LEGENDS FOR SUPPLEMENTARY DATA

Supplementary Fig. 1. A. Expression of ROCK1 and ROCK2 in $CD4^+$ T cells as assessed by real-time PCR. Purified $CD4^+$ T cells from $Def6^{+/+}$ or $Def6^{trap/trap}$ mice were subjected to a primary stimulation and then either left unstimulated (unst) or restimulated (st) with αCD3 and αCD28 mAbs for 48 hrs. The mRNA expression of ROCK1 (left panel) and ROCK2 (right panel) genes was measured by real-time RT-PCR. The data are representative of three independent experiments. The error bars represent Mean ± S.D. *P≤0.006. ns= not statistically significant. **B.** Purified $CD4^+$ T cells from $Def6^{+/+}$ or $Def6^{trap/trap}$ mice were subjected to a primary stimulation and then either left unstimulated or restimulated with αCD3 and αCD28 mAbs for 48 hrs. Nuclear and cytoplasmic extracts were then prepared and analyzed by Western blot analysis using either a ROCK1 (left upper panel) or a ROCK2 (right upper panel) antibody as indicated. The blots were later stripped and reprobed with anti-lamin B (middle panels) or anti-β-tubulin (lower panels) antibody to assess the purity of the different fractions. Data are representative of three independent experiments. **C.** Intracellular staining of pERM in $CD4^+CD44^{int}CD62L^{hi}$ and $CD4^+CD44^{hi}CD62L^{lo}$ T cells from *Def6*$^{+/+}$ and *Def6*$^{trap/trap}$ mice was performed and analyzed by FACS. Results are demonstrated as overlaid histograms after gating on $CD4^+$ T cells. Data are representative of three independent experiments.

Supplementary Figure 2. A. Purified $CD4^+$ T cells from *Def6*$^{+/+}$ (white bars) or *Def6*$^{trap/trap}$ (black bars) DO11.10 mice were subjected to a primary stimulation

and then restimulated with αCD3 and αCD28 for 48 hrs in presence or absence of either 10 or 30 μM of Y27632 (Y(10) and Y(30), respectively) or 10 or 30 μM of Fasudil (F(10) or F(30), respectively) as indicated. Supernatants were then collected and assayed for IL-2 (left upper panel), IFN-γ (right upper panel), IL-4 (left lower panel), and IL-10 (right lower panel) production by ELISA. The data are representative of three independent experiments. The error bars represent Mean ± S.D. *P≤0.03, ns= not significant. **B.** Purified $CD4^+$ T cells from *Def6*$^{+/+}$ (white bars) or *Def6*$^{trap/trap}$ (black bars) mice were subjected to a primary stimulation and then restimulated with αCD3 and αCD28 for 48 hrs in the presence or absence of 30 μM of Y27632 (Y(30)) or 30 μM of Fasudil (F(30)). Supernatants were then collected and assayed for IL-17 (left panel) and IL-21 (right panel) production by ELISA. The data are representative of three independent experiments. The error bars represent Mean ± S.D. *P≤0.0009. **C.** Purified $CD4^+$ T cells from *Def6*$^{+/+}$ (white bars) or *Def6*$^{trap/trap}$ (black bars) mice were stimulated as indicated above. 24 hrs later cells were collected and the mRNA expression of IL-17 (left panel), IL-21 (middle panel) and RORγt (right panel) genes measured by real-time RT-PCR. The data are representative of three independent experiments. The error bars represent Mean ± S.D. *P≤0.002. **D.** $CD4^+$ T cells from *Def6*$^{+/+}$ or *Def6*$^{trap/trap}$ mice were infected with control YFP-RV (VECTOR) or DN-ROCK2-expressing retroviruses. Cells were harvested after 6 days, sorted for YFP^+ cells and restimulated with αCD3 and αCD28 for 48 hrs. Supernatants were then collected and assayed for IL-10 production by ELISA. The data are representative of three independent experiments. The error bars represent Mean ± S.D. **E.** $CD4^+$ T cells

from *Def6*$^{+/+}$ or *Def6*$^{trap/trap}$ mice were infected with control GFP-RV (VECTOR) or DN-RhoA-expressing retroviruses. Cells were harvested after 6 days, sorted for GFP^{+} cells and restimulated with αCD3 and αCD28 for 48 hrs. Supernatants were then collected and assayed for IL-10 production by ELISA. The data are representative of three independent experiments. The error bars represent Mean ± S.D.

Supplementary Figure 3. A. Detection of a Phosphorylated Tryptic Peptide of IRF4 by LC-MS. A tryptic digest of IRF4 was analyzed by LC-MS/MS on a Micromass Qtof mass spectrometer with a Dionex Ultimate nanoflow LC. The inserted panel shows the MS spectrum of the ion 568.79 corresponding to the phosphorylated peptide SIRHp[SS]IQE and the resulting MS/MS spectrum. The detected y and b series ions are indicated along the MH-98, b_6-98, b_7-98, and y_6-98 ions which reflect the neutral loss of phosphoric acid from the Ser(P) side chain. Although phosphorylation is shown on Ser6 in the figure, the MS and MS/MS spectra are consistent with phosphorylation on either Ser5 or Ser6. **B.** Detection of a Phosphorylated Asp-N Peptide of IRF4 by LC-MS. An endoproteinase Asp-N digest of IRF4 was analyzed by LC-MS/MS on a Micromass Qt of mass spectrometer with a Dionex Ultimate nanoflow LC. The MS spectrum of the phosphorylated peptide ion at 569.99 is shown along with the MS/MS spectrum. The MH-98, b_{12}-98, and b_{11}-98 ions reflect the neutral loss of phosphoric acid from the Ser(P) side chain.

Supplementary Figure 4. A. Expression of IRF4 and IRF4AA in the transduced T cells shown in Fig. 4C as assessed by FACS. Results are represented as histograms. UNTX=untransfected control. **B.** $CD4^+$ T cells from *Def6*$^{+/+}$*Irf4*$^{+/+}$ (Wt) or *Def6*$^{trap/trap}$*Irf4*$^{-/-}$ mice were infected with control YFP-RV (Vector), Wt IRF4, IRF4AA (AA), IRF4A446 (A446) single mutant or IRF4A447 (A447) single mutant expressing retroviruses. Cells were harvested after 6 days, sorted for YFP^+ cells and restimulated with PMA and ionomycin for 4 hrs. **C.** Expression of IRF4, IRF4D446, and IRF4D447 in the transduced $CD4^+$ T cells shown in Fig. 4D as assessed by FACS. Results are represented as histograms. **D.** 293T cells were transfected with an empty vector (MOCK), with an expression vector for wt IRF4 (IRF4), or with expression vectors for IRF4 mutants in which Serine 446 and Serine 447 are mutated to alanines (AA) or in which Serine 446 and Serine 447 are separately mutated to aspartic acid (D446 and D447, respectively). Nuclear extracts were analyzed by Western blotting using an antibody generated against an IRF4 peptide containing phosphorylated forms of Serine 446 and Serine 447 (pIRF4) (upper panel). The blot was later stripped and reprobed with an antibody against total IRF4 (lower panel). **E.** 293T cells were transiently transfected with an empty vector (MOCK), or with an expression vector for IRF4ΔDBD mutant. Whole cell lysates were then prepared and immunoprecipitated with an IRF4 antibody. The immunoprecipitates were subjected to *in vitro* kinase reactions with or without purified constitutively active ROCK2 kinase (CAROCK2). 30 μM of Y27632 (Y(30)) was added in selected reaction samples as indicated. The reaction samples were resolved by 10% SDS-PAGE and analyzed by Western

blotting using an antibody that recognizes phosphorylated IRF4 (pIRF4) (upper panel). The blot was later stripped and reprobed with an antibody against total IRF4 (lower panel). The white line indicates that the lanes were run on the same gel but were noncontiguous.

