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Supplemental Information

Orphan Nuclear Receptor NR2F6 Suppresses T Follicular Helper Cell Accumulation through Regulation of IL-21

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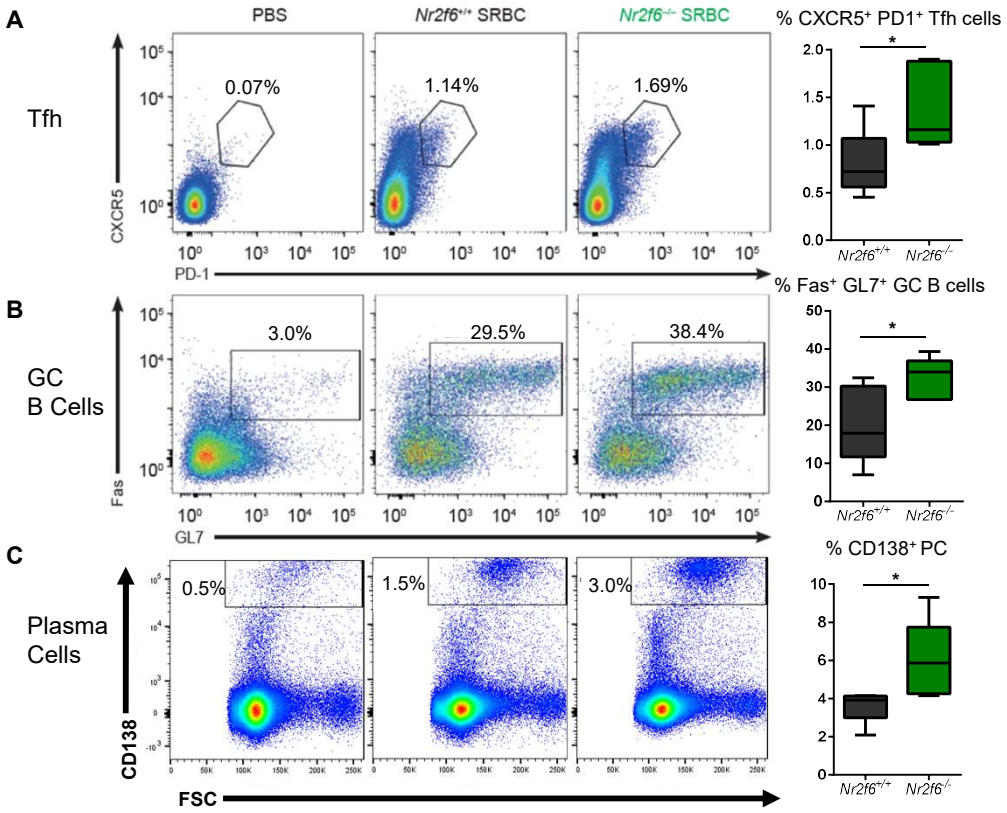
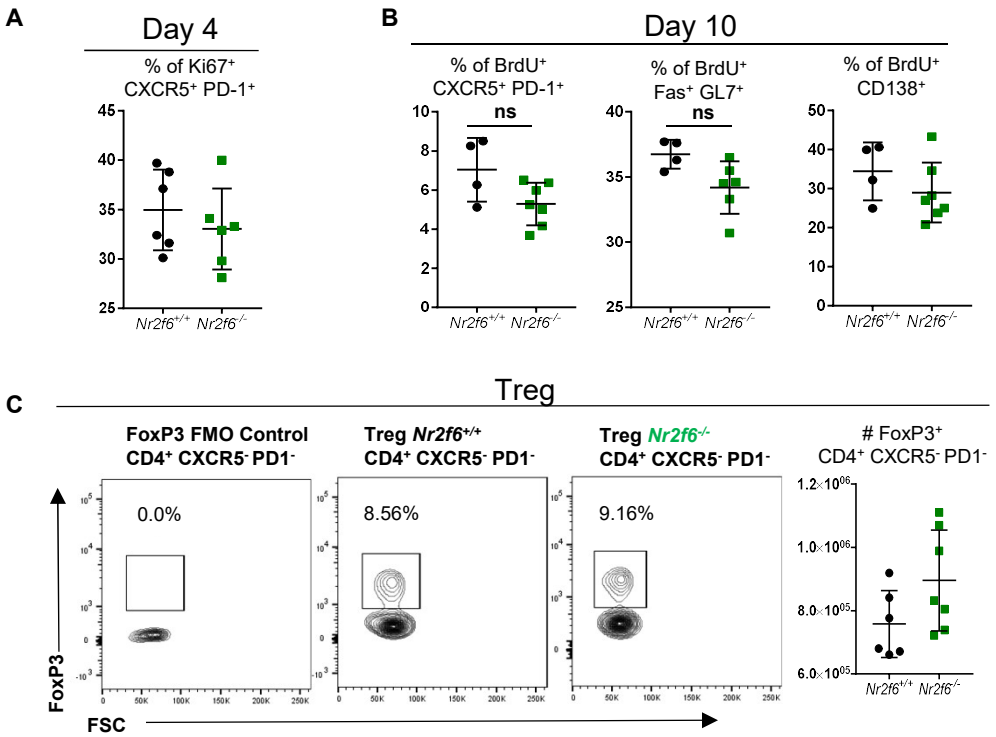
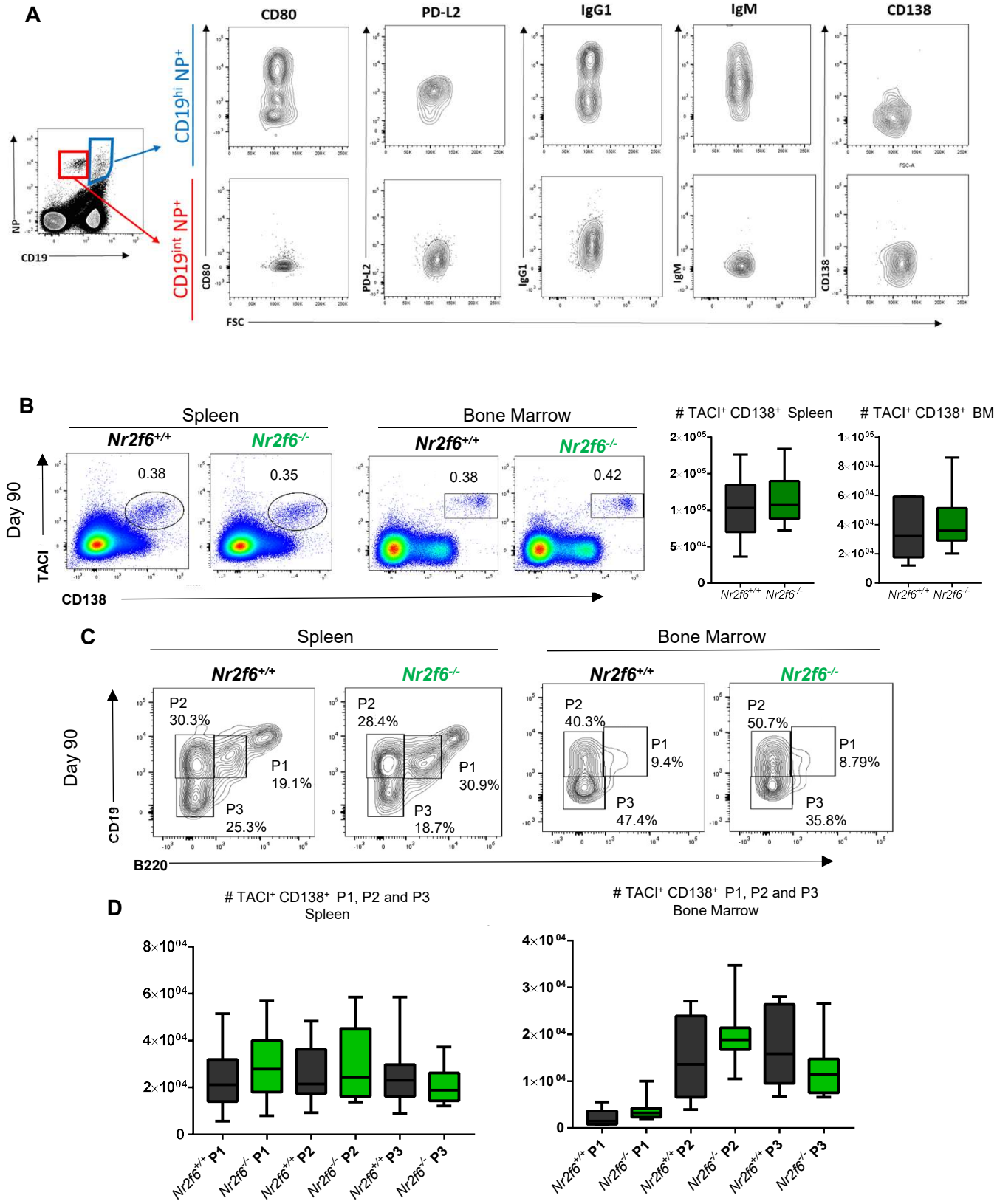


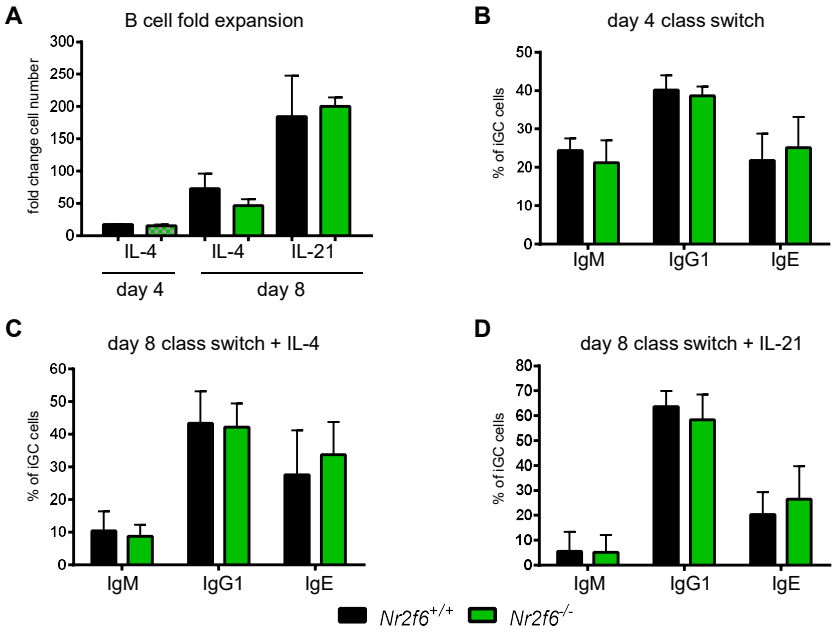
Supplementary Figure 1 (related to Fig. 1): *Nr2f6* loss results in increased GC response to SRBC. *Nr2f6*$^{+/+}$ or *Nr2f6*$^{-/-}$ mice