EGFR-HIF1α signaling positively regulates the differentiation of IL-9 producing T helper cells

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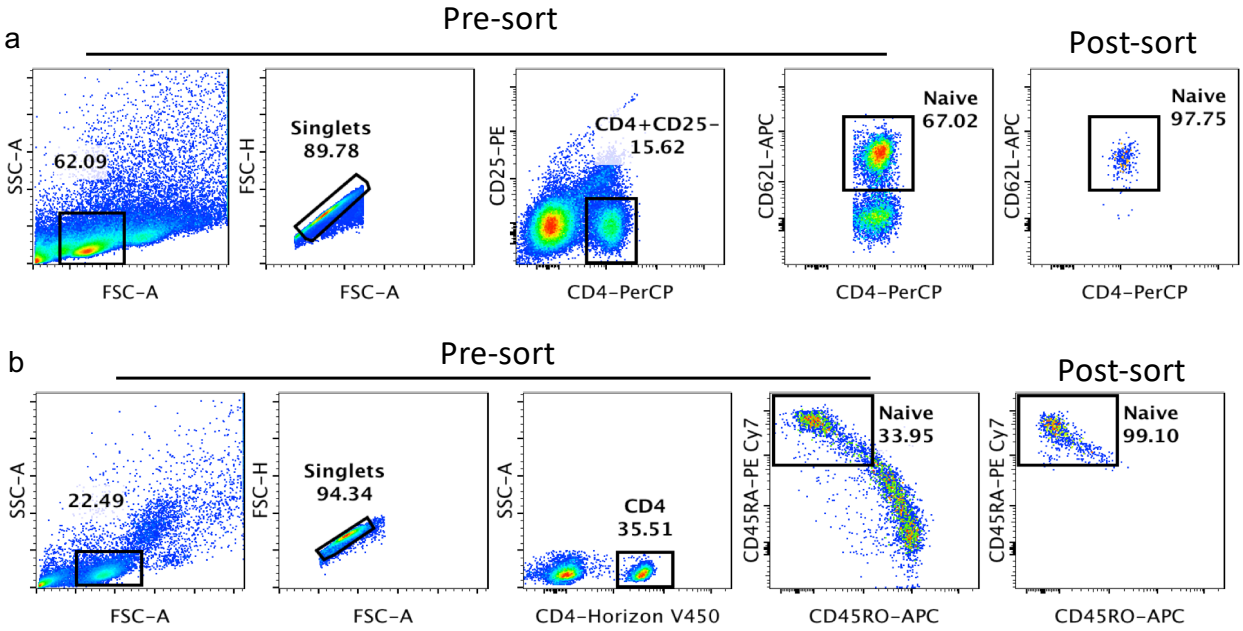
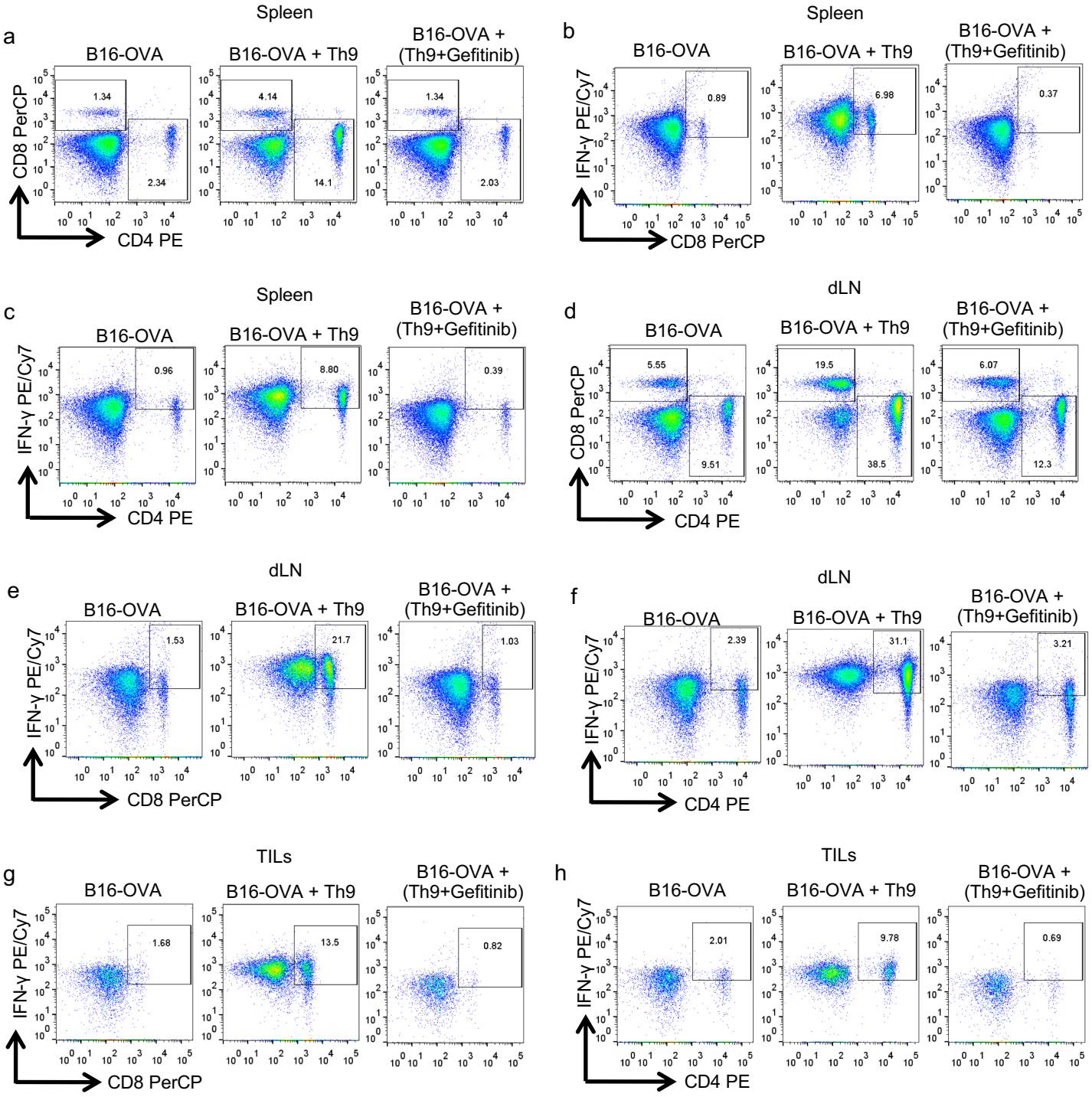