Supplementary Figure 5. Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from wt mice were stimulated either in the absence or in the presence of TGF-β, IL-6, or both TGF-β and IL-6 for 5 days. ROCK2 kinase activity was assayed by incubating the immunoprecipitated ROCK2 with purified recombinant MYPT1 (rMYPT1) as ROCK2 substrate. Phosphorylated rMYPT1 (pMYPT1) was detected by Western blot analysis using an anti-phospho-MYPT1 antibody (upper panel). Lower panel shows the total ROCK2 levels in the input samples. Data are representative of three independent experiments. **B.** Expression of IRF4 and IRF4AA in the transduced T cells shown in Fig. 5D as assessed by FACS. Results are represented as histograms. UNTX=untransfected control.

Supplementary Figure 6. A. Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from *Rock2*$^{+/+}$ or *Rock2*$^{+/-}$ mice were stimulated under T_H0 or T_H-17 conditions for 5 days. Cells were harvested, stimulated with PMA and ionomycin for 5 hrs and expression of IL-17 and IL-4 assessed by intracellular FACS. Data are representative of two independent experiments. **B.** Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from *Rock2*$^{+/+}$ or *Rock2*$^{+/-}$ mice were stimulated under T_H0 or T_H-17 conditions for 5 days. Cells were harvested and

expression of FoxP3 assessed by FACS. Data are representative of two independent experiments. **C.** Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from *Rock2*$^{+/+}$ or *Rock2*$^{+/-}$ mice were stimulated under T_H1 conditions for 5 days. Cells were harvested, stimulated with PMA and ionomycin for 5 hrs and expression of IFN-γ assessed by intracellular FACS. Data are representative of two independent experiments. **D.** Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from *Rock2*$^{+/+}$ or *Rock2*$^{+/-}$ mice were stimulated under T_H2 conditions for 5 days. Cells were harvested, stimulated with PMA and ionomycin for 5 hrs and expression of IL-4 assessed by intracellular FACS. Data are representative of two independent experiments. E. Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from *Rock2*$^{+/+}$ or *Rock2*$^{+/-}$ mice were stimulated under either T_H0, T_H2, or T_H-17 conditions for 3 days. Production of IL-21 (left panel) and IL-10 (right panel) was assessed by ELISA. Data are representative of two independent experiments. **F.** Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells from *Rock2*$^{+/+}$ or *Rock2*$^{+/-}$ mice were stimulated under either T_H0, T_H2, or T_H-17 conditions for 3 days. Cells were then harvested and ChIP assays performed with either an IRF4 antibody or a control Ab. Quantification of IRF4 binding to the IL-17A, IL-21, and RORγt promoters was performed using qPCR and is shown as percent input. The data are representative of two independent experiments.

Supplementary Figure 7. Percentages of splenic $CD4^{+}$ T cells from *Def6*$^{+/+}$ DO11.10 mice (grey circles), *Def6*$^{trap/trap}$ DO11.10 mice (black circles), and *Def6*$^{trap/trap}$ DO11.10 mice treated with Fasudil (white circles) expressing effector

markers ($CD44^{hi}CD62L^{lo}$) (**A)**, ICOS **(B)**, CD69 **(C)** or Treg markers **(D)** as assessed by FACS analysis. **E.** Percentages of splenic $B220^{+}PNA^{+}$ cells (left panel), $B220^{+}sIgG1^{+}$ (middle panel), or $B220^{lo}CD138^{+}$ cells (right panel) from *Def6*$^{+/+}$DO11.10 mice (grey circles), *Def6*$^{trap/trap}$DO11.10 mice (black circles), and *Def6*$^{trap/trap}$DO11.10 mice treated with Fasudil (white circles). *P≤0.006.

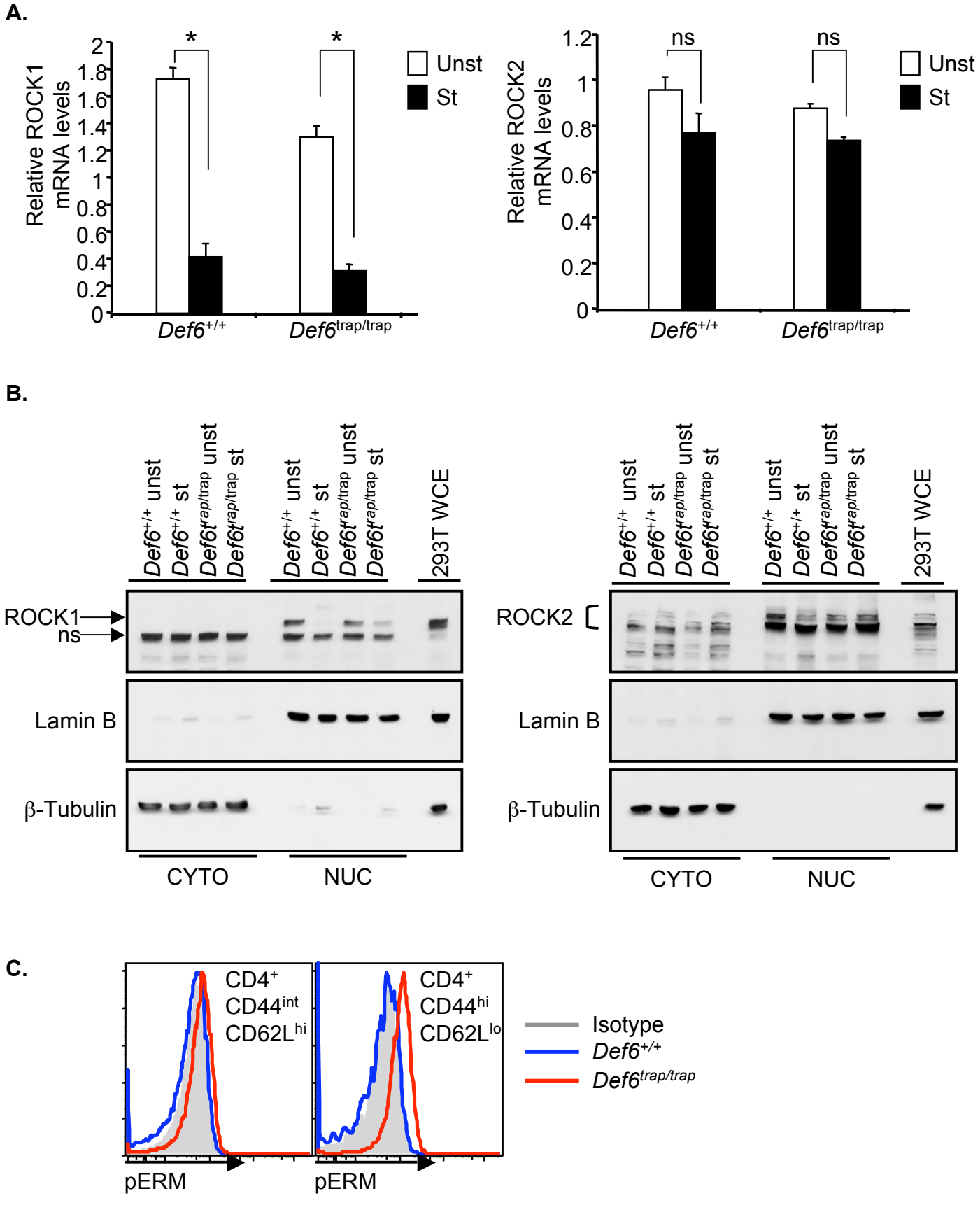
Supplementary Figure 8. A. Percentages of $CD4^{+}$ T cells from MRL/lpr mice (black circles) and MRL/lpr mice treated with Fasudil (white circles) expressing effector markers ($CD44^{hi}CD62L^{lo}$) (left panel) or ICOS (right panel) as assessed by FACS analysis. **B.** Left panel: Percentages of $CD8^{+}$ T cells from MRL/lpr mice (black circles) and MRL/lpr mice treated with Fasudil (left panel) (white circles). ns= not statistically significant. Right panel: Percentages of DN T cells from MRL/lpr mice (black bar) and MRL/lpr mice treated with Fasudil (white bar) as assessed by FACS analysis. ns= not statistically significant. **C.** Histopathologic analysis (hematoxylin/eosin staining) of the kidneys of MRL/lpr mice and MRL/lpr mice treated with Fasudil (magnification 200X). The graph shows the renal histopathologic score of kidneys of MRL/lpr mice (n=7) (black circles) and MRL/lpr mice treated with Fasudil (n=5) (white circles). ns= not statistically significant. **D.** Deposition of immunoglobulin complexes in glomeruli of MRL/lpr mice and MRL/lpr mice treated with Fasudil. Left panels show representative photomicrographs of a kidney from either an untreated MRL/lpr mouse or a Fasudil treated MRL/lpr mouse as detected by immunofluorescence with anti-IgG

