Title: Reversal of type 1 diabetes through therapeutic elevation of ω-3 PUFAs

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Supplemental Materials

Supplemental Figure 1. Representative hematoxylin/eosin images of four categories based on the relative degree of islets immune infiltration, related to Figure 1B and 6D.

Supplemental Figure 2. The ratio of Th1/Th2 cells was polarized towards Th1 in diabetic NOD mice.

Supplemental Figure 3. Modulation of Th cells by ω-3 and ω-6 PUFAs in vivo, related to Figure 2.

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Supplemental Table 1. Distribution of ω-3 and ω-6 PUFA Species in Diets.

Supplemental Table 2. Analysis of ω-3 and ω-6 PUFA Species in the Blood Samples of NOD mice with Dietary Intervention.

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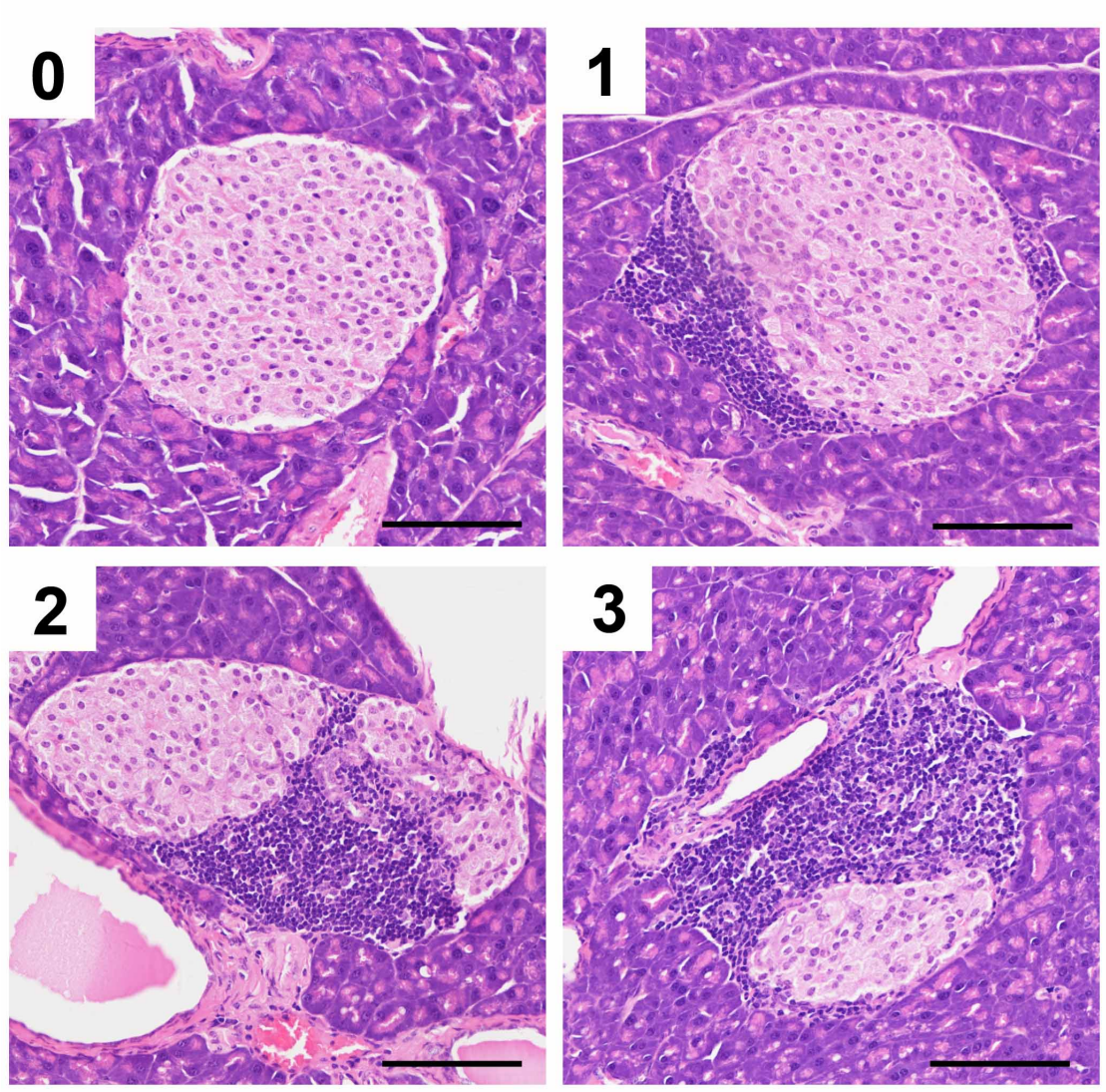
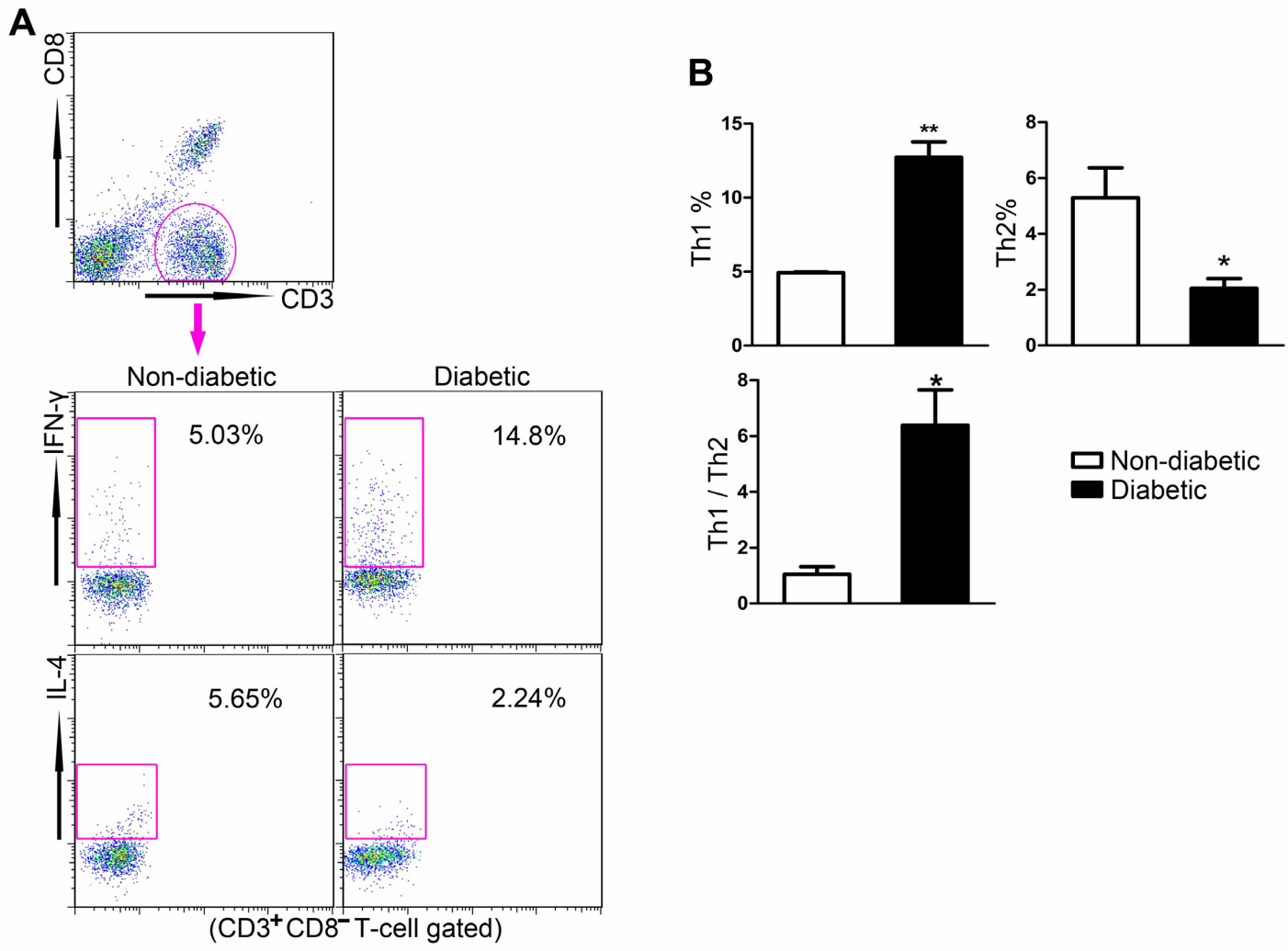
Supplemental Figure 1