were immunized with 5x10^{8} sheep red blood cells or given PBS as a negative control. On day 7 post-immunization, spleens were harvested and stained for (A) Tfh cells (CD4^{+} CXCR5^{+} PD-1^{+}) frequency of all mice is shown in the right panel. (B) GC B cells (IgD^{neg} B220^{+} Fas^{+} GL7^{+}) and (C) plasma cells (B220^{int} CD45^{+} CD138^{+}) along with total frequency of each population to the right. Data shown are from two independent experiments with n≥6. Error bars represent SD, an asterisk (*) indicates statistically significant differences calculated using Student's t-test. A p-value of < 0.05 was considered statistically significant. * p <0.05; ** p <0.01; *** p <0.001.



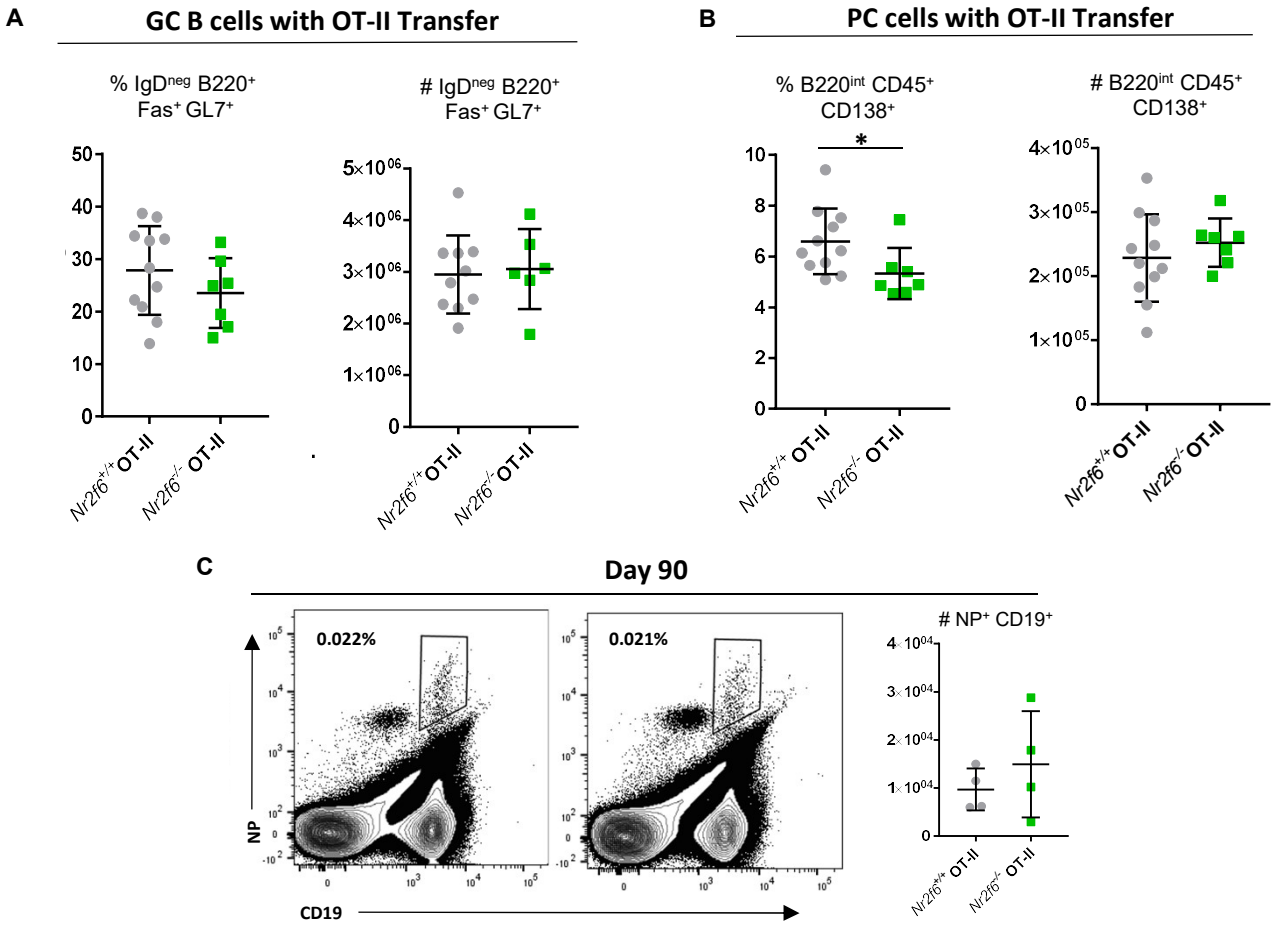
Supplementary Figure 2 (related to Fig. 1): *Nr2f6*-deficiency does not alter proliferation or total Treg cell number after OVA immunization. *Nr2f6*$^{+/+}$ or *Nr2f6*$^{-/-}$ mice were immunized with OVA precipitated in 10% alum 4 days later (A) Tfh cells (CD4^{+} CXCR5^{+} PD-1^{+}) were co-stained for Ki67 frequency. (B) Frequency of BrdU incorporation on day 10 after 7-12 hours pulse labelling of mice was performed on Tfh (p=0.056), GC B (IgD^{neg} B220^{+} Fas^{+} GL7^{+}) (p=0.0506) and plasma cells (B220^{int} CD45^{+} CD138^{+}). (C) Representative day 10 staining of Treg cells (CXCR5- PD-1- CD4+ FoxP3+) and total Treg numbers. Data shown are from at least two separate experiments with n≥5. Middle bar represents average for the data set. Error bars represent SD, an asterisk (*) indicates statistically significant differences calculated using two-tailed Student's t-test.