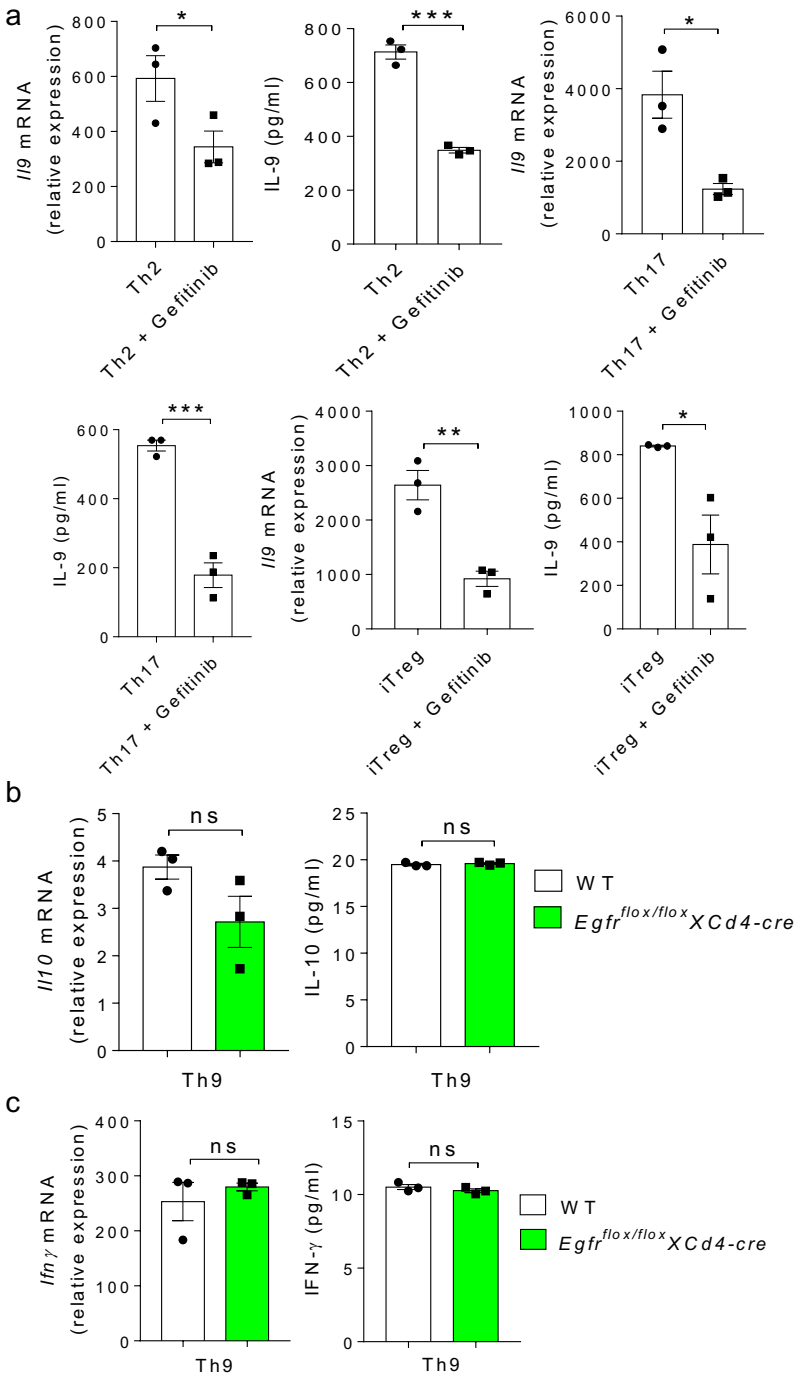
Supplementary Fig. 1. Gating and sorting strategy of mouse and human naïve $CD4^+$ T cells.

a. Spleen and lymph nodes were harvested from mice and single cell suspension were prepared. In the first step, lymphocytes were gated in forward and side scatter (SSC-A vs. FSC-A), and lymphocytes were further selected as singlets using the size discrimination parameters (FSC-H vs. FSC-A). Using the CD4, CD25 and CD62L fluorochrome labelled antibodies, $CD25^+$ and $CD4^-$ populations were excluded resulting in the selection of $CD4^+$ T cells, which was further sorted into $CD4^+CD62L^+$ (naïve T cells) cells while excluding the $CD4^+CD62L^-$ population and the purity of post-sort population, $CD4^+CD62L^+$ T cells, was determined as indicated. b. Blood from healthy individuals were collected and PBMCs were subjected to lymphocytes gating based on the forward and side scatter (SSC-A vs. FSC-A). Cells were further selected as singlets using the size discrimination parameter (FSC-H vs. FSC-A). Using the fluorochrome labelled anti-human CD4, CD45RA and CD45RO antibodies, naive $CD4^+CD45RA^+$ T cells were sorted with a 99% post-sort purity.



