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Supplemental Data

IRF-4-Binding Protein Inhibits Interleukin-17 and Interleukin-21 Production by Controlling the Activity of IRF-4 Transcription Factor

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Supplementary Figure 1

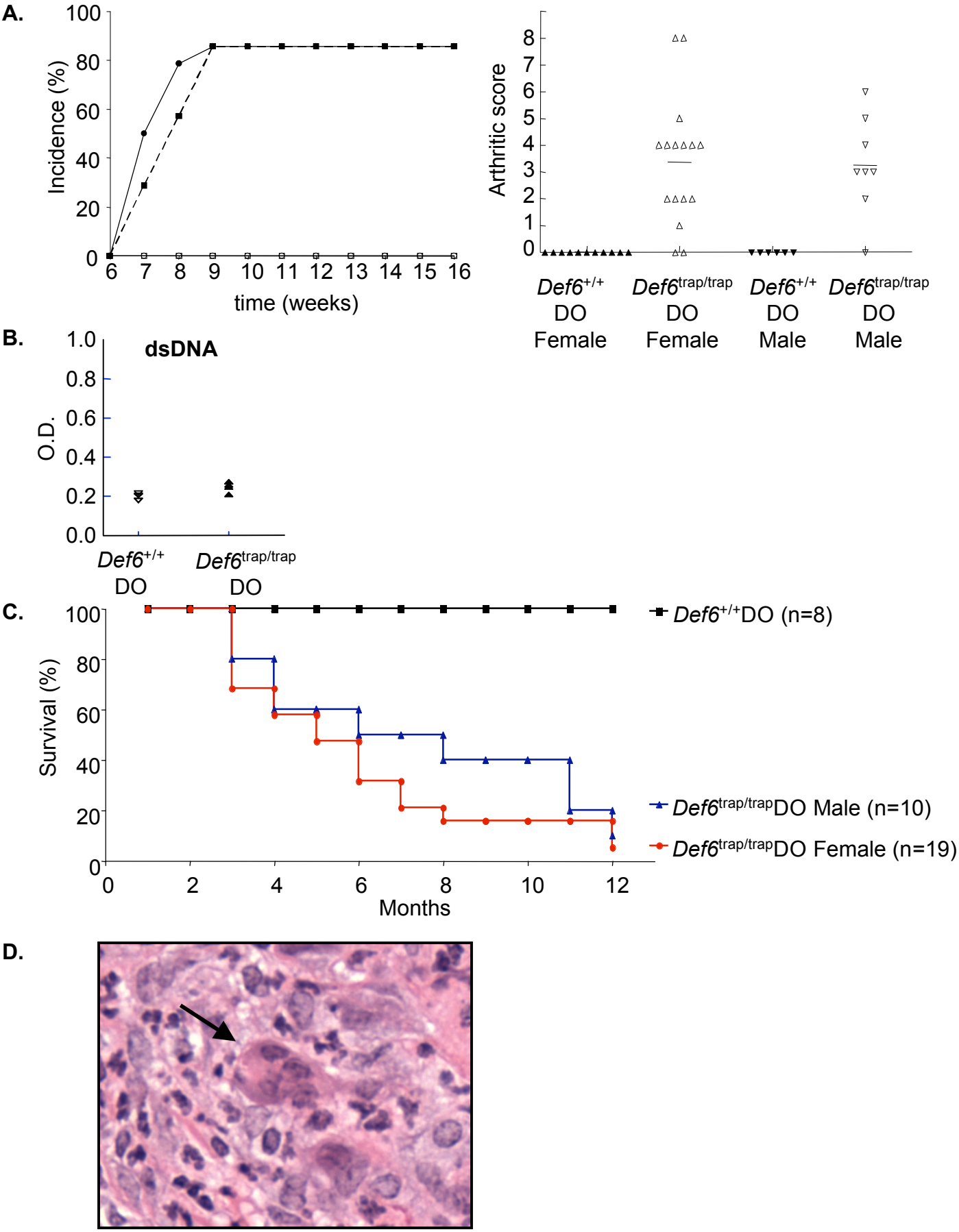

Figure S1. **A.** Arthritis incidence and severity. Left panel: time course of joint swelling in *Def6*$^{trap/trap}$DO11.10 mice (females: closed circle, solid line, n=15; males: closed square, dotted line, n=8) and *Def6*$^{+/+}$DO11.10 (females: open circle, solid line, n=11; males: open square, dotted line, n=6). Right panel: *Def6*$^{+/+}$DO11.10 and *Def6*$^{trap/trap}$DO11.10 mice were scored for arthritis severity at 12 weeks. **B.** dsDNA Ab levels in *Def6*$^{+/+}$DO11.10 and *Def6*$^{trap/trap}$DO11.10 mice. Sera from *Def6*$^{+/+}$DO11.10 and *Def6*$^{trap/trap}$DO11.10 mice (6-25 weeks old, male and female, n=6) were collected and levels of dsDNA antibodies analyzed by ELISA. **C.** Early mortality in *Def6*$^{trap/trap}$DO11.10 mice. Time course of the survival of *Def6*$^{+/+}$DO11.10 (males and females, n=8) and *Def6*$^{trap/trap}$DO11.10 mice (females, n=19 and males, n=10). **D.** Hematoxylin-eosin (HE) stained tissue sections of the root of the aorta of a 16 wks old *Def6*$^{trap/trap}$DO11.10 female mouse showing a multinucleated giant cell (light microscopy, magnification 100X).