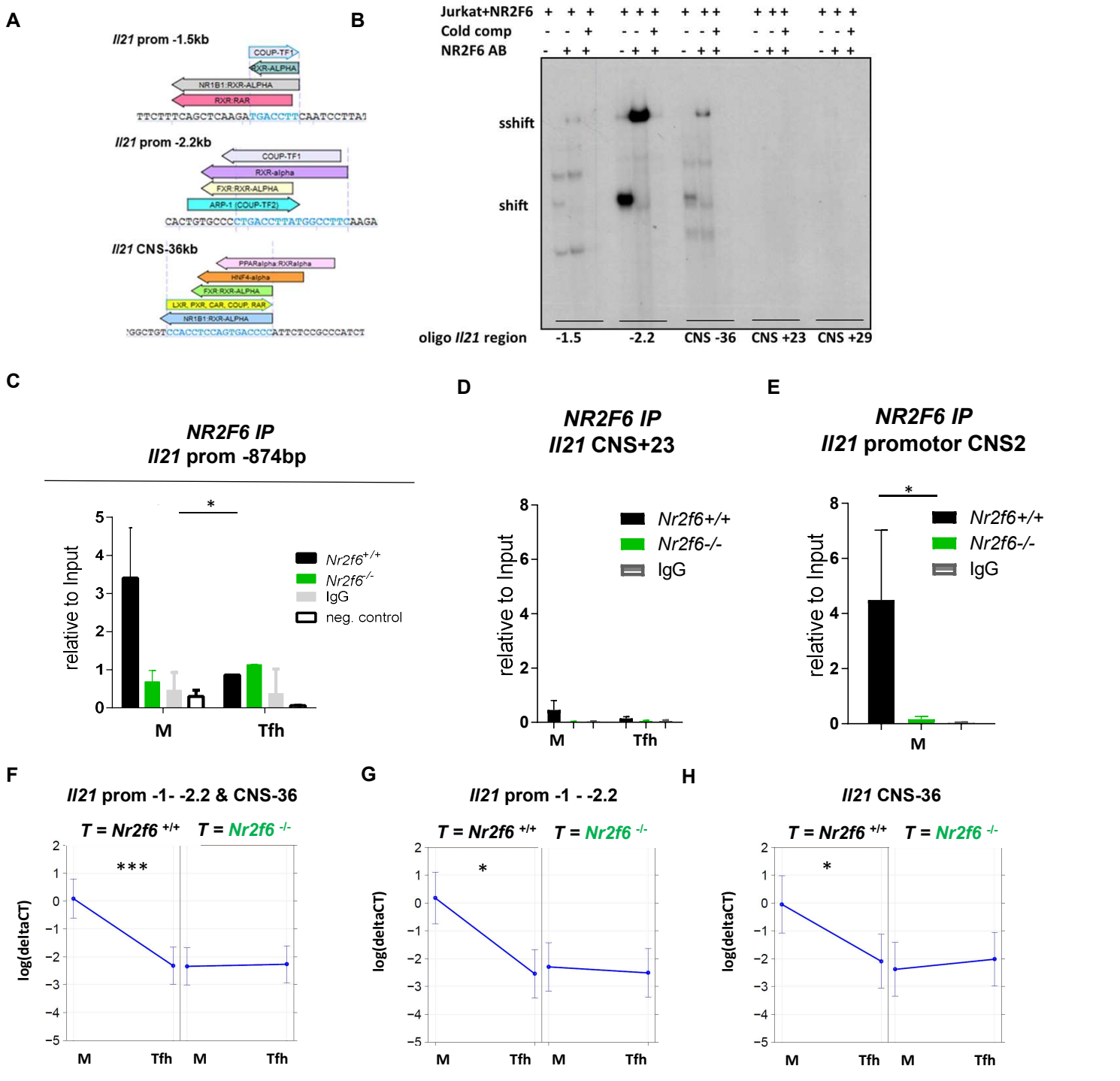
Supplementary Figure 3 (related to Fig. 2): The total *Nr2f6*$^{-/-}$ LLPC population is largely unaffected. (A) Justification for exclusion of CD19^{int} NP^{+} population for memory B cell analysis, showing no expression of CD80, PD-L2, IgM, CD138 and only intermediate IgG1 in this population. (B) *Nr2f6*$^{+/+}$ or *Nr2f6*$^{-/-}$ mice were immunized with NP-CGG precipitated in 10% alum, and 90 days later spleens and bone marrow were stained for long-lived plasma cells (TACI^{+} CD138^{+}) and total cell numbers calculated (right two panels). (C) LLPC cells were further stained for CD19 and B220 expression and (D) total cell numbers from these populations were calculated. Error bars represent SD, data shown are from two independent experiments with n≥6.



Supplementary Figure 4 (related to Fig. 3): In vitro generation of iGC B cells and class switching is not compromised by lack of NR2F6. B cells from wild-type or *Nr2f6*-deficient mice were cultured to induce GC B differentiation *in vitro*. Cells were harvested on day 4, (A) counted and stained with (B) frequency, measured by flow cytometry, of IgM, IgG1 and IgE positive iGC B cells are shown. Cells were re-cultured in either (C) IL-4 or (D) IL-21 (PC differentiating conditions) for 4 more days and stained with anti-IgM, anti-IgG1 and anti-IgE as in B (n=4 per group). Error bars represent SD. Data shown are from two independent experiments with n≥6.



