figure 7

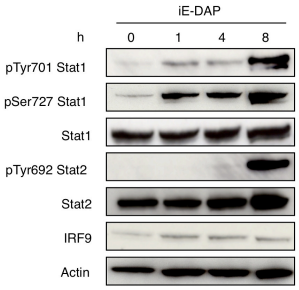


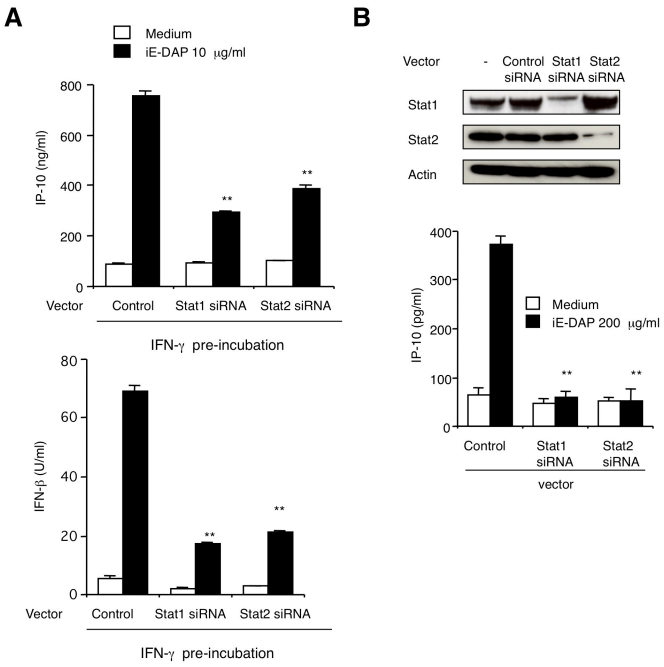


Supplementary Figure 8



Supplementary Figure 9

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IFN-β (U/ml)
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60
40
20
0
**
**
Vector
Control
Stat1 siRNA
Stat2 siRNA
IFN-γ pre-incubation
B
Vector
-
Control siRNA
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Stat2 siRNA
Stat1
Stat2
Actin
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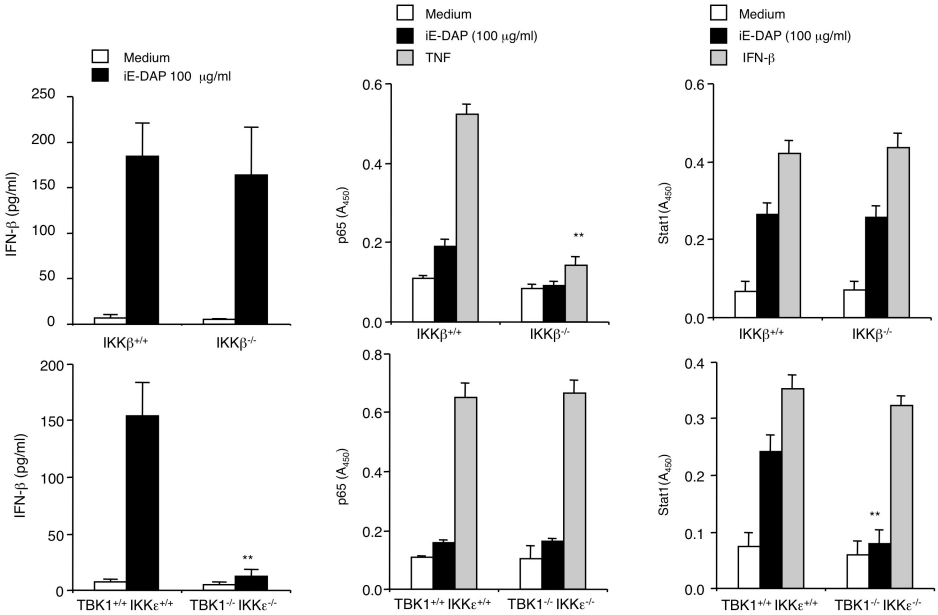
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50
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100
50
0
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TBK1+/+ IKKε+/+