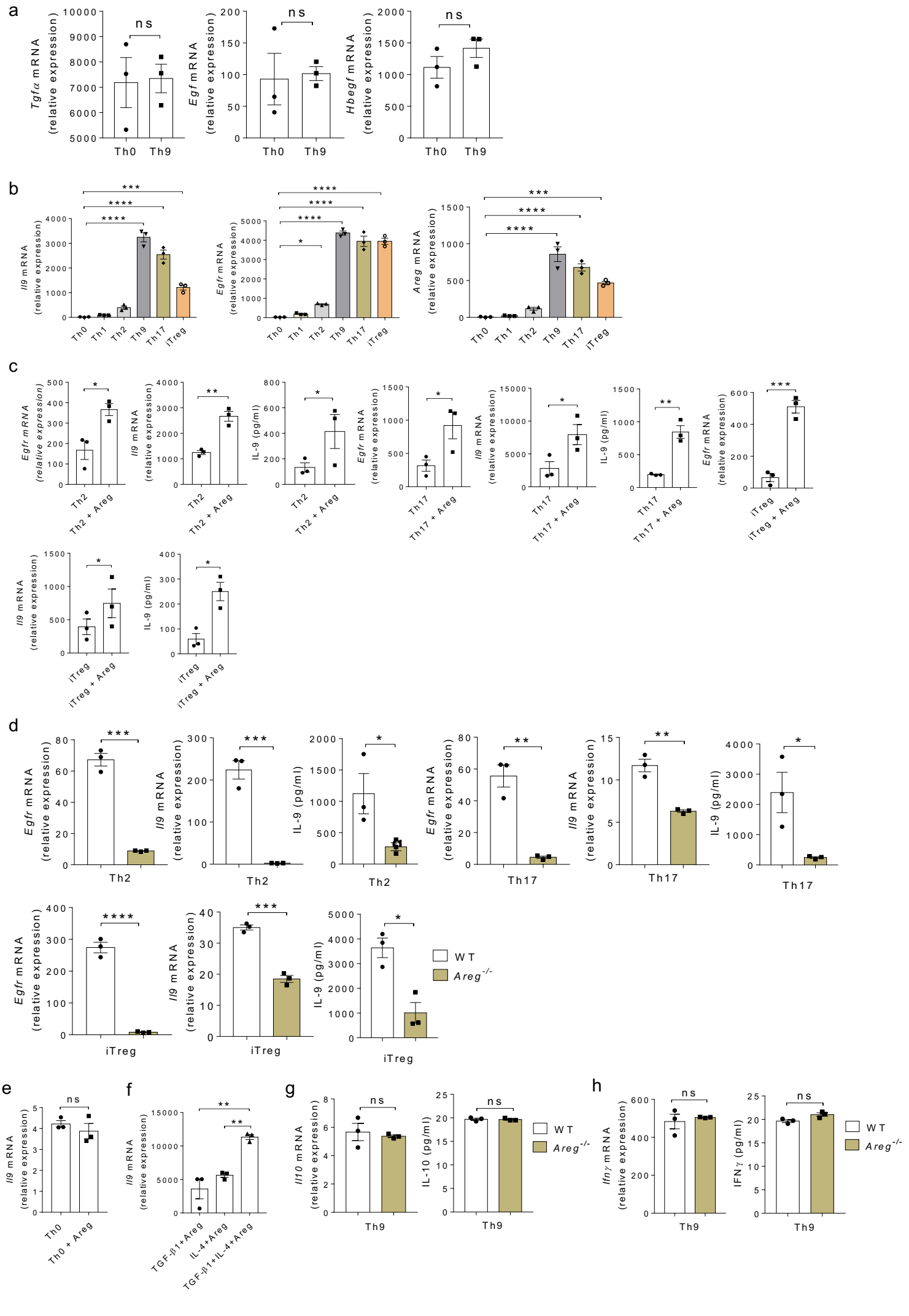
Supplementary Fig. 2. EGFR inhibition attenuates Th9 cell-mediated anti-tumor response *in vivo*.

a-h. Naïve $CD4^+$ T cells from OT-II TCR transgenic mice were *in vitro* differentiated into Th9 with or without 1.0 μM gefitinib for 3 days. Cells were then adoptively transferred into B16-OVA tumor bearing WT mice, randomized into three groups (n=5 mice per group). a-f. Representative FACS analysis for $CD4^+$, $CD8^+$, IFN-γ^+ populations in spleen and tumor draining lymph nodes (dLN). g,h. Tumor-infiltrating lymphocytes (TILs) were isolated from the tumor followed by FACS analysis of intracellular staining for $CD8^+$IFN-γ^+ and $CD4^+$IFN-γ^+ cell populations. Data are representative from three independent experiments.



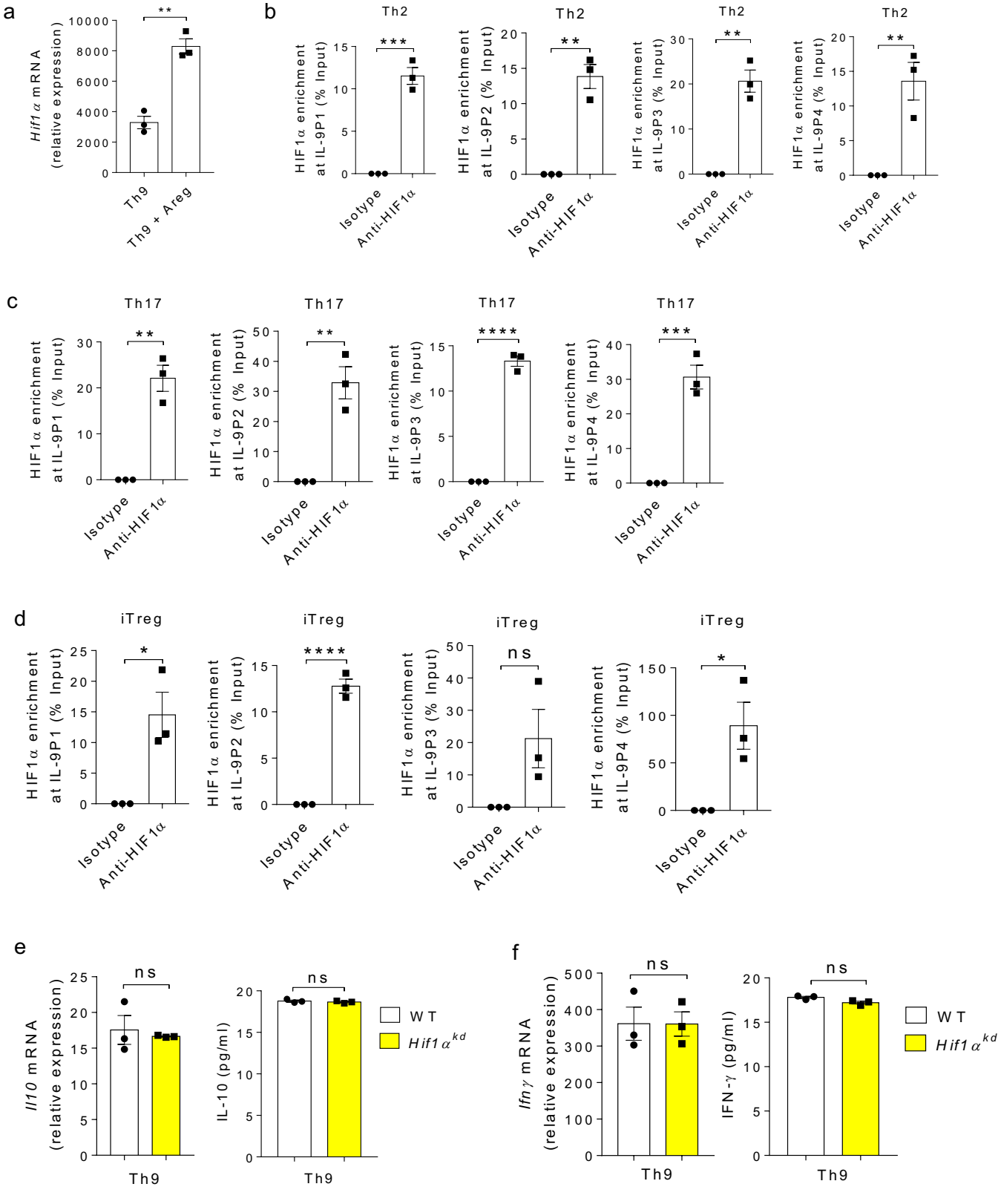
Supplementary Fig. 3. Blocking EGFR signaling abrogates IL-9 induction in Th2, Th17, iTregs.