Supplementary Figure 5 (related to Fig. 4): Adoptive transfer of *Nr2f6*-deficient OT-II T cells does not significantly affect the GC or PC responses on day 10 or NP$^+$ CD19$^+$ B cell numbers on day 90. (A) GC B cell frequency and total numbers (B) Frequency and total PCs from CD45.1/CD45.2 heterozygous wild-type mice receiving either 3x10^6 *Nr2f6*$^{+/+}$ or *Nr2f6*$^{-/-}$ OT-II CD45.2 cells and immunized with NP-OVA. (C) Frequency and total NP$^+$ CD19$^+$ B cells from CD45.1 wild-type mice that received either 3x10^6 *Nr2f6*$^{+/+}$ or *Nr2f6*$^{-/-}$ OT-II CD45.2 cells and were NP-CGG immunized one day later. Memory B cells were investigated on day 90 post immunization. Error bars represent SD. Data shown are from two independent experiments with n≥6.



Supplementary Figure 6 (related to Fig. 6): In resting CD4 T cells NR2F6 binds to the *Il21* promoter and the CNS-36 region. (A) Putative NR2F6 (COUP) binding sites of the mouse *Il21* promoter and CNS-36 regions are shown in detail. (B) NR2F6 binding to the *Il21* promotor at -1.5kb, -2.2kb, CNS-36, CNS+23, and CNS+29 were investigated by EMSA in Jurkat nuclear extracts overexpressing NR2F6. (C) NR2F6 binding at the -0.874 *Il21* promoter (D) or CNS+23 or (E) the *Il17* CNS2 region as positive control (Hermann-Kleiter et al., 2012) were investigated by ChIP. Resting *Nr2f6*$^{+/+}$ or *Nr2f6*$^{-/-}$ CD4 or activated under Tfh-differentiating conditions were used with anti-NR2F6 or IgG control. *Il21 or Il17* cytokine promoter region sequences was quantified by RT-PCR. (C), (D), (E) The data are presented as percent of input samples before immunoprecipitation. Error bars represent SD. Differences between genotypes were investigated with log-transformed data following by a linear mixed-effects model by analysis of variance. (F), (G), (H) Interaction-plot Tfh Stimulation (no/yes) x *Nr2f6* $^{+/+}$/ *Nr2f6* $^{-/-}$. Error bars indicate 95% confidence intervals for indicated regions are shown for the different regions. For details see Material and Method section. An asterisk (*) indicates statistically significant differences between genotypes. *p*-value of < 0.05 was considered statistically significant. * p <0.05; ** p <0.01; *** p <0.001.

Table S1. Oligonucleotide sequences for EMSA assay, related to STAR methods section Oligonucleotide sequences for EMSA.

Oligo -1.0kb F- ACTTTCTCCAGCAGGCAAAGAACCCGAAGGTCAGCAAA	Synthesized by Eurofins
Oligo -1.0kb R- TTTGCTGACCTTCGGGTTCTTTGCCTGCTGGAGAAAGT	Synthesized by Eurofins
Oligo -1.5kb F- TTCTTTCAGCTCAAGATGACCTTCAATCCTT	Synthesized by Eurofins
Oligo -1.5kb R- AAGGATTGAAGGTCATCTTGAGCTGAAAGAA	Synthesized by Eurofins
Oligo -2.2kb F- ACTGTGCCCCTGACCTTATGGCCTTCAAGATA	Synthesized by Eurofins
Oligo -2.2kb R- TATCTTGAAGGCCATAAGGTCAGGGGCACAGT	Synthesized by Eurofins
Oligo -36 CNS: F- TTCATGGCTGTCCACCTCCAGTGACCCCATTCTCC	Synthesized by Eurofins
Oligo -36 CNS: R- GGAGAATGGGGTCACTGGAGGTGGACAGCCATGAA	Synthesized by Eurofins
Oligo +29 CNS: F- GATCTGATGAGGAGAGAAAGGGTCAGGGTGGGGCAG	Synthesized by Eurofins
Oligo +29 CNS: R- CTGCCCCACCCTGACCCTTTCTCTCCTCATCAGATC	Synthesized by Eurofins
Oligo +23 CNS: F- TGCTGTGAAGGGGTCAGAACAGGCCACTCAAAA	Synthesized by Eurofins
Oligo +23 CNS: R- TTTTGAGTGGCCTGTTCTGACCCCTTCACAGCA	Synthesized by Eurofins