Supplementary Figure 2

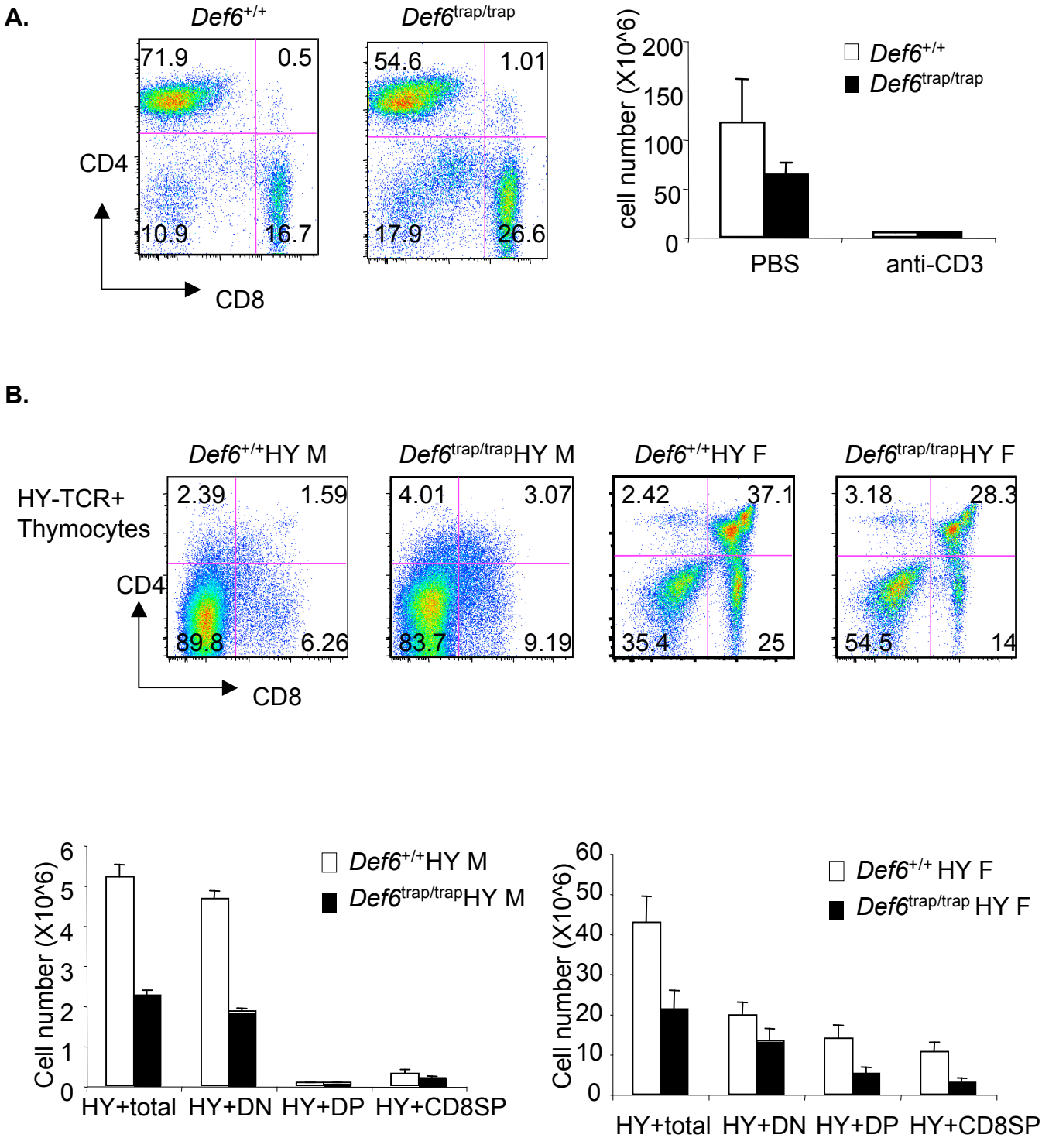

Figure S2. Thymic T cell selection in IBP-deficient mice. **A.** 6-week-old *Def6*$^{+/+}$ and *Def6*$^{trap/trap}$ Balb/c mice were injected with 250μg of anti-CD3 or PBS as control. 72 hours later thymocytes were harvested, counted, and stained with anti-CD4 and anti-CD8 for FACS analysis. A representative FACS of the thymi of *Def6*$^{+/+}$ and *Def6*$^{trap/trap}$ mice injected with anti-CD3 is depicted on the left. A summary of the total number of thymocytes from mice that had received either PBS or anti-CD3 is shown on the right (3 mice/group). **B.** Thymocyte selection in *Def6*$^{trap/trap}$HY transgenic mice. Thymocytes from *Def6*$^{+/+}$HY and *Def6*$^{trap/trap}$HY mice (5 wk old) were analyzed by flow cytometry using anti-CD4, anti-CD8, and T3.70 (anti-HY-TCR). Male (M) and female (F) mice are as labeled. The top panel shows representative CD4 versus CD8 dot plots gated on T3.70^{+} (HY-TCR^{+}) cells. The lower panel shows a summary of the number of thymocytes at each developmental stage (HY-TCR^{+} total thymocytes, HY-TCR^{+} CD4^{-}CD8^{-} double negative (DN), HY-TCR^{+} CD4^{+}CD8^{+} double positive (DP), and HY-TCR^{+} CD4^{-}CD8^{+} single positive (SP) thymocytes) (3 mice/group). All graphs show mean ± SD.