a. Naïve CD4$^+$ T cells from WT mice were *in vitro* differentiated under Th2, Th17 and iTreg polarizing conditions with or without 1.0 μM gefitinib; analysis of IL-9 expression was done by qPCR and ELISA. Data are representative of mean ± SEM from three independent experiments. b,c. Naïve CD4$^+$ T cells from WT and *Egfr$^{flox/flox}$XCd4-cre* mice were differentiated under Th9 polarizing conditions for 3 days followed by qPCR and ELISA for IL-10 and IFN-γ. Data are representative of mean ± SEM from three independent experiments. a-c. *P < 0.0332, **P < 0.0021, ***P < 0.0002, *P* = ns (not significant), using two-tailed unpaired student's t test.


a
Tgfα mRNA (relative expression)
Egf mRNA (relative expression)
Hbegf mRNA (relative expression)
ns
Th0
Th9
b
Il9 mRNA (relative expression)
Egfr mRNA (relative expression)
Areg mRNA (relative expression)
Th0
Th1
Th2
Th9
Th17
iTreg
c
Egfr mRNA (relative expression)
Il9 mRNA (relative expression)
IL-9 (pg/ml)
Th2
Th2 + Areg
Th17
Th17 + Areg
iTreg
iTreg + Areg
d
Egfr mRNA (relative expression)
Il9 mRNA (relative expression)
IL-9 (pg/ml)
Th2
Th17
iTreg
WT
Areg-/-
e
Il9 mRNA (relative expression)
Th0
Th0 + Areg
f
Il9 mRNA (relative expression)
TGF-β1+Areg
IL-4+Areg
TGF-β1+IL-4+Areg
g
Il10 mRNA (relative expression)
IL-10 (pg/ml)
Th9
WT
Areg-/-
h
Ifnγ mRNA (relative expression)
IFNγ (pg/ml)
Th9
WT
Areg-/-

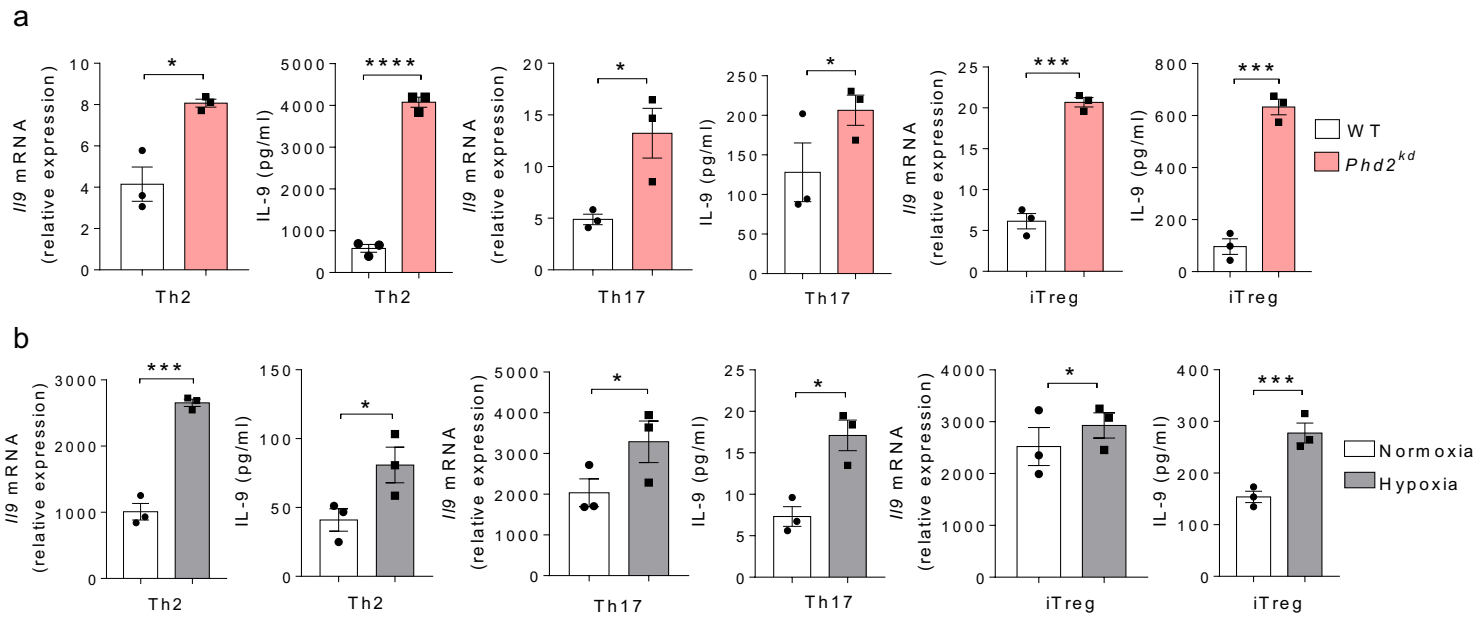
Supplementary Fig. 4. Areg amplifies IL-9 induction in Th2, Th17, iTregs.