(upper panel) and anti-C3 (lower panel) staining. The graphs on the right represent the renal immune complex deposition scores. *P≤0.04.

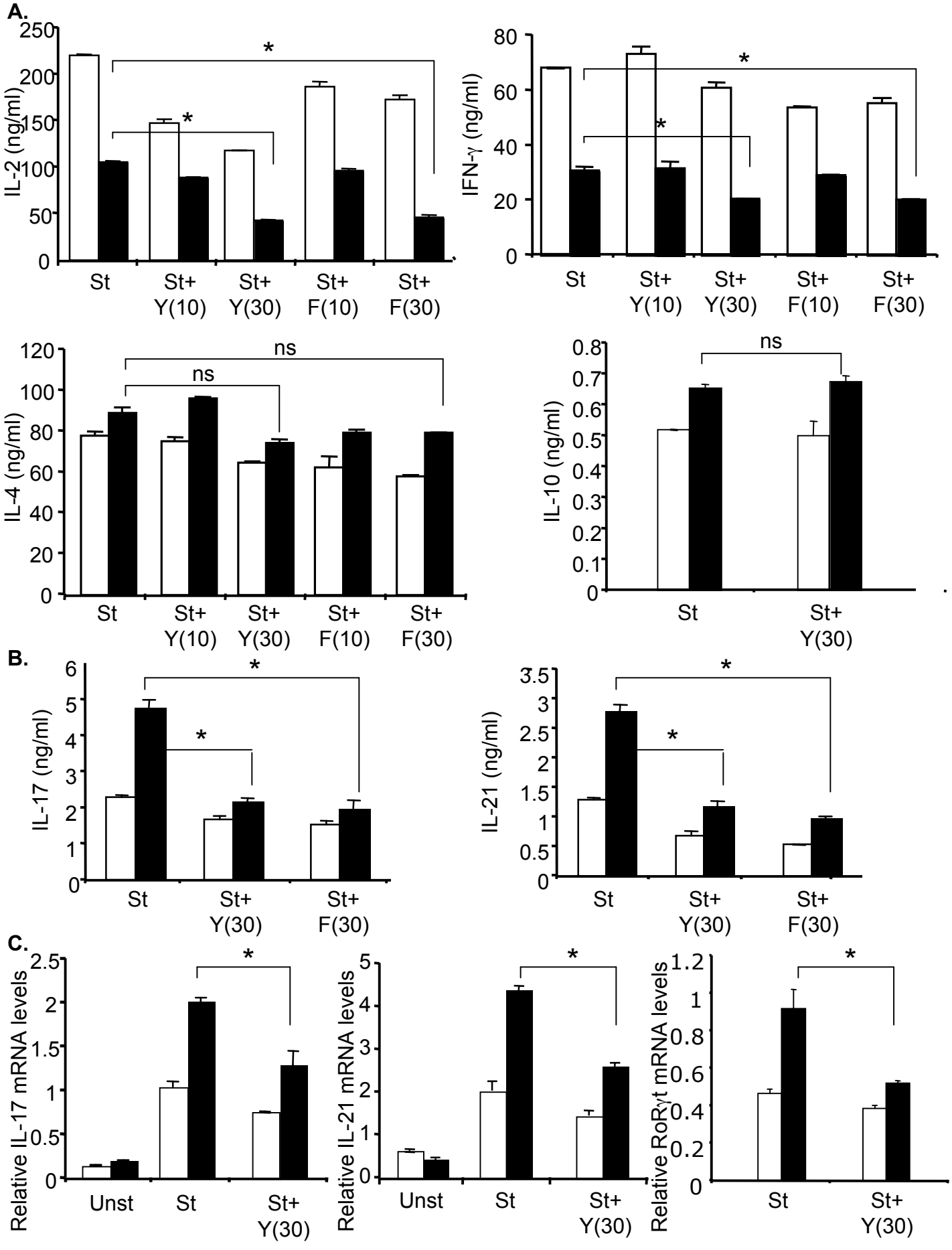
Supplementary Figure 9. Model of the role of ROCK2 in $CD4^+$ T helper cells.

Supplementary Figure 10. Effects of ROCK inhibition on B cell proliferation. **A.** Cells were stimulated with soluble anti-CD40 (5 μg/ml) and anti-IgM (10 μg/ml) and IL-21 (50ng/ml) in the presence/ absence of 30 μM of Y27632 (Y(30)) or 30 μM of Fasudil (F(30)) for 48h. The culture was then pulsed with [^{3}H]thymidine for 12 hours. Data are representative of three independent experiments. **B.** Effects of ROCK inhibition on B cell differentiation. Cells were stimulated with soluble anti-CD40 (5 μg/ml) and anti-IgM (10 μg/ml) and IL-21 in the presence/ absence of 30 μM of Y27632 (Y(30)) or 30 μM of Fasudil (F(30)) for 4 days and percentages of $CD138^+$ cells were assessed by FACS. The data are representative of three independent experiments. The error bars represent Mean ± S.D.

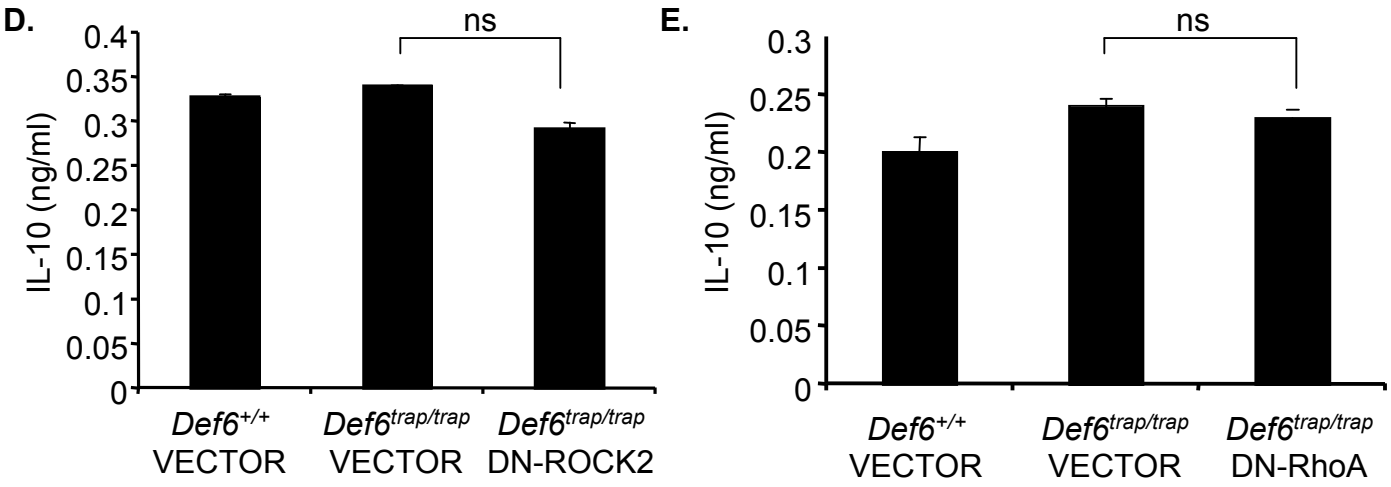
Supplementary Figure 1 Biswas et al.