Supplementary Figure 3

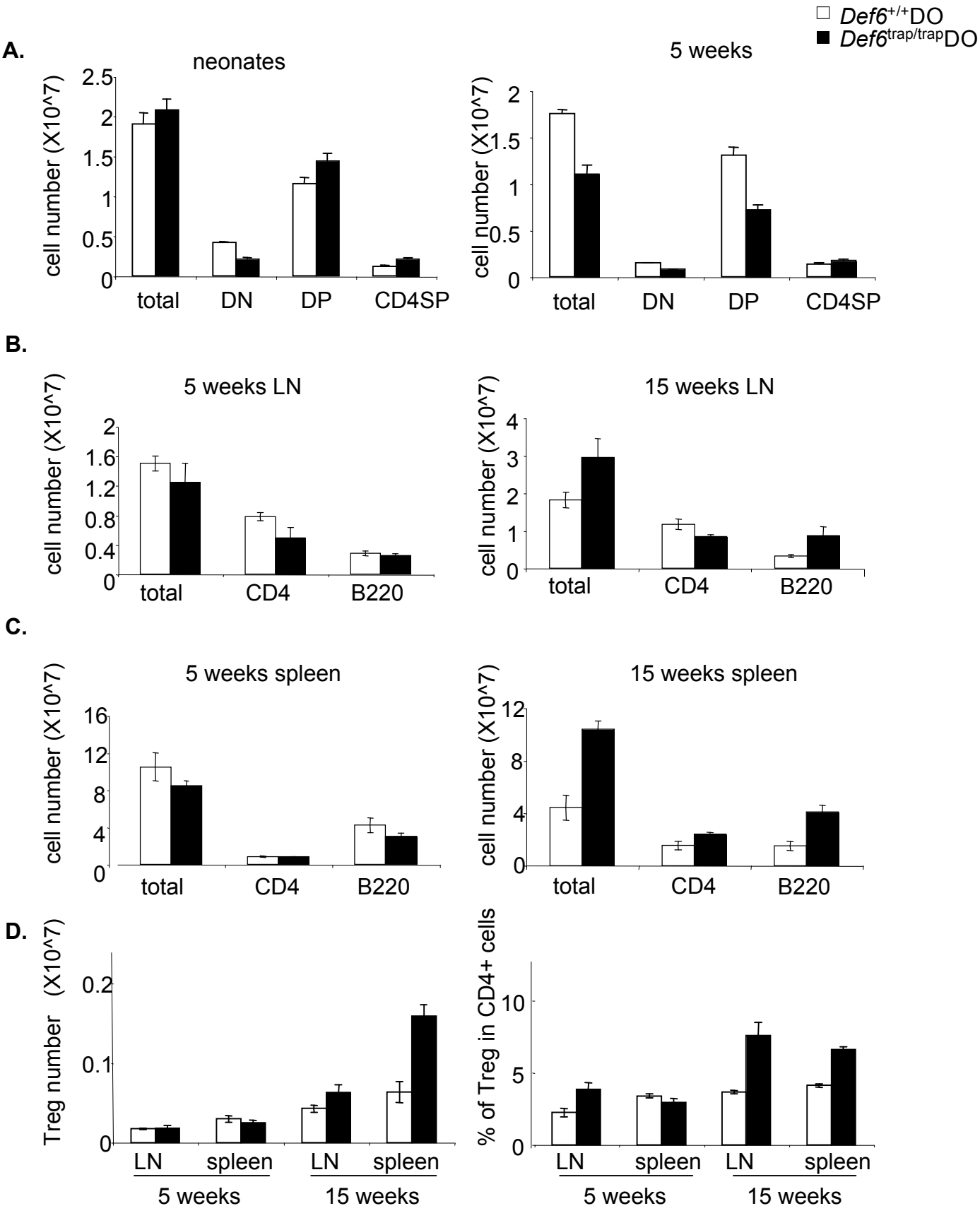

Figure S3. Flow cytometric analysis of lymphocyte populations from *Def6*$^{+/+}$DO11.10 and *Def6*$^{trap/trap}$DO11.10 mice. **A.** Thymic development in *Def6*$^{+/+}$DO11.10 and *Def6*$^{trap/trap}$DO11.10 mice. Thymocytes from *Def6*$^{+/+}$DO11.10 and *Def6*$^{trap/trap}$ DO11.10 mice were counted, stained with anti-CD4 and anti-CD8, and analyzed by FACS. Thymocyte numbers in the different categories (total thymocytes, CD4^{-}CD8^{-} double negative (DN), CD4^{+}CD8^{+} double positive (DP), and CD4^{+}CD8^{-} single positive (SP) thymocytes) for 3-day-old mice (neonates) and 5-week-old mice are shown. **B.** CD4^{+} and B220^{+} populations in the lymph nodes of 5-week-old (left panel) and 15-week-old (right panel) female *Def6*$^{+/+}$DO11.10 (white bar) and *Def6*$^{trap/trap}$DO11.10 (black bar) mice. **C.** Populations in the spleens of 5-week-old (left panel) and 15-week-old (right panel) female *Def6*$^{+/+}$DO11.10 (white bar) and *Def6*$^{trap/trap}$DO11.10 (black bar) mice. **D.** CD4^{+}CD25^{+}Foxp3^{+} T cell numbers (left panel) and percentages of Tregs to CD4^{+} T cells (right panel) in 5-week-old female and 15-week-old female *Def6*$^{+/+}$ DO11.10 (white bar) and *Def6*$^{trap/trap}$ DO11.10 (black bar) mice. Data are from 3-4 mice per group.