TBK1-/- IKKε-/-
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TNF
p65 (A450)
0.6
0.4
0.2
0.0
IKKβ+/+
IKKβ-/-
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0.6
0.4
0.2
0.0
TBK1+/+ IKKε+/+
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Medium
iE-DAP (100 μg/ml)
IFN-β
Stat1(A450)
0.6
0.4
0.2
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IKKβ+/+
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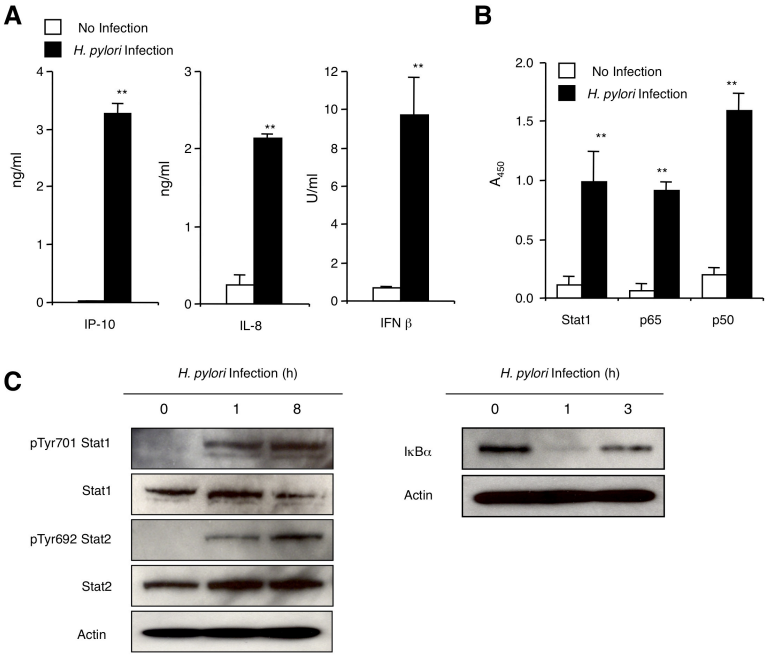
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Stat1
p65
p50
C
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IκBα
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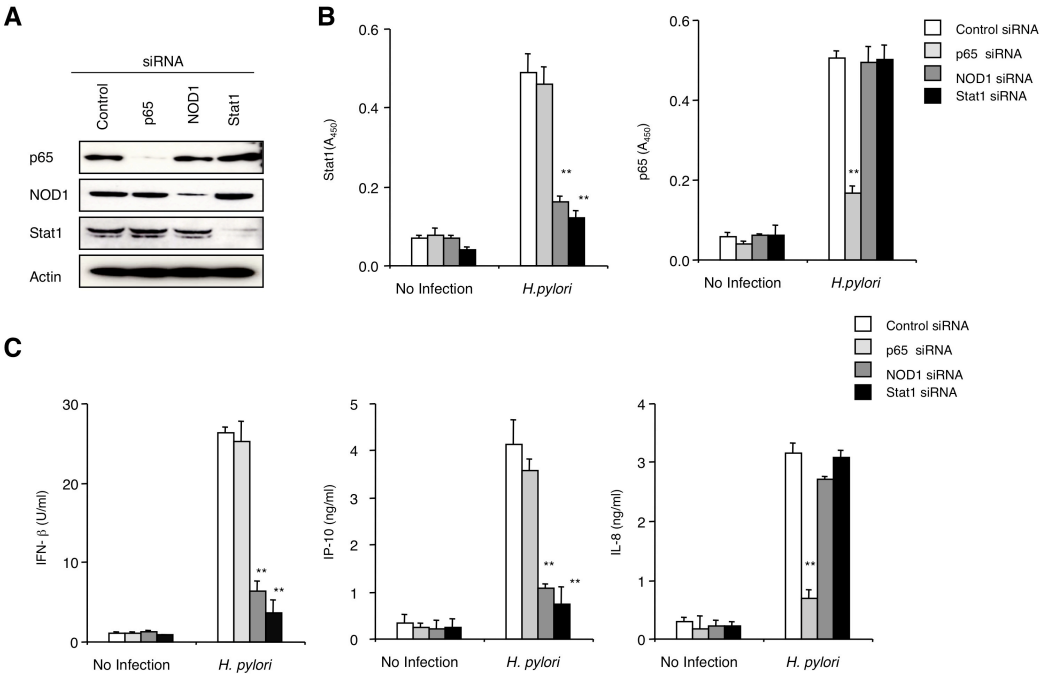
Supplementary Figure 13



Supplementary Figure 14