Supplementary Figure 2 Biswas et al.

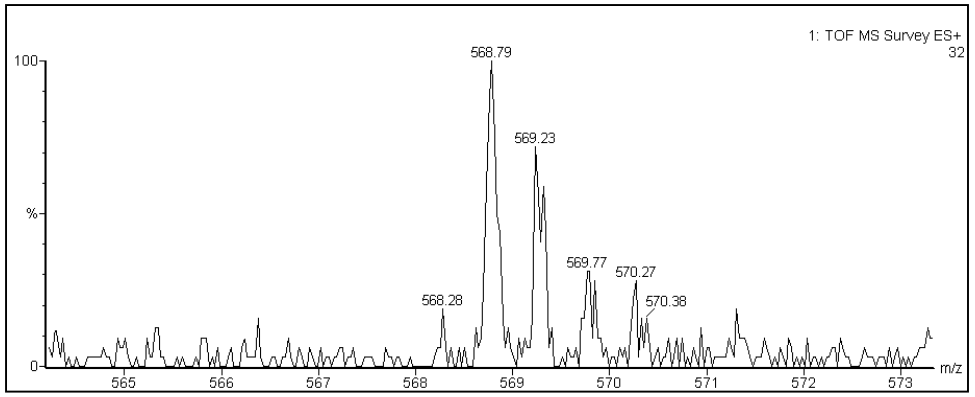


Supplementary Figure 2 Biswas et al.



Supplementary Figure 3 Biswas et al.

A.

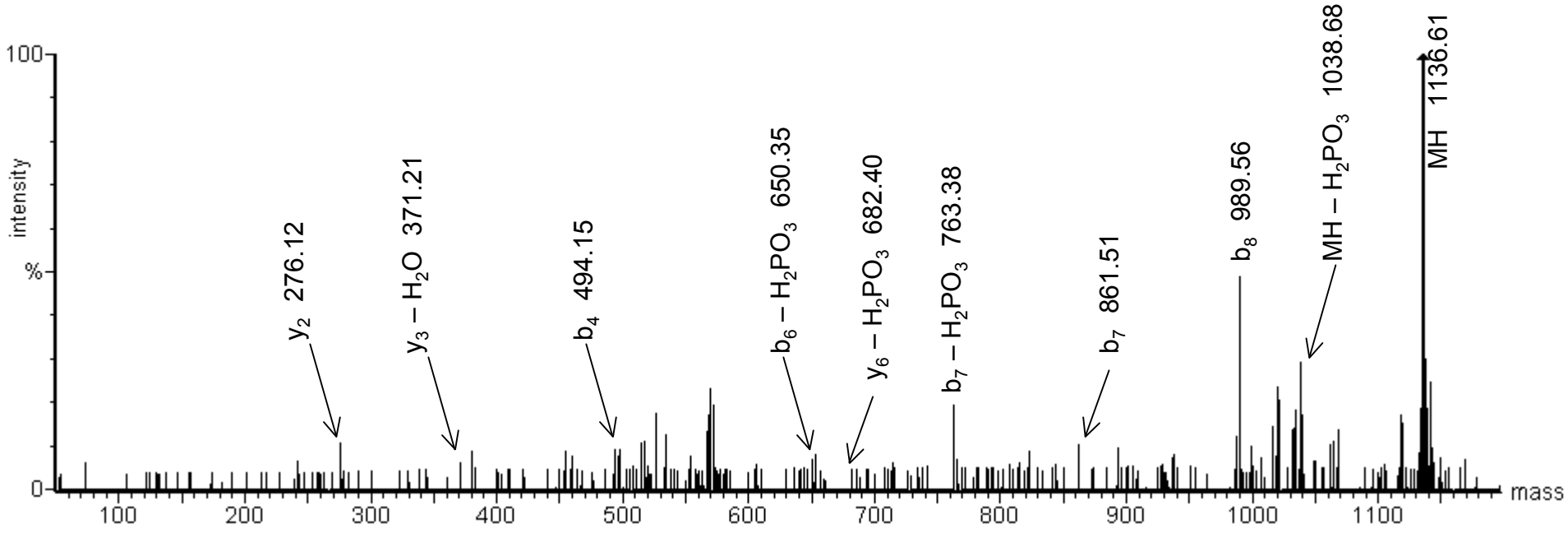


b_4 b_6 b_7 b_8

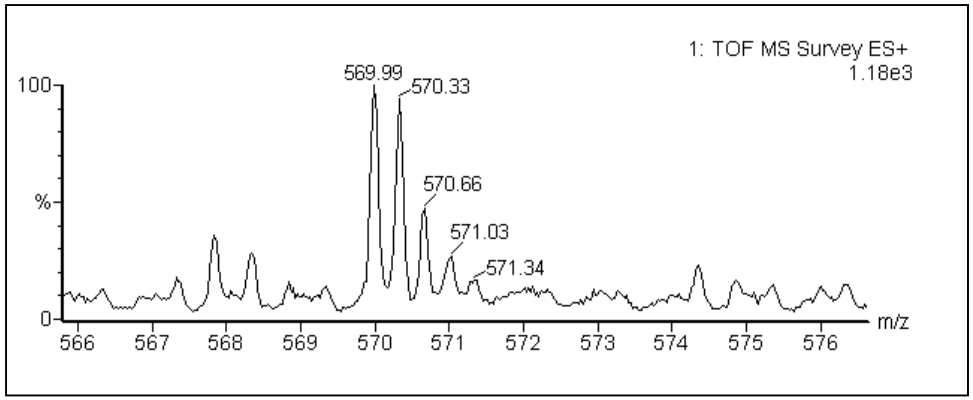
S I R [H] S pS [I] [Q] E

y_6 y_3 y_2

m/z 568.79
mass 1135.58



B.