Supplementary Figure 4

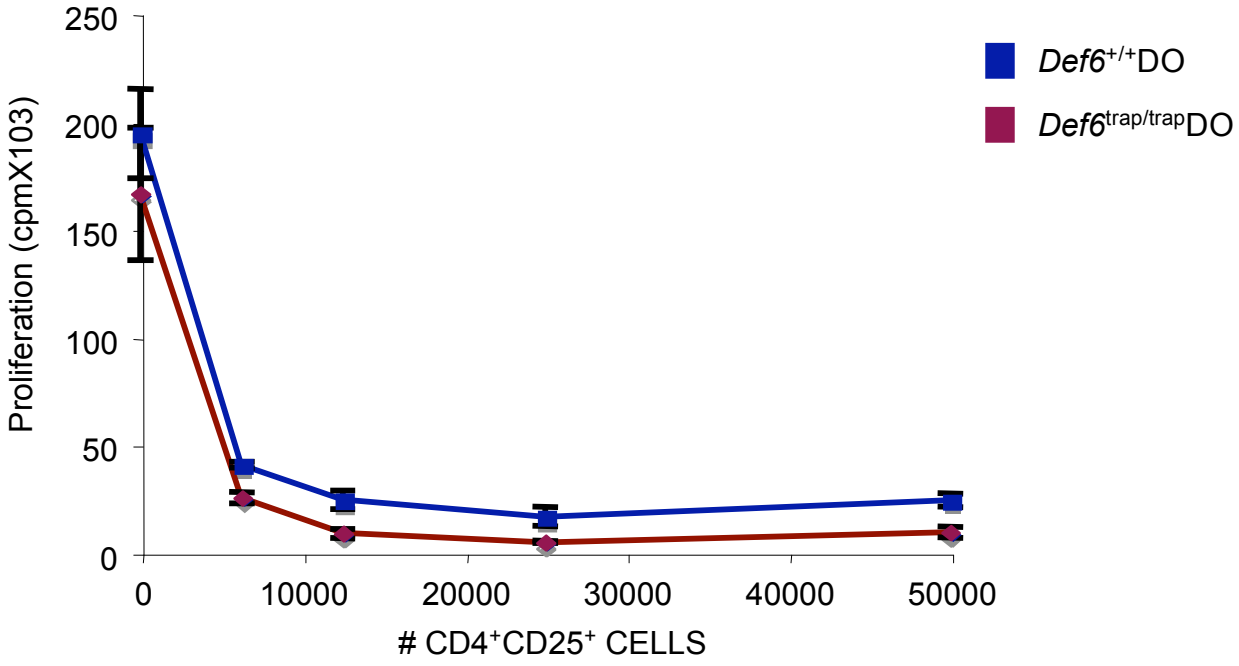

Figure S4. The absence of IBP does not affect Treg function. Total $CD4^+$ cells (5 x 10^4) from *Def6*$^{+/+}$DO11.10 mice were cultured with T cell depleted APCs (2 x 10^5) pulsed with $OVA_{323-339}$ (1 μM) in presence or absence of increasing numbers of FACS sorted $CD4^+CD25^+$ T cells derived either from *Def6*$^{+/+}$ DO11.10 (blue line) or *Def6*$^{trap/trap}$ DO11.10 (red line) mice. Proliferation was measured by thymidine incorporation. The data are presented as Mean ± S.D. and are representative of three independent experiments.

Supplementary Figure 5

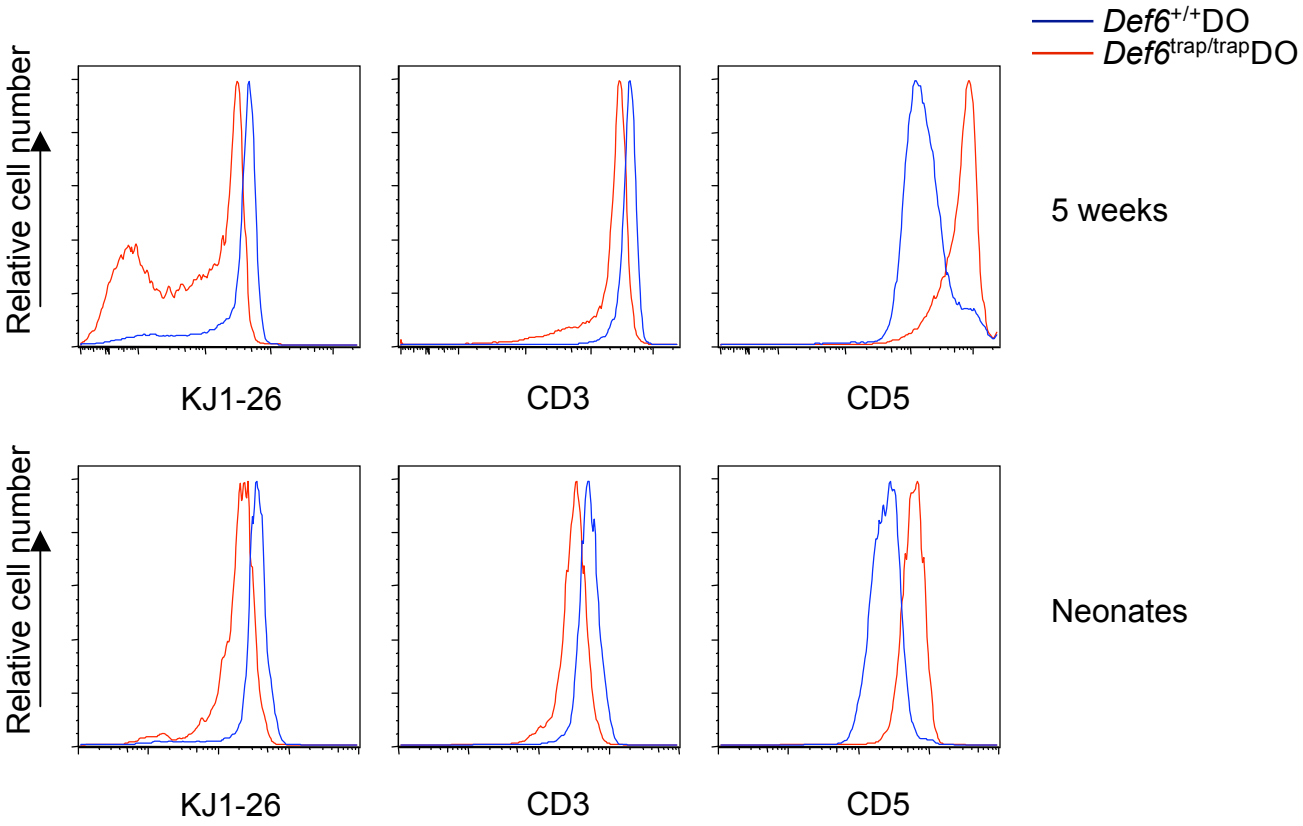

Figure S5. Lack of IBP leads to changes in the expression of CD5, CD3, and of the DO11.10 TCR (KJ1-26). Single cell suspensions from lymph nodes of 5wk. old *Def6*$^{+/+}$DO11.10 (blue) and *Def6*$^{trap/trap}$DO11.10 (red) mice (top panels) or from thymocytes of 3-day-old *Def6*$^{+/+}$DO11.10 (blue) and *Def6*$^{trap/trap}$DO11.10 (red) neonates (lower panels) were stained with antibodies against the OVA-specific TCR (KJ1-26), CD3ε, and CD5 (as indicated) and analyzed by FACS. Histograms are representative of 3 mice/group (for 5 wk old mice) or 7 mice/group (for neonates).