a. Naïve $CD4^+$ T cells from WT mice were differentiated into Th0 and Th9 followed by qPCR analysis of *Tgfα, Egf* and *Hbegf* expression. Data are representative of mean ± SEM from three independent experiments. b. Naïve $CD4^+$ T cells from WT mice were differentiated into Th0, Th1, Th2, Th9, Th17, iTregs followed by qPCR analysis of *Il9, Egfr* and *Areg* expression. Data are representative of mean ± SEM from three independent experiments c. Naïve $CD4^+$ T cells from WT mice were *in vitro* differentiated under Th2, Th17 and iTreg polarizing conditions with or without 100 ng/ml Areg followed by qPCR analysis of *Egfr* and *Il9* expression and ELISA for IL-9. Data are representative of mean ± SEM from three independent experiments. d. Naïve $CD4^+$ T cells from WT and $Areg^{-/-}$ mice were differentiated under Th2, Th17, iTreg polarizing conditions for 3 days followed by qPCR analysis of *Il9* and *Egfr* expression and ELISA for IL-9. Data are representative of mean ± SEM from three independent experiments e. Naïve $CD4^+$ T cells from WT mice were cultured as Th0 in the presence or absence of 100 ng/ml Areg followed by qPCR analysis of *Il9* expression. Data are representative of mean ± SEM from three independent experiments. f. Naïve $CD4^+$ T cells from WT mice were differentiated in the presence of TGF-β1 or IL-4 or TGF-β1+IL-4 with 100 ng/ml Areg supplementation followed by qPCR analysis of *Il9* expression. Data are representative of mean ± SEM from three independent experiments. g,h. Naïve $CD4^+$ T cells from WT and $Areg^{-/-}$ mice were differentiated under Th9 polarizing conditions for 3 days followed by qPCR and ELISA for IL-10 and IFN-γ. Data are representative of mean ± SEM from three independent experiments. a,c,d,e,g,h. *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001 , P = ns (not significant), using two-tailed unpaired student's t test. b,f. *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001, using one-way ANOVA followed by Tukey's multiple comparison test.


a
Hif1α mRNA (relative expression)
10000
8000
6000
4000
2000
0
**
Th9
Th9 + Areg
b
Th2
HIF1α enrichment at IL-9P1 (% Input)
***
HIF1α enrichment at IL-9P2 (% Input)
**
HIF1α enrichment at IL-9P3 (% Input)
**
HIF1α enrichment at IL-9P4 (% Input)
**
Isotype
Anti-HIF1α
c
Th17
HIF1α enrichment at IL-9P1 (% Input)
**
HIF1α enrichment at IL-9P2 (% Input)
**
HIF1α enrichment at IL-9P3 (% Input)
****
HIF1α enrichment at IL-9P4 (% Input)
***
Isotype
Anti-HIF1α
d
iTreg
HIF1α enrichment at IL-9P1 (% Input)
*
HIF1α enrichment at IL-9P2 (% Input)
****
HIF1α enrichment at IL-9P3 (% Input)
ns
HIF1α enrichment at IL-9P4 (% Input)
*
Isotype
Anti-HIF1α
e
Il10 mRNA (relative expression)
ns
IL-10 (pg/ml)
ns
WT
Hif1α kd
Th9
f
Ifnγ mRNA (relative expression)
ns
IFN-γ (pg/ml)
ns
WT
Hif1α kd
Th9

Supplementary Fig. 5. HIF1α binds to IL-9 promoter in Th2, Th17, iTregs.

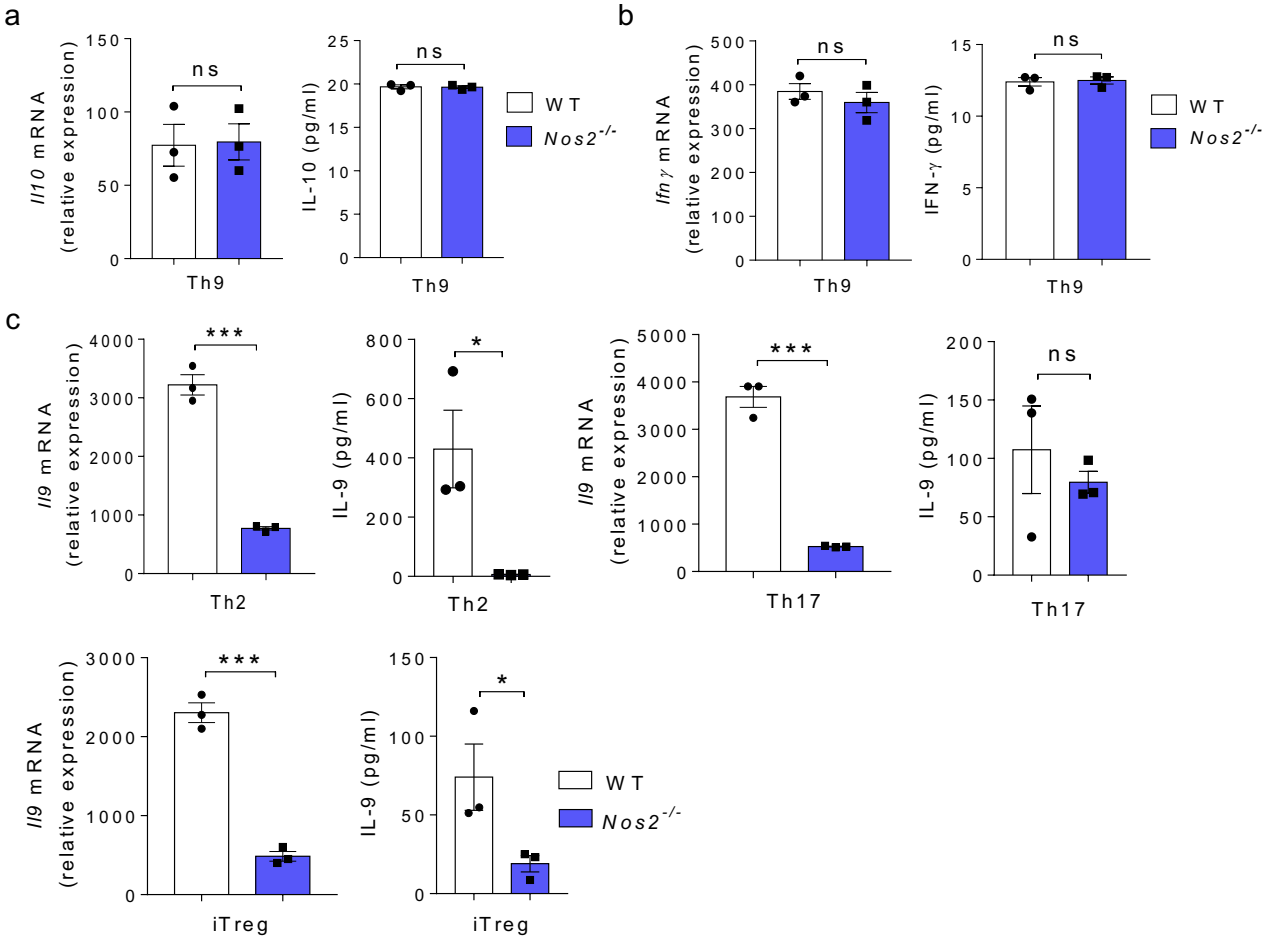
a. Naïve $CD4^+$ T cells from WT mice were differentiated to Th9 in the presence or absence of 100 ng/ml Areg followed by qPCR analysis of *Hif1α* expression. Data are representative of mean ± SEM from three independent experiments. b-d. ChIP analysis of HIF1α binding to IL-9 promoter in Th2, Th17, iTregs represented as enrichment of HIF1α on IL-9 promoter relative to input. Data are representative of mean ± SEM from three independent experiments. e,f. Naïve $CD4^+$ T cells from WT and $Hif1\alpha^{kd}$ mice were differentiated under Th9 polarizing conditions for 3 days followed by qPCR and ELISA for IL-10 and IFN-γ. Data are representative of mean ± SEM from three independent experiments. a-f. *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001, *P* = ns (not significant), using two-tailed unpaired student's t test.