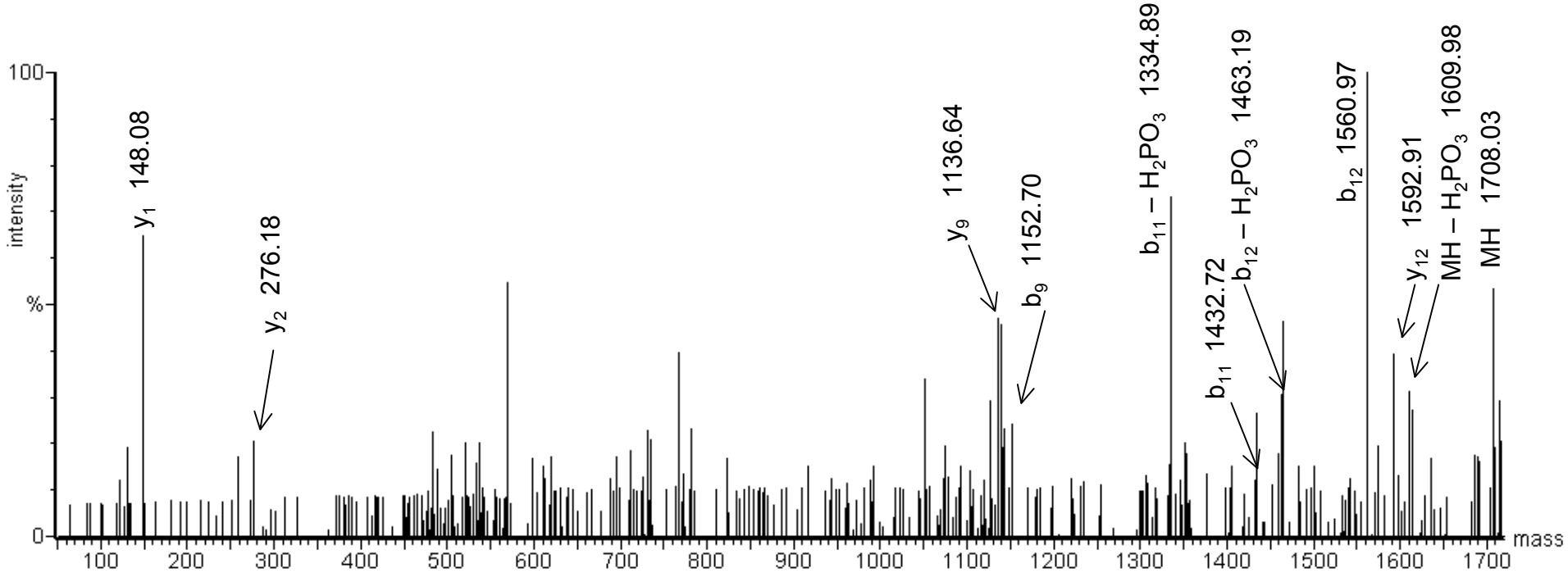


b_9 b_{11} b_{12}

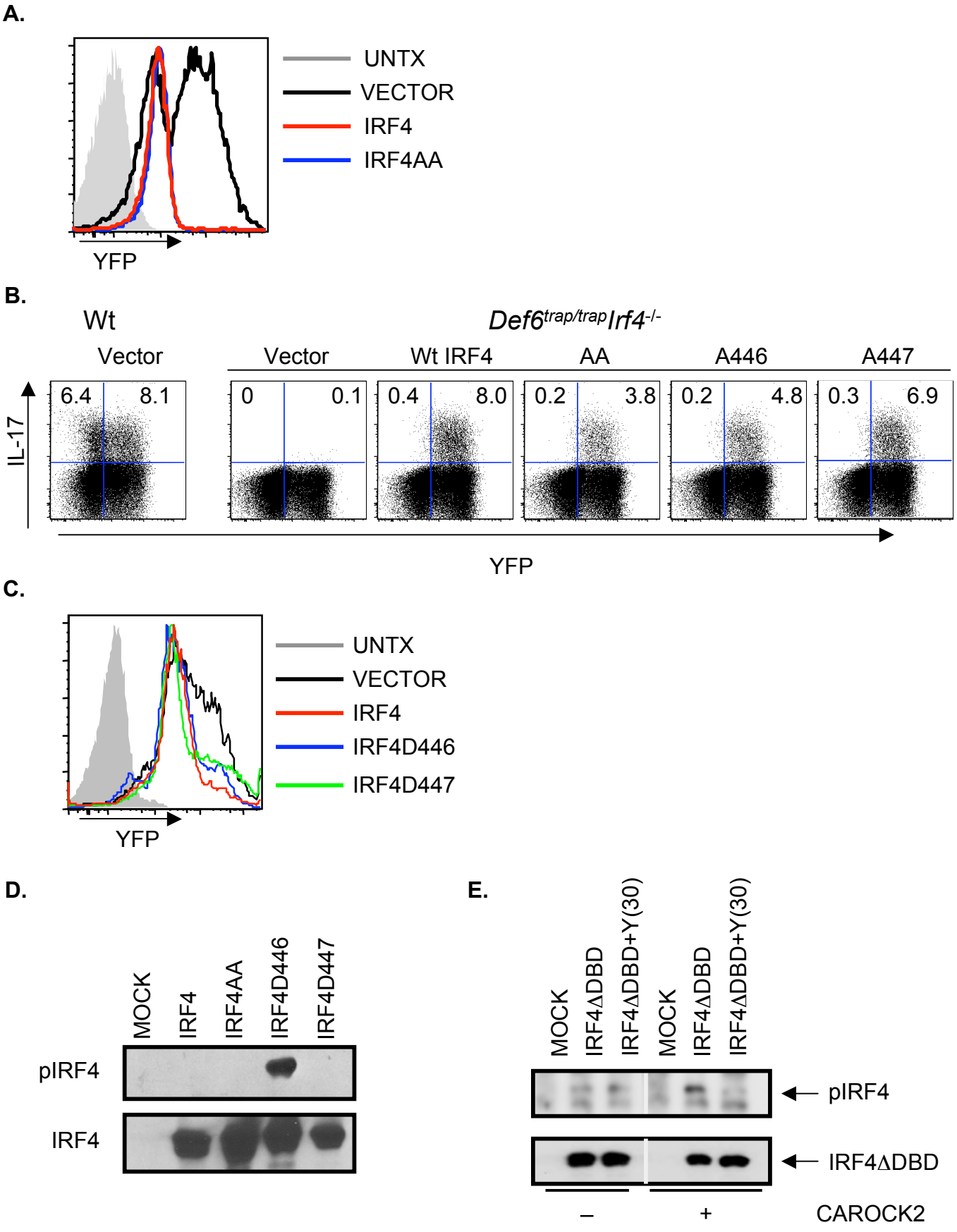
D Y H R S I R H S pS I Q E

y_{12} y_9 y_2 y_1

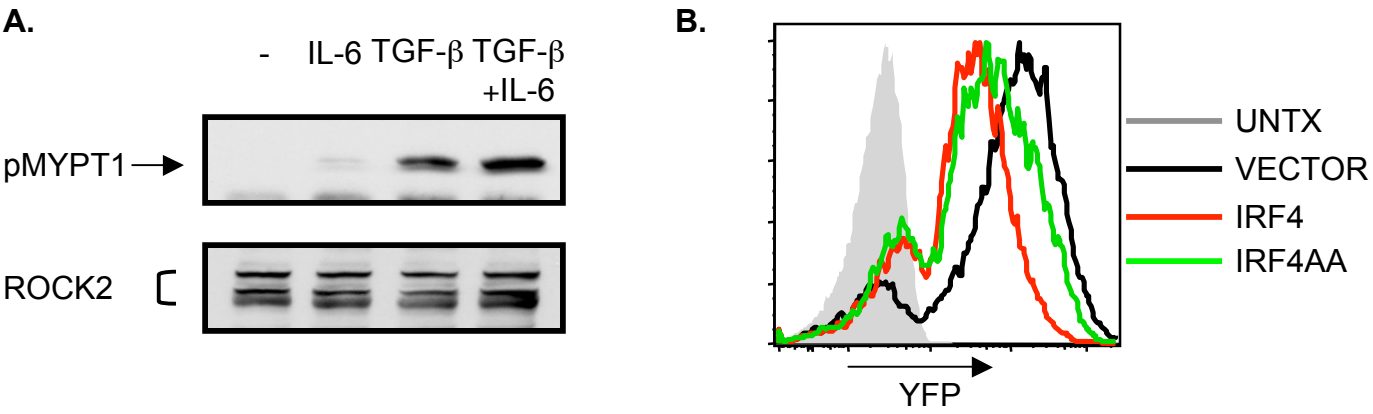