Supplementary Figure 6

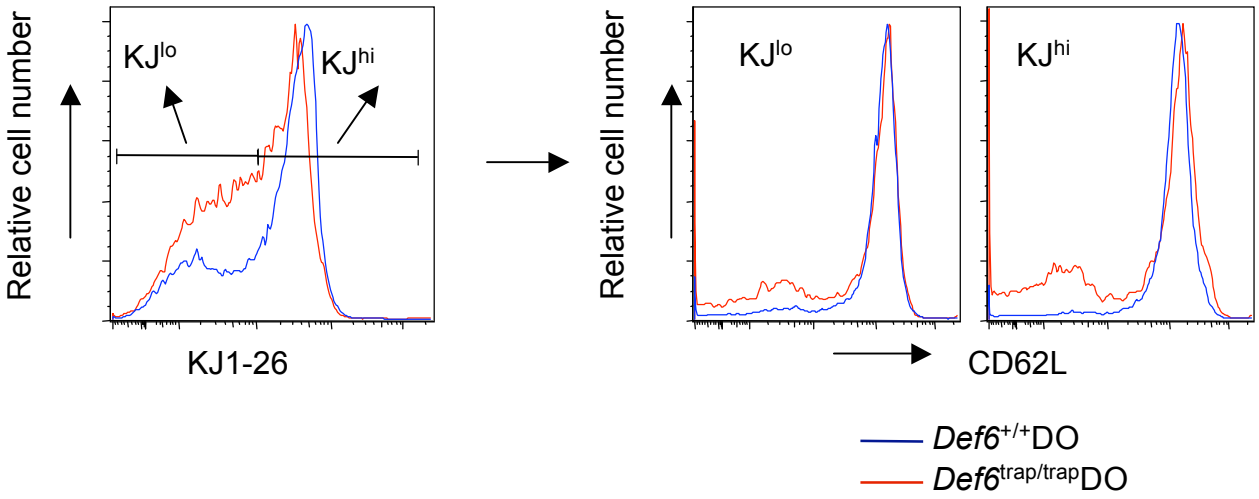

Figure S6. The expression of the DO11.10 TCR (KJ1-26) on $CD4^+$ T cells derived from *Def6*$^{+/+}$DO11.10 (blue) and *Def6*$^{trap/trap}$DO11.10 (red) mice was analyzed by FACS (left). The expression level of CD62L on gated KJ1-26^{hi} (KJ^{hi}) and KJ1-26^{lo} (KJ^{lo}) populations was then assessed as shown on the right.

Supplementary Figure 7

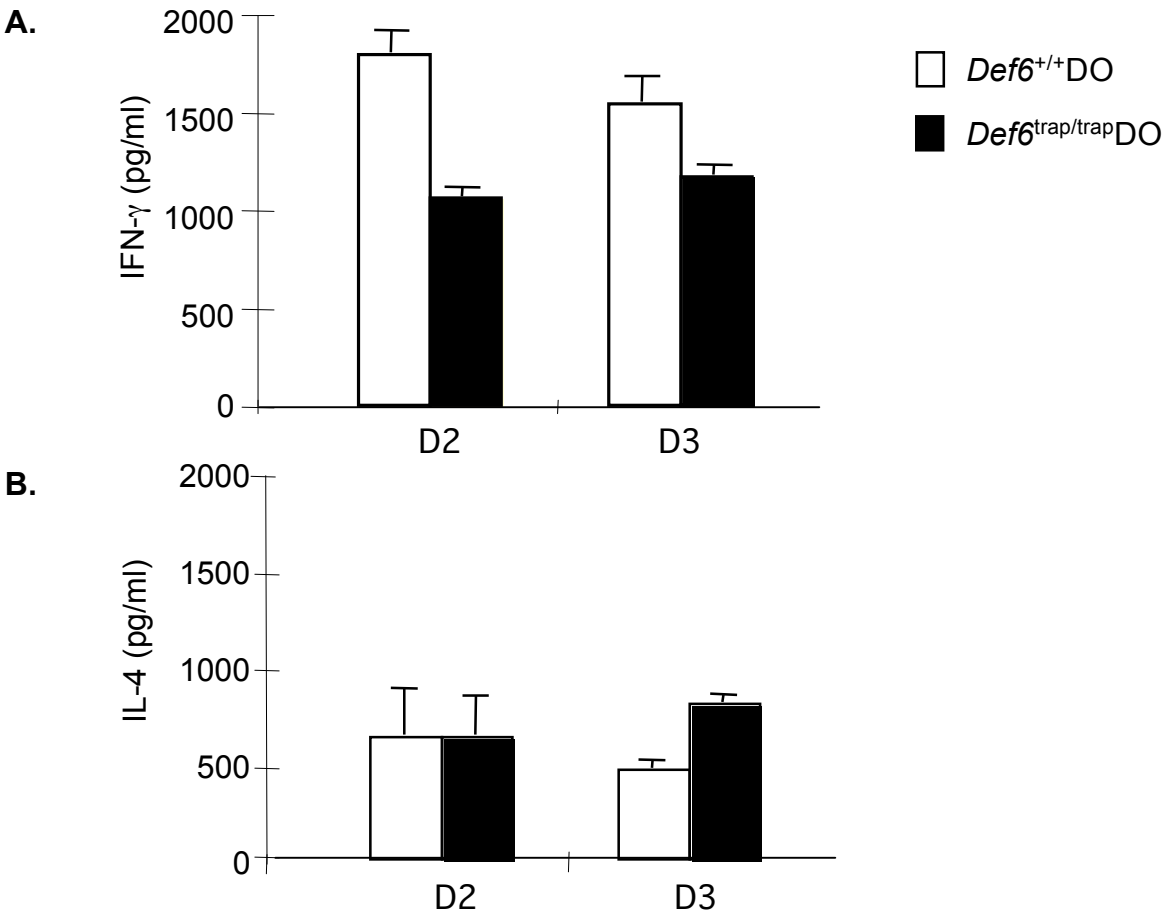

Figure S7. Production of IFN-γ and IL-4 by *Def6*$^{trap/trap}$DO11.10 T cells **A.** Naïve CD4^{+} T cells derived from 6 wks. old *Def6*$^{+/+}$DO11.10 (white bars) or *Def6*$^{trap/trap}$DO11.10 (black bars) mice were cultured with *Def6*$^{+/+}$ APCs pulsed with 1 μM $OVA_{323-339}$ peptide for the times indicated. The production of IFN-γ in the supernatants was measured by ELISA. **B.** Naïve CD4^{+} T cells derived from 6 wks. old *Def6*$^{+/+}$DO11.10 (white bars) or *Def6*$^{trap/trap}$DO11.10 (black bars) mice were cultured as above. The production of IL-4 in the supernatants was measured by ELISA. All graphs show mean ± SD.