Supplementary Fig. 6. PHD2 and Hypoxia enhances IL-9 induction in Th2, Th17, iTregs.

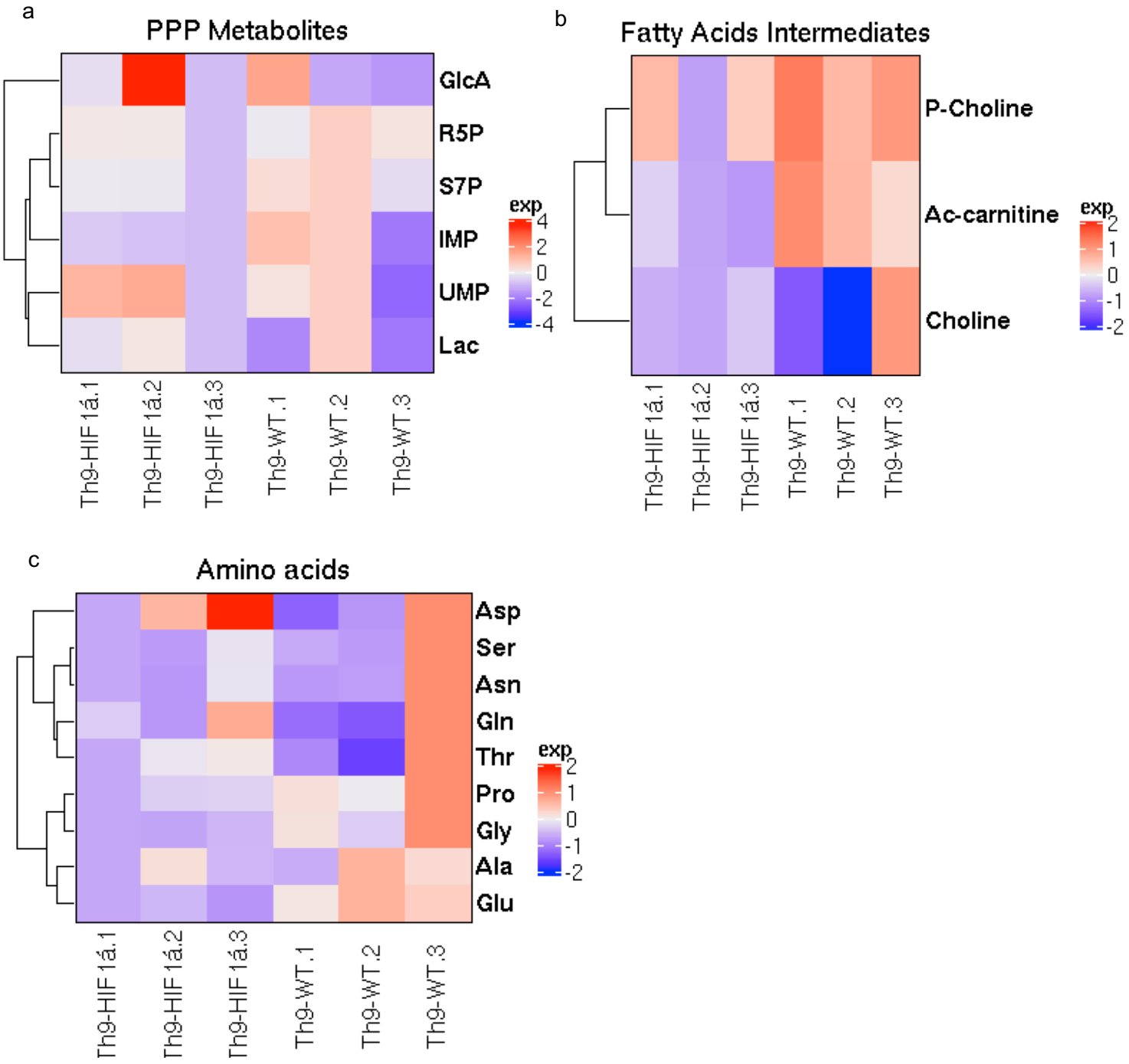
a. Naïve CD4$^+$ T cells from WT and *Phd2^{kd}* mice were differentiated under Th2, Th17, iTreg polarizing conditions with daily treatment of 1.0 μg/ml Dox for 3 days followed by qPCR and ELISA for IL-9. Data are representative of mean ± SEM from three independent experiments.

b. Naïve CD4$^+$ T cells from WT mice were differentiated into Th2, Th17, iTregs under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions for 3 days. qPCR analysis of *Il9* expression and ELISA for IL-9 production. Data are representative of mean ± SEM from three independent experiments. a.b. *P < 0.0332, ***P < 0.0002, ****P < 0.0001, using two-tailed unpaired student's t test.