m/z 569.99
mass 1706.97



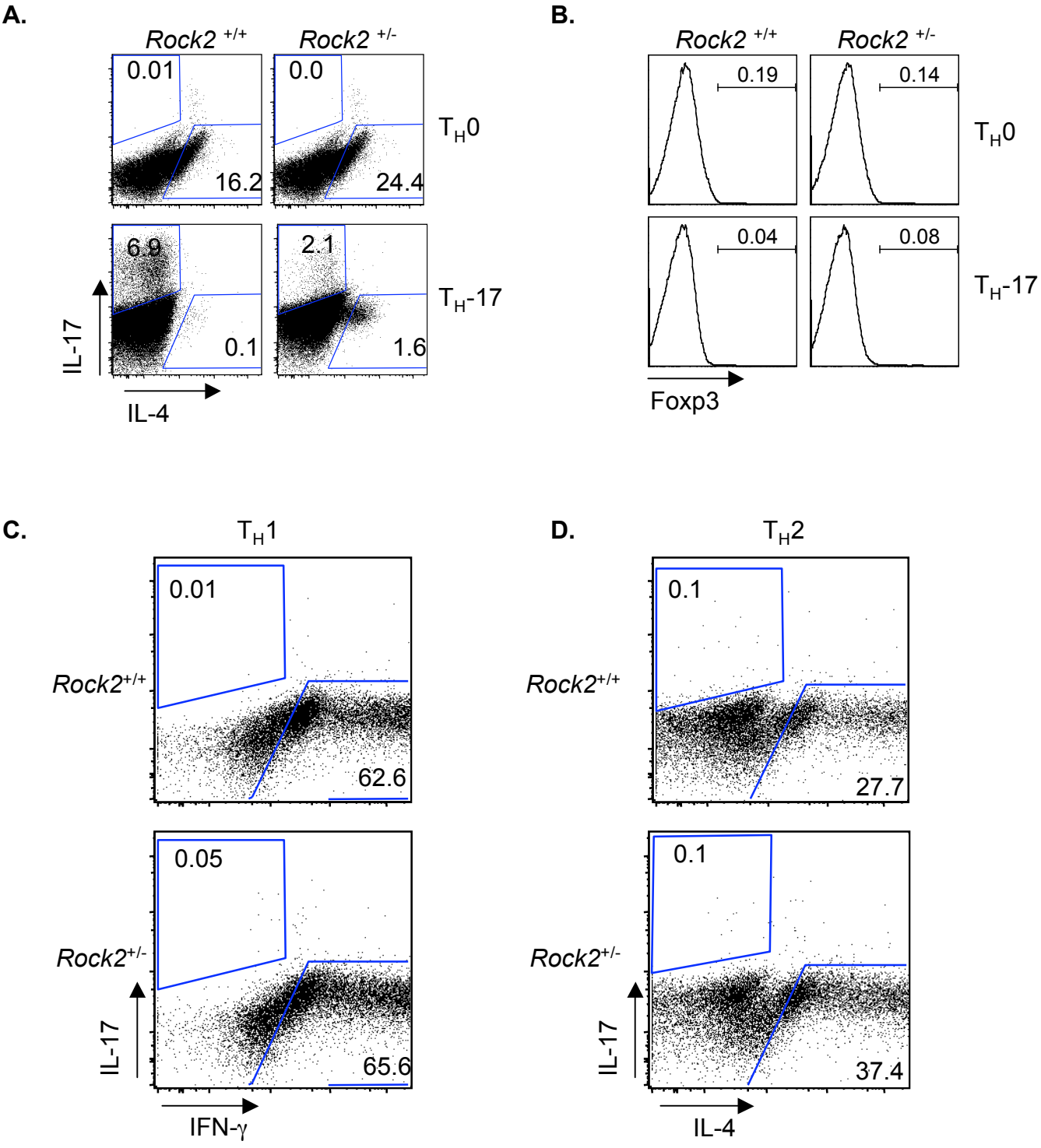
Supplementary Figure 4 Biswas et al.



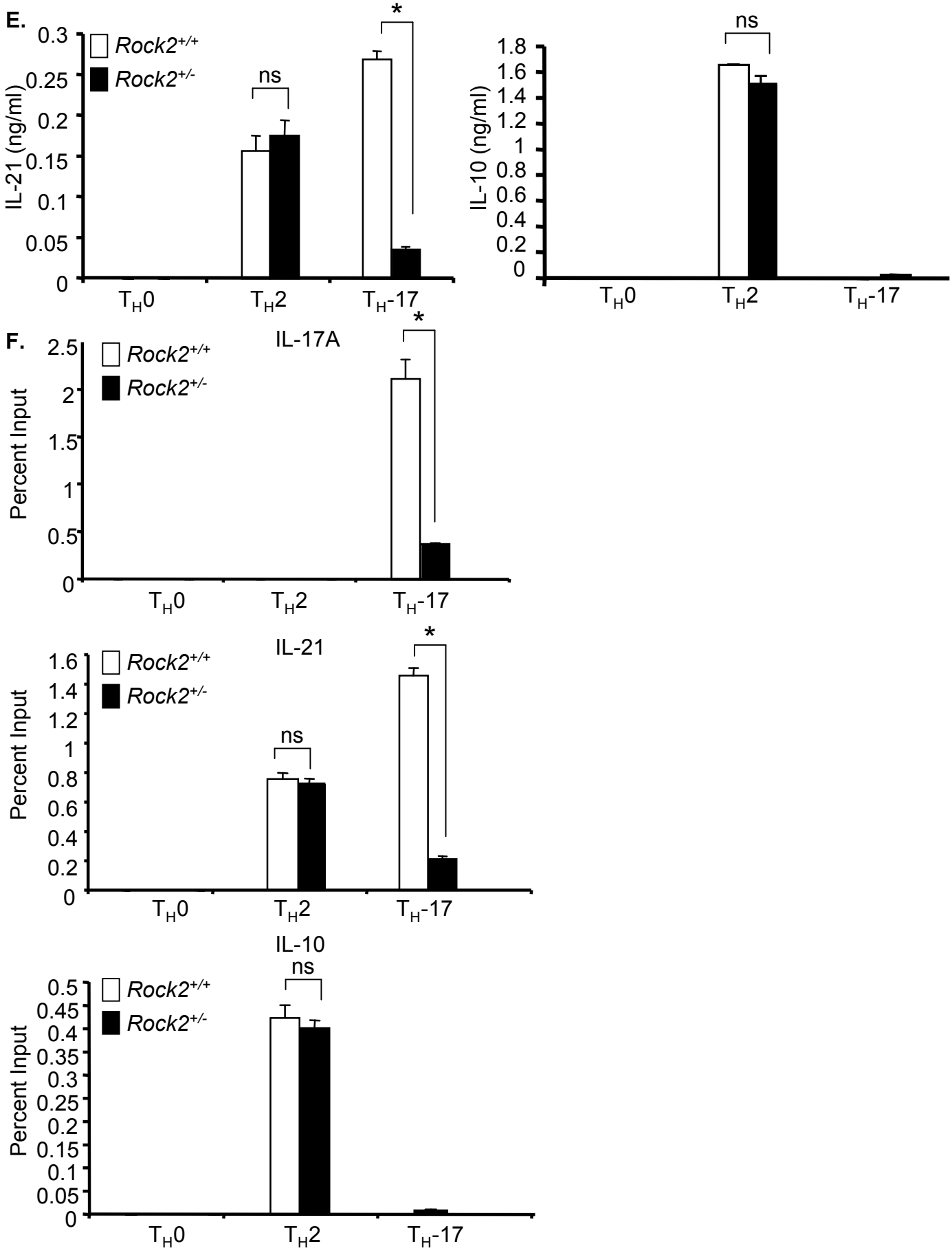
Supplementary Figure 5 Biswas et al.