Supplementary Figure 8

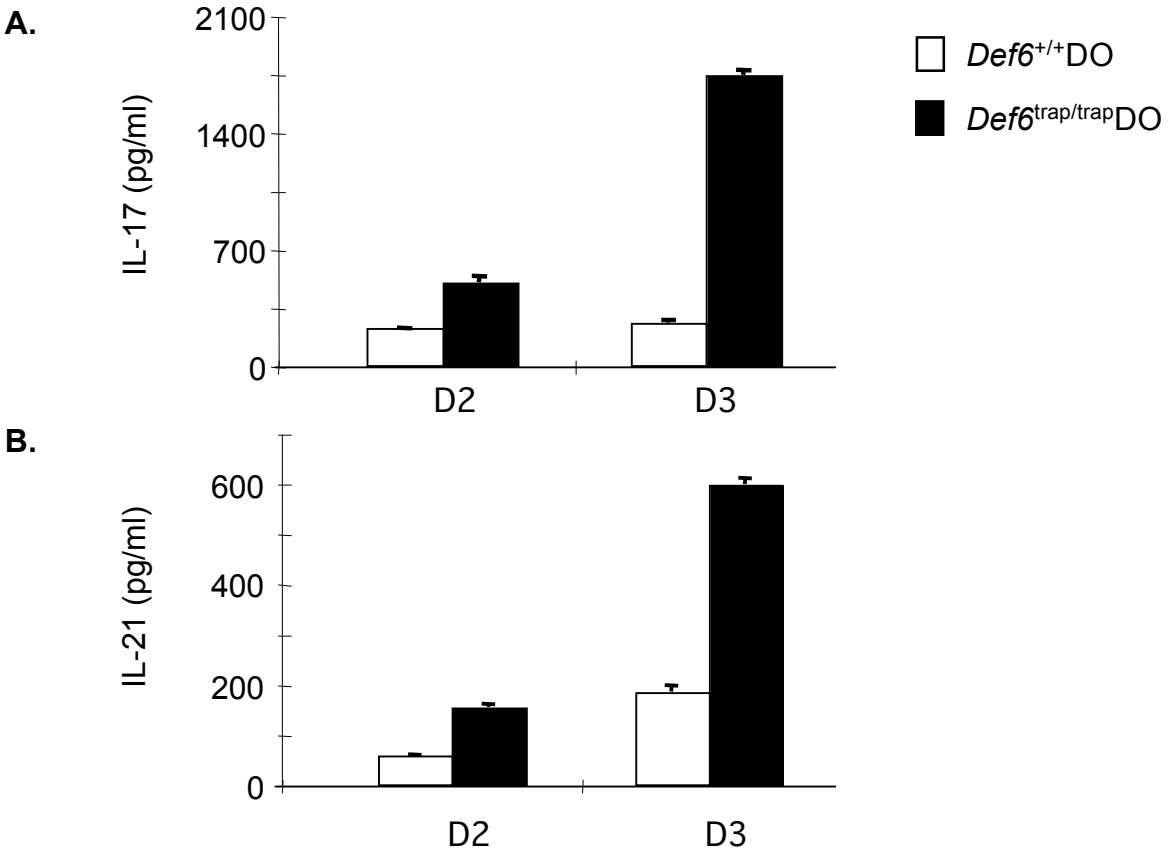

Figure S8. Production of IL-17 and IL-21 by FACS sorted $CD44^{lo}CD62L^{hi}CD25^{-}CD4^{+}$ *Def6*$^{trap/trap}$DO11.10 T cells. **A.** FACS sorted $CD44^{lo}CD62L^{hi}CD25^{-}CD4^{+}$ T cells derived from 6 wks. old *Def6*$^{+/+}$DO11.10 (white bars) or *Def6*$^{trap/trap}$DO11.10 (black bars) mice were cultured with T cell depleted *Def6*$^{+/+}$ APCs pulsed with 1 μM $OVA_{323-339}$ peptide for the times indicated. The production of IL-17 in the supernatants was measured by ELISA. **B.** FACS sorted $CD44^{lo}CD62L^{hi}CD25^{-}CD4^{+}$ T cells derived from 6 wks. old *Def6*$^{+/+}$ DO11.10 (white bars) or *Def6*$^{trap/trap}$ DO11.10 (black bars) mice were cultured with *Def6*$^{+/+}$ APCs pulsed with 1 μM $OVA_{323-339}$ peptide for the times indicated. The production of IL-21 in the supernatants was measured by ELISA. All graphs show mean ± SD.