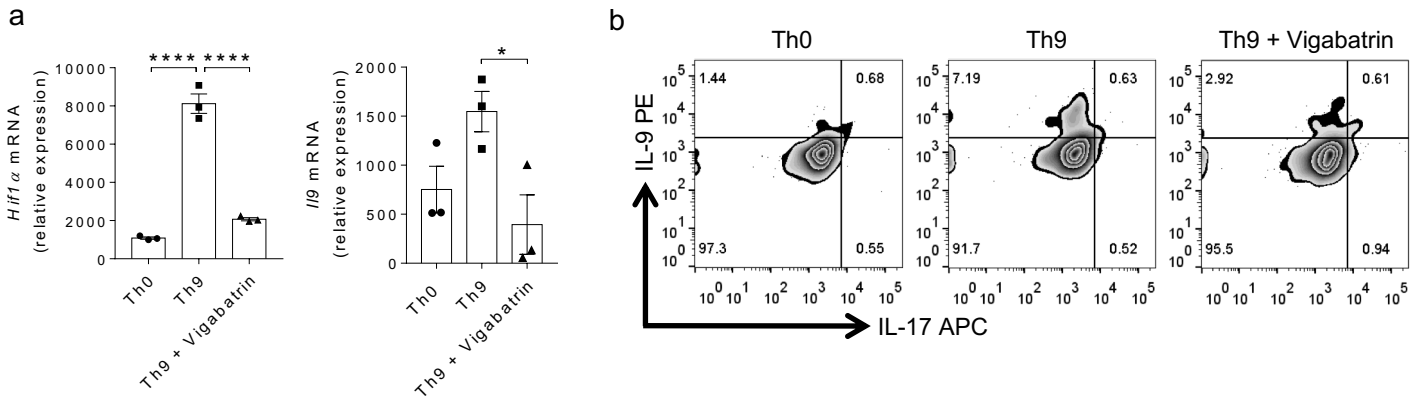
Supplementary Fig. 7. NO augments IL-9 induction in Th2, Th17, iTregs.

a,b. Naïve CD4$^+$ T cells from WT and *Nos2*$^{-/-}$ mice were differentiated to Th9 followed by qPCR and ELISA for IL-10 and IFN-γ. Data are representative of mean ± SEM from three independent experiments. c. Naïve CD4$^+$ T cells from WT and *Nos2*$^{-/-}$ mice were differentiated to Th2, Th17 and iTregs followed by qPCR and ELISA for IL-9. Data are representative of mean ± SEM from three independent experiments. a-c. *P < 0.0332, ***P < 0.0002, *P* = ns (not significant), using two-tailed unpaired student's t test.



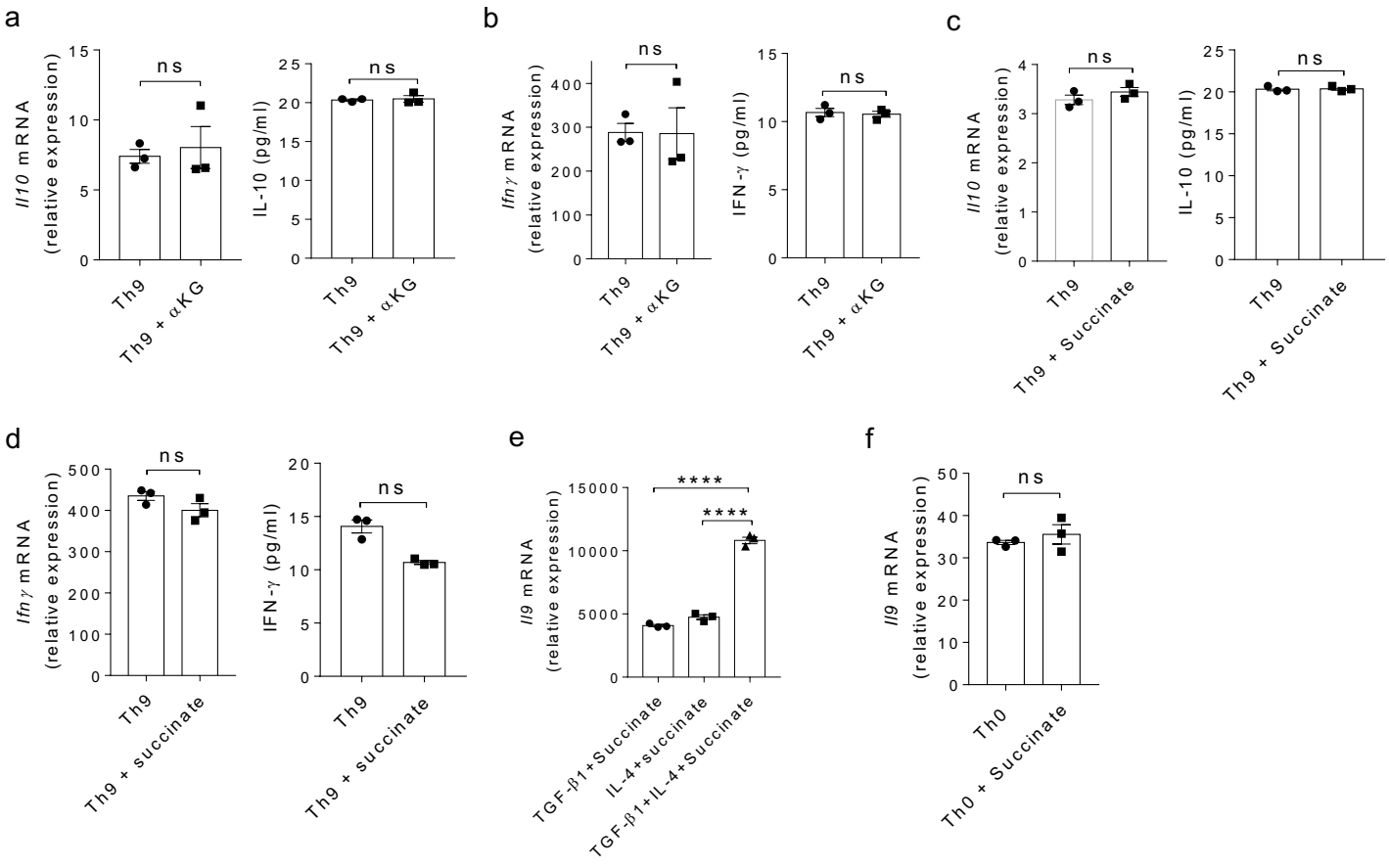
Supplementary Fig. 8. Metabolomics profiling of Th9 cells.

Naïve $CD4^+$ T cells from WT and *Hif1α*^{kd} mice were differentiated under Th9 polarizing conditions. Cell extracts were prepared and subjected to metabolomics profiling. Heat-maps showing (a) metabolites of Pentose Phosphate Pathway (PPP), (b) fatty acid intermediates and (c) amino acids.



Supplementary Fig. 9. Vigabatrin inhibits HIF1α-mediated Th9 cell differentiation.