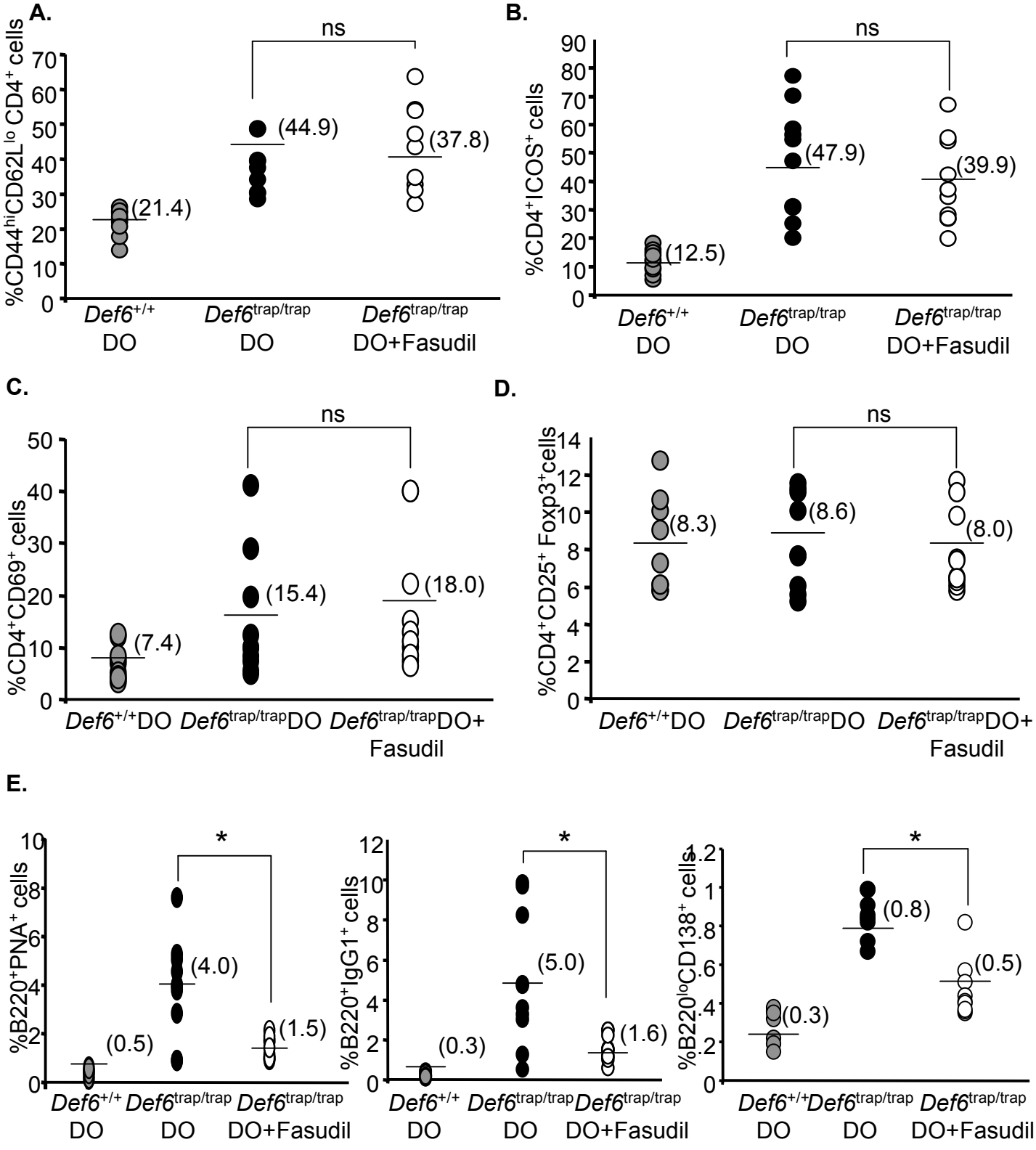
Supplementary Figure 6 Biswas et al.



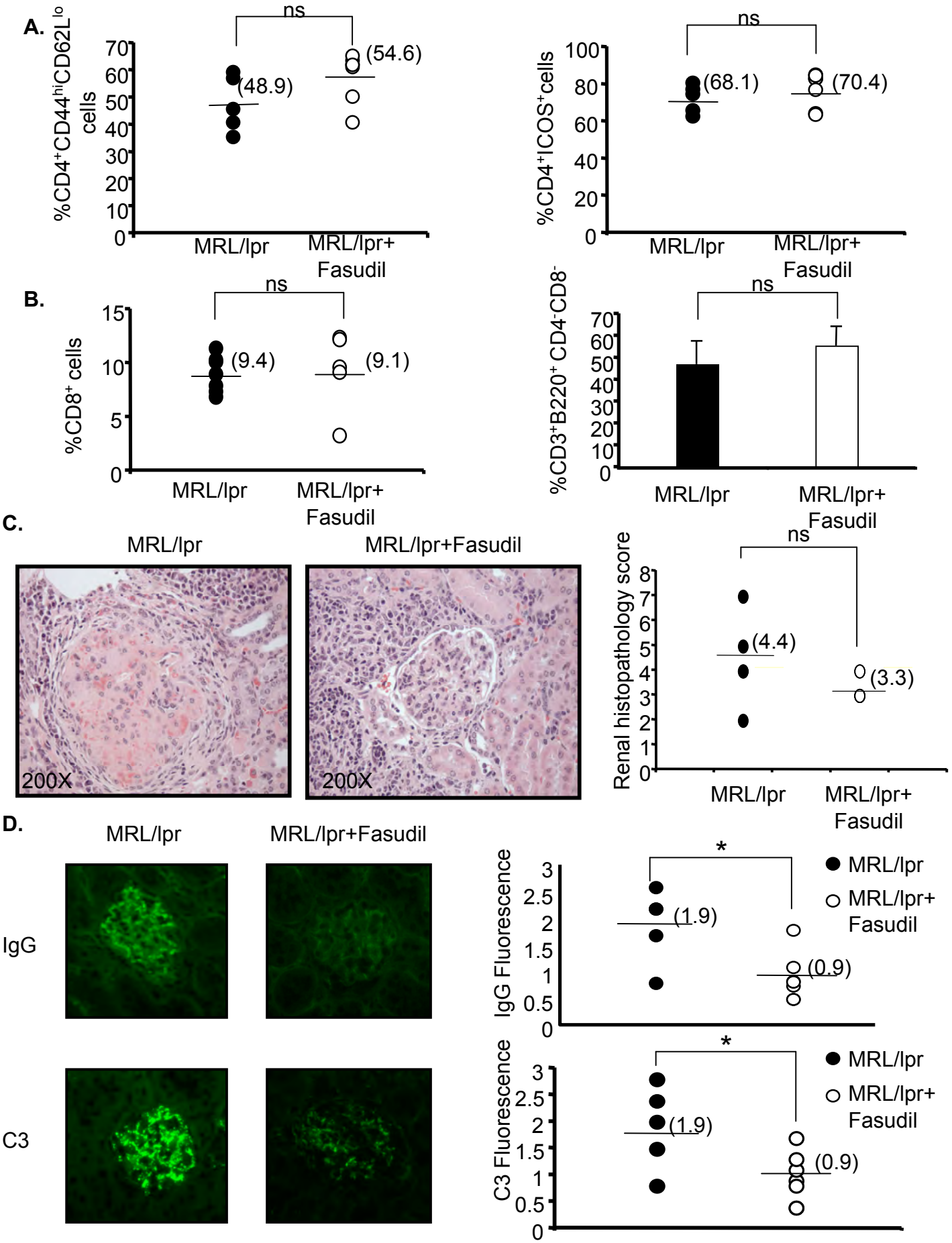
Supplementary Figure 6 Biswas et al.