Supplementary Figure 9

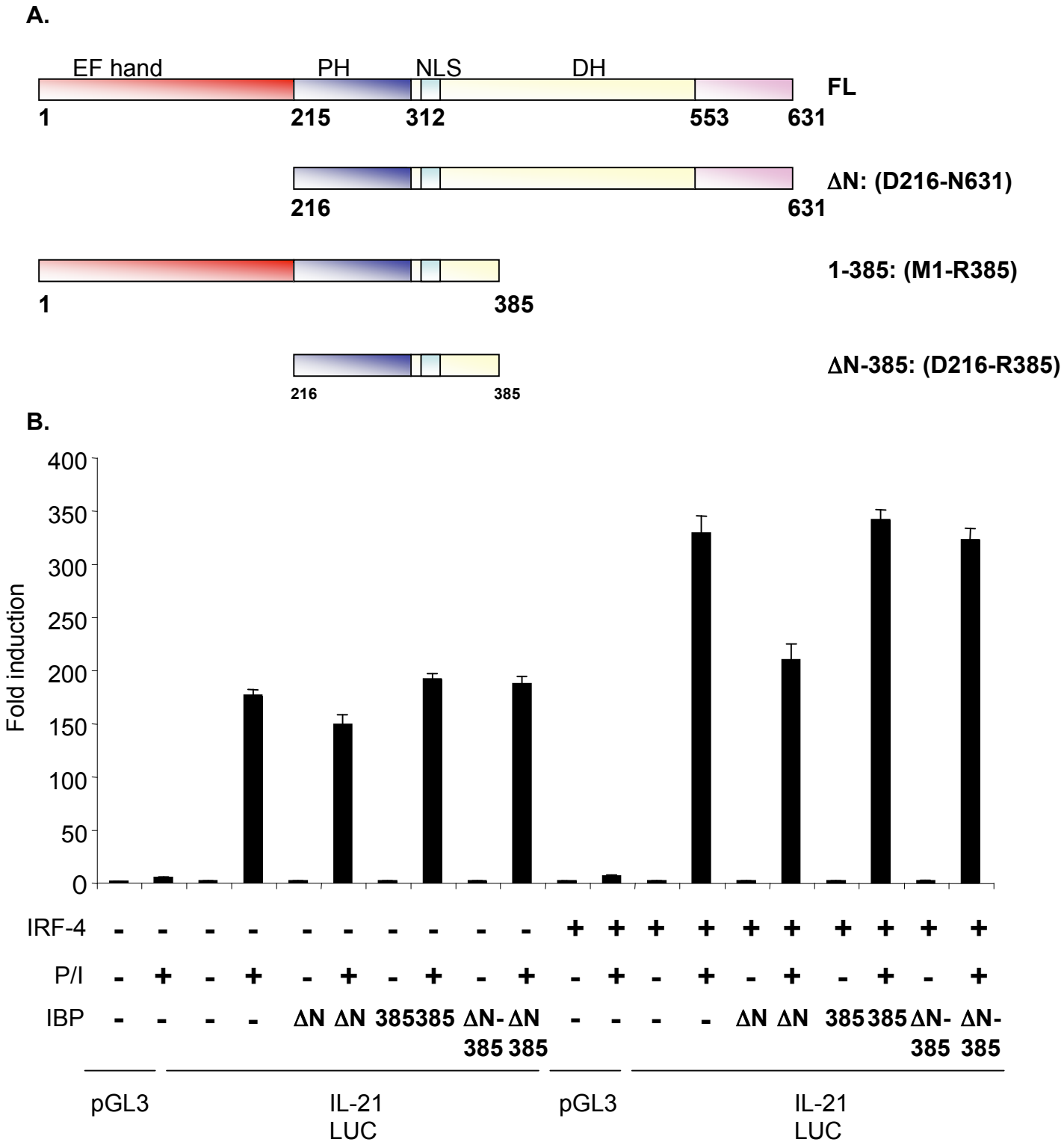

Figure S9. The ability of IBP1-385 to interfere with the transactivating activity of IRF-4 is not due to the presence of the autoinhibitory N-terminal domain. **A.** Diagram of the IBP mutants utilized in the experiment. **B.** Jurkat cells that express an empty vector or Jurkat cells expressing IRF-4 were transfected with a luciferase reporter construct driven by the murine IL-21 promoter (IL-21 LUC) together with an empty vector, HA-tagged IBPΔN, HA-tagged IBP 1-385 or HA-tagged IBPΔN-385 as indicated. Cells were either left unstimulated or stimulated with PMA and ionomycin as indicated. Transfections with a control luciferase reporter construct (pGL3) in the presence or absence of PMA and ionomycin were also carried out. Cotransfection with a renilla-luciferase construct was performed to normalize the transfection efficiency of the different samples. All graphs show mean ± SD. Data are representative of 3 independent experiments.