Naïve $CD4^+$ T cells from WT mice were differentiated under Th0 and Th9 polarizing conditions with or without 500 μM vigabatrin followed by a. qPCR analysis of *Hif1α* and *Il9* expression. Data are representative of mean ± SEM from three independent experiments. b. FACS analysis of intracellular IL-9 and IL-17. *P < 0.0332, ****P < 0.0001, using one-way ANOVA followed by Tukey's multiple comparison test.



Supplementary Fig. 10. The effects of αKG and succinate are central to IL-9 and Th9 cells.

a,b. Naïve $CD4^+$ T cells from WT mice were differentiated under Th9 polarizing conditions with or without 1.0 mM αKG followed by qPCR and ELISA for IL-10 and IFN-γ. Data are representative of mean ± SEM from three independent experiments. c,d. Naïve $CD4^+$ T cells from WT mice were differentiated under Th9 polarizing conditions with or without 5.0 mM succinate followed by qPCR and ELISA for IL-10 and IFN-γ. Data are representative of mean ± SEM from three independent experiments e. Naïve $CD4^+$ T cells from WT mice were differentiated in the presence of TGF-β1 or IL-4 or TGF-β1+IL-4 with 5.0 mM succinate supplementation followed by qPCR analysis of *Il9* expression. Data are representative of mean ± SEM from three independent experiments. f. Naïve $CD4^+$ T cells from WT mice were cultured as Th0 with or without 5.0 mM succinate followed by qPCR analysis of *Il9* expression. Data are representative of mean ± SEM from three independent experiments. a,b,c,d,f. *P* = ns (not significant), using two-tailed unpaired student's t test. e. ****$P < 0.0001$, using one-way ANOVA followed by Tukey's multiple comparison test.

Supplementary Table 1. SYBR Primers for qPCR

Gene	Gene Sequence
mβactin F	5’-GATGTATGAAGGCTTTGGTC-3’
mβactin R	5’-TGTGCACTTTTATTGGTCTC-3’
mHif1α F	5’-CGATGACACAGAAACTGAAG-3’
mHif1α R	5’-GAAGGTAAAGGAGACATTGC-3’
mIl9 F	5’-CTGATGATTGTACCACACGTGC-3’
mIl9 R	5’-GCCTTTGCATCTCTGTCTTCTGG-3’
mIl10 F	5’-CAGGACTTTAAGGGTTACTTG-3’
mIl10 R	5’-ATTTTCACAGGGGAGAAATC-3’
mIfnγ F	5’-TGAGTATTGCCAAGTTTGAG-3’
mIfnγ R	5’-CTTATTGGGACAATCTCTTCC-3’
mSpi1 F	5’-CATGAGGTGAAATGTGAGAG-3’
mSpi1 R	5’-AGTTGGTTGAAATGGATCAC-3’
mIrf4 F	5’-ACGCTGCCCTCTTCAAGGCTT-3’
mIrf4 R	5’-TGGCTCCTCTCdCAATTCC-3’
mGata3 F	5’-TATTAACAGACCCCTGACTATG-3’
mGata3 R	5’-CACCTTTTTGCACTTTTTCG-3’
mBatf F	5’-AAAATGACAAGTCAACCCTG-3’
mBatf R	5’-TTAGAAAACTATCCACCCCC-3’
mIrf1 F	5’-TCTGTATAACCTACAGGTGTC-3’
mIrf1 R	5’- CAGACTGTTCAAAGAGCTTC -3’
mEgfr F	5'-TCTTCAAGGATGTGAAGTGTG-3’
mEgfr R	5'-TGTACGCTTTCGAACAATGT-3'
mAreg F	5’-GCCATTATGCAGCTGCTTTGGAGC-3’
mAreg R	5’-TGTTTTTCTTGGGCTTAATCACCT-3’
mIl33r F	5’- GGTTGCTCTGTTCTGGAGAGAT-3’
mIl33r R	5’-CTGCATCTTGCCCAGGTAAC-3’
mNos2 F	5’-TTTTGCATGACACTCTTCAC-3’
mNos2 R	5’-ACTGGTTGATGAACTCAATG-3’
mEgln1 F	5’-CCAAATGGAGATGGAAGATG-3’
mEgln1 R	5’-AGAATACCTCCACTTACCTTG-3’
mTgfα F	5’-AGCCAGAAGAAGCAAGCCATCACT-3’
mTgfα R	5’-CTCATTCTCGGTGTGGGTTAGCAA-3’
mEgf F	5’-AGATGAGTGTGTGCTGGCTAGATC-3’
mEgf R	5’-TCCGAGTCCTGTAGTAGTAAGTCC -3’
mHbegf F	5’-CTCCCACTGGATCCACAAAC-3’
mHbegf R	5’-GGCATGGGTCTCTCTTCTTC-3’
hGapdh F	5’-TGCACCACCAACTGCTTAGC-3’
hGapdh R	5’-GGCATGGACTGTGGTCATGAG-3’
hIl9 F	5’- GTGCCACTGCAGTGCTAATGT-3’
hIl9 R	5’-CTCTCACTGAAGCATGGTCTGG-3’
hEgfr F	5'-GCCAAGGCACGAGTAACAAGC-3'
hEgfr R	5'-AGGGCAATGAGGACATAACC-3'

Supplementary Table 2. SYBR Primers for ChIP-PCR

Gene	Gene Sequence
IL9P_HIF1α_site 1 RP	5’-TGCTGTAACCACTGAGCCAT-3’
IL9P_HIF1α_site 1 LP	5’-GTGACAGCAGACAAGAGCAG-3’
IL9P_HIF1α_site 2 RP	5’-ACTGCGGTGGGATATGTAGG-3’
IL9P_HIF1α_site 2 LP	5’-GAAGTGTGAGAGCAACGTGG-3’
IL9P_HIF1α_site 3 RP	5’-TGCCAGTGTATCAGTCAGGG-3’
IL9P_HIF1α_site 3 LP	5’-AGAATATGCATGCCTGGTGG-3’
IL9P_HIF1α_site 4 RP	5’-ACCACAGACCAGAGTTAGGC-3’
IL9P_HIF1α_site 4 LP	5’-CTCTCCCCTTCTACCTGCTG-3’
Nos2P_HIF1α_site 1 RP	5’-CATGCAAGGCCATCTCTTCC-3’
Nos2P_HIF1α_site 1 LP	5’-TCCGTGCCCAGAACAAAATC-3’