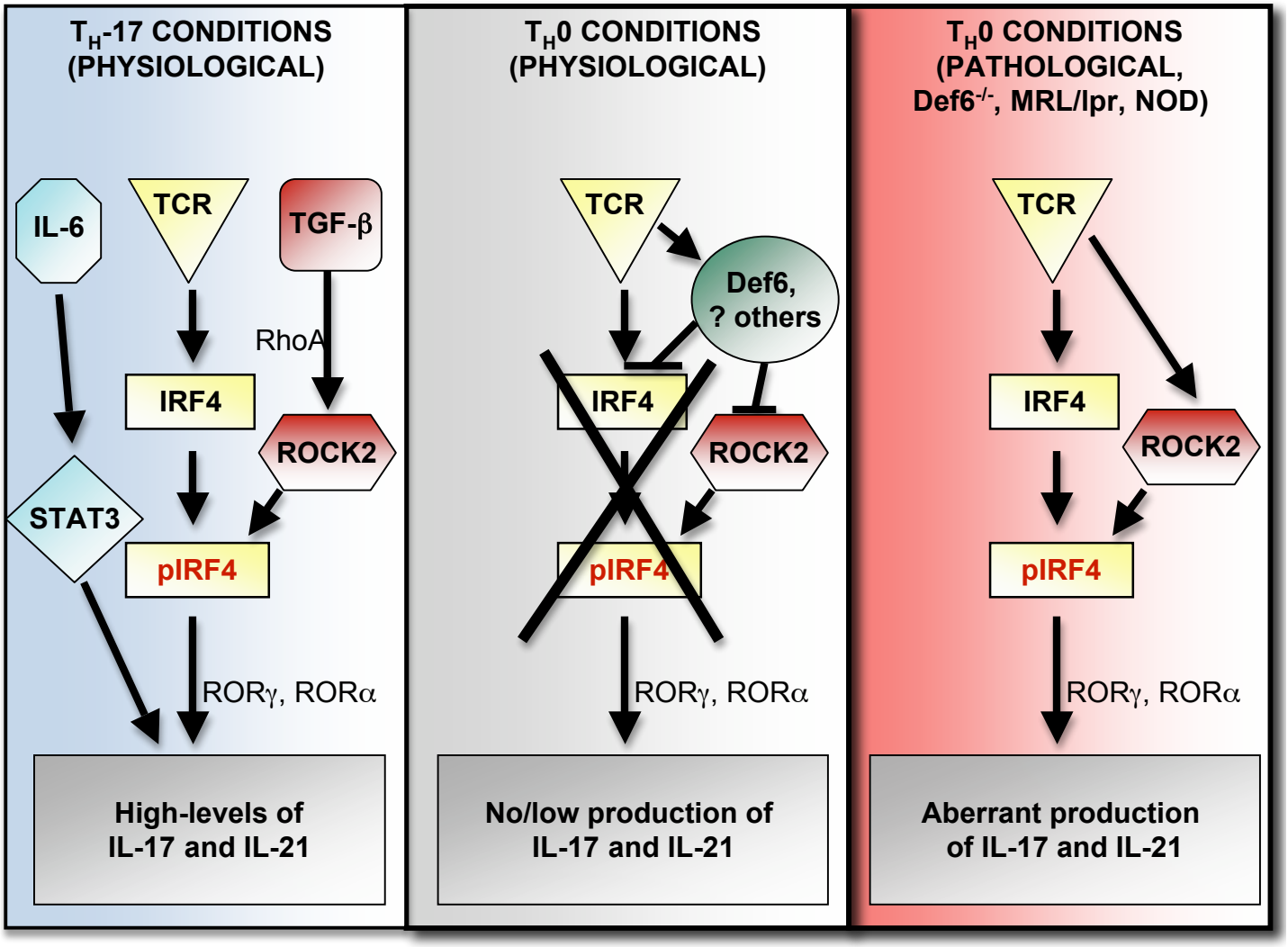
Supplementary Figure 7 Biswas et al.



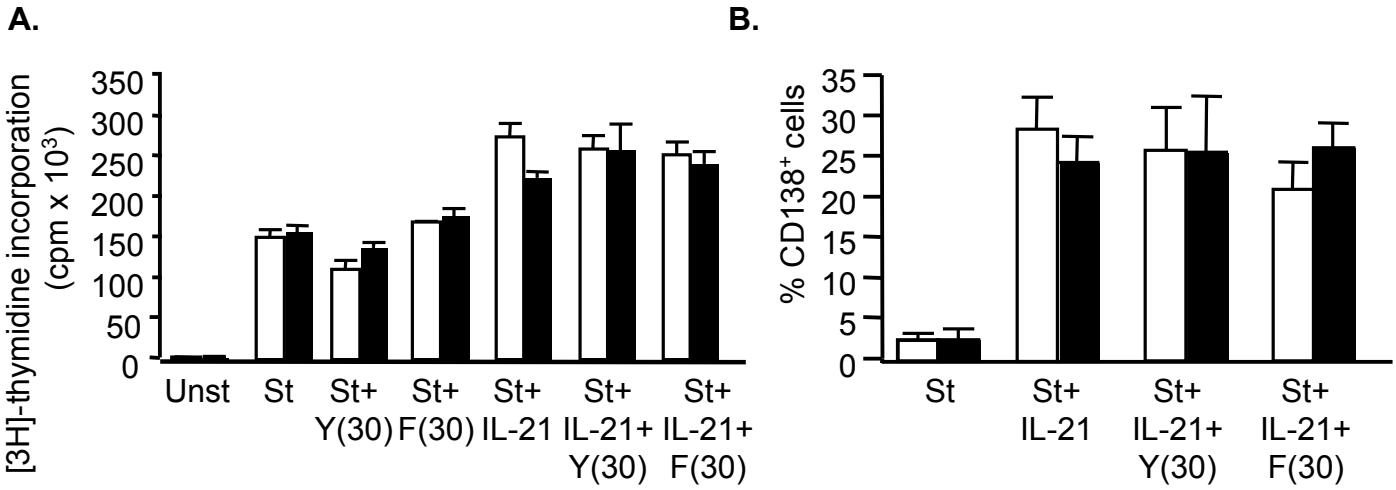
Supplementary Figure 8 Biswas et al.



Supplementary Figure 9 Biswas et al.



Supplementary Figure 10 Biswas et al.



Supplementary Table I Biswas et al.

Hematopoietic cellularity in *Rock2*$^{+/-}$ mice[a&b]

Tissue	Wt	*Rock2*$^{+/-}$
Thymus		
Total cell number (X10^6)	196.2 ± 13.8	197.2 ± 17.3
CD4+CD8+ (%)	80.4 ± 2.9	79.7 ± 3.2
CD4+CD8- (%)	10.0 ± 2.1	10.1 ± 2.0
CD4-CD8+ (%)	3.6 ± .88	2.3 ± .32
Spleen		
Total cell number (X10^6)	94.2 ± 8.8	83.3 ± 7.8
CD4+CD8- (%)	19.9 ± 1.4	23.1 ± 3.0
CD4-CD8+ (%)	12.3 ± .75	12.8 ± 1.3
CD3+ (%)	29.8 ± 5.4	32.7 ± 7.4
B220+ (%)	54.5 ± 5.9	48.3 ± 11.1
Lymph node		
Total cell number (X10^6)	2.2 ± .6	1.8 ± .4
CD4+CD8- (%)	33.1 ± 3.9	37.3 ± 2.0
CD4-CD8+ (%)	26.8 ± 2.8	22.0 ± 1.6
CD3+ (%)	55.1 ± 8.5	53.1 ± 11.4
B220+ (%)	35.5 ± 8.6	34.4 ± 11.0
Bone Marrow		
Total cell number (X10^6)	19.4 ± 2.4	19.6 ± 2.8

[a] Mean values± one standard deviation are shown

[b] Total lymphocytes, splenocytes, lymph node cells and bone marrow cells from Wt and *Rock2*$^{+/-}$ were isolated from 8 wk old mice and counted. Percentage of cells stained with antibodies to CD4, CD8, CD3, and B220 were determined by flow cytometry.