Supplementary Figure 10

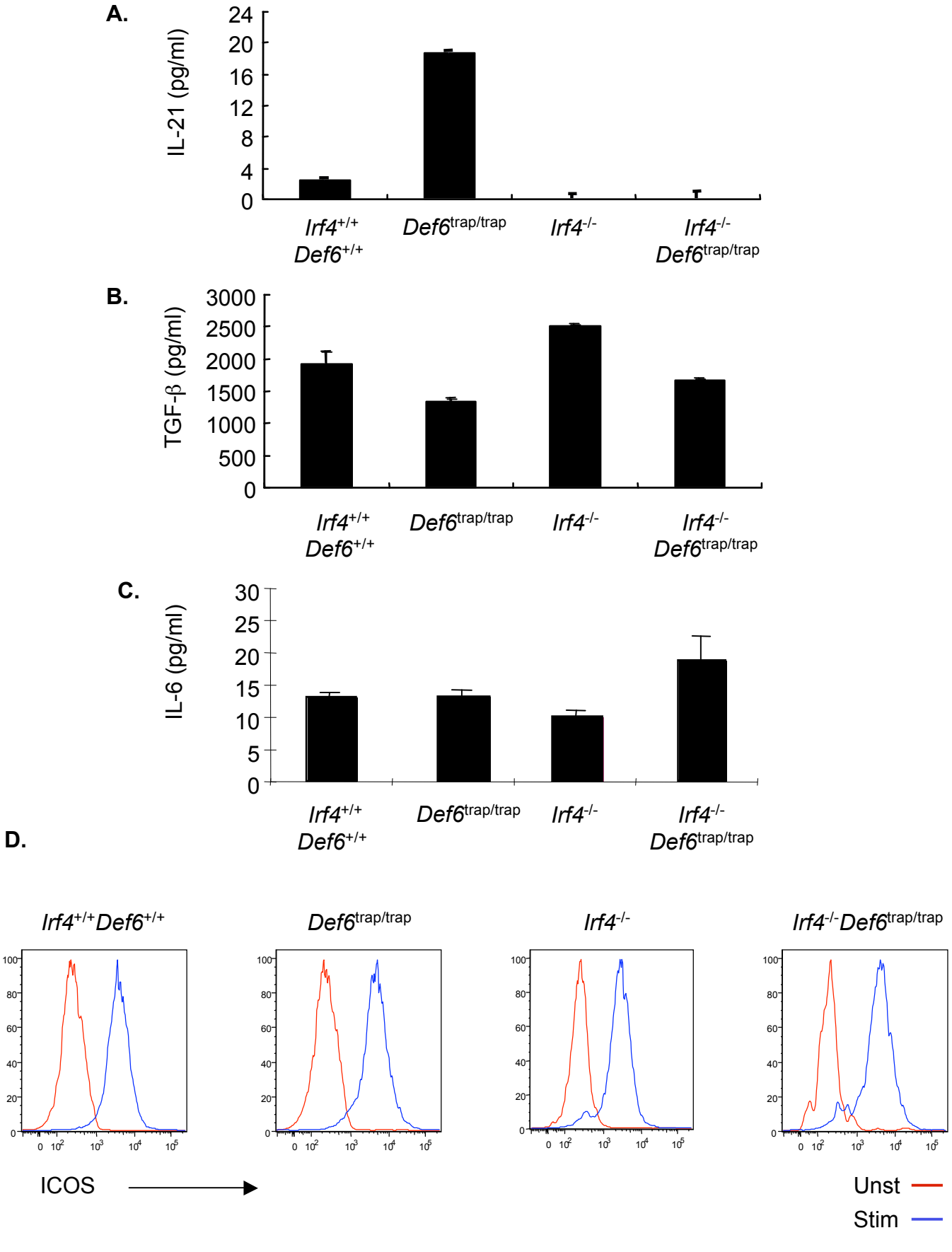

Figure S10. A. IL-21 production by *Irf4*$^{+/+}$*Def6*$^{+/+}$, *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$ *Def6*$^{trap/trap}$ cultured under Th0 cell conditions. Purified naïve CD4^{+} T cells were stimulated under Th0 cell conditions and IL-21 levels in the supernatants determined by ELISA. **B.** TGF-β production by *Irf4*$^{+/+}$*Def6*$^{+/+}$, *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$*Def6*$^{trap/trap}$. Purified naïve CD4^{+} T cells were stimulated as above and TGF–β levels in culture supernatants determined by ELISA. **C.** IL-6 production by *Irf4*$^{+/+}$*Def6*$^{+/+}$, *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$*Def6*$^{trap/trap}$. Purified naïve CD4^{+} T cells were stimulated as above and IL-6 levels in the supernatants determined by ELISA. All graphs show mean ± SD. **D.** ICOS expression on *Irf4*$^{+/+}$*Def6*$^{+/+}$, *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$*Def6*$^{trap/trap}$. Purified naïve CD4^{+} T cells were stimulated as above and the surface expression of ICOS determined by FACS.

Supplemental Table 1

Hematopoietic cellularity in *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$*Def6*$^{trap/trap}$ [a&b]

Tissue	*Irf4*$^{+/+}$*Def6*$^{+/+}$	*Def6*$^{trap/trap}$	*Irf4*$^{-/-}$	*Irf4*$^{-/-}$*Def6*$^{trap/trap}$
Thymus				
Total cell number (X10^6)	164.8 ± 23.3	126.4 ± 23.2	205.0 ± 23.2	126.7 ± 12.9
CD4+CD8+ (%)	74.4 ± 7.4	67.9 ± 11.1	78.8 ± 1.3	73.6 ± 6.8
CD4+CD8- (%)	13.1 ± 5.6	14.9 ± 6.2	8.9 ± 2.1	10.8 ± 4.6
CD4-CD8+ (%)	4.0 ± 0.2	5.2 ± 1.1	3.2 ± 1.6	4.1 ± 2.6
Spleen				
Total cell number (X10^6)	146.2 ± 1.3	137.7 ± 12.5	190.5 ± 35.0	142.6 ± 11.1
CD4+CD8- (%)	28.4 ± 6.1	23.6 ± 5.6	27.0 ± 4.6	25.6 ± 3.3
CD4-CD8+ (%)	13.3 ± 2.0	13.8 ± 1.9	11.9 ± 1.7	9.8 ± 0.9
CD3+ (%)	46.7 ± 7.0	39.9 ± 6.8	42.7 ± 4.4	37.5 ± 4.9
B220+ (%)	43.4 ± 3.4	44.2 ± 5.8	37.7 ± 8.1	39.6 ± 12.3
Lymph node				
Total cell number (X10^6)	47.5 ± 12.7	52.0 ± 19,3	115.6 ± 28.1	72.8 ± 14.5
CD4+CD8- (%)	42.1 ± 6.7	35.2 ± 5.0	50.0 ± 4.9	43.1 ± 6.7
CD4-CD8+ (%)	24.6 ± 2.1	24.8 ± 1.9	28.7 ± 7.2	24.3 ± 3.5
CD3+ (%)	69.4 ± 5.3	61.6 ± 4.1	81.5 ± 3.0	70.9 ± 6.4
B220+ (%)	26.3 ± 8.0	32.4 ± 6.3	15.9 ± 3.7	25.9 ± 6.4
Bone Marrow				
Total cell number (X10^6)	69.0 ± 4.2	59.1 ± 11.5	70.7 ± 3.3	61.6 ± 17.5

[a] Mean values ± SD are shown

[b] Total thymocytes, splenocytes, lymph node cells (mesenteric, axillary, and inguinal), and bone marrow cells from 6-8 wks old *Irf4*$^{+/+}$*Def6*$^{+/+}$, *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$*Def6*$^{trap/trap}$ mice were isolated and counted. Percentages of cells stained with antibodies to CD4, CD8, CD3, and B220 were determined by flow cytometry.

Table S1. Hematopoietic cellularity in *Irf4*$^{+/+}$*Def6*$^{+/+}$, *Def6*$^{trap/trap}$, *Irf4*$^{-/-}$ and *Irf4*$^{-/-}$ *Def6*$^{trap/trap}$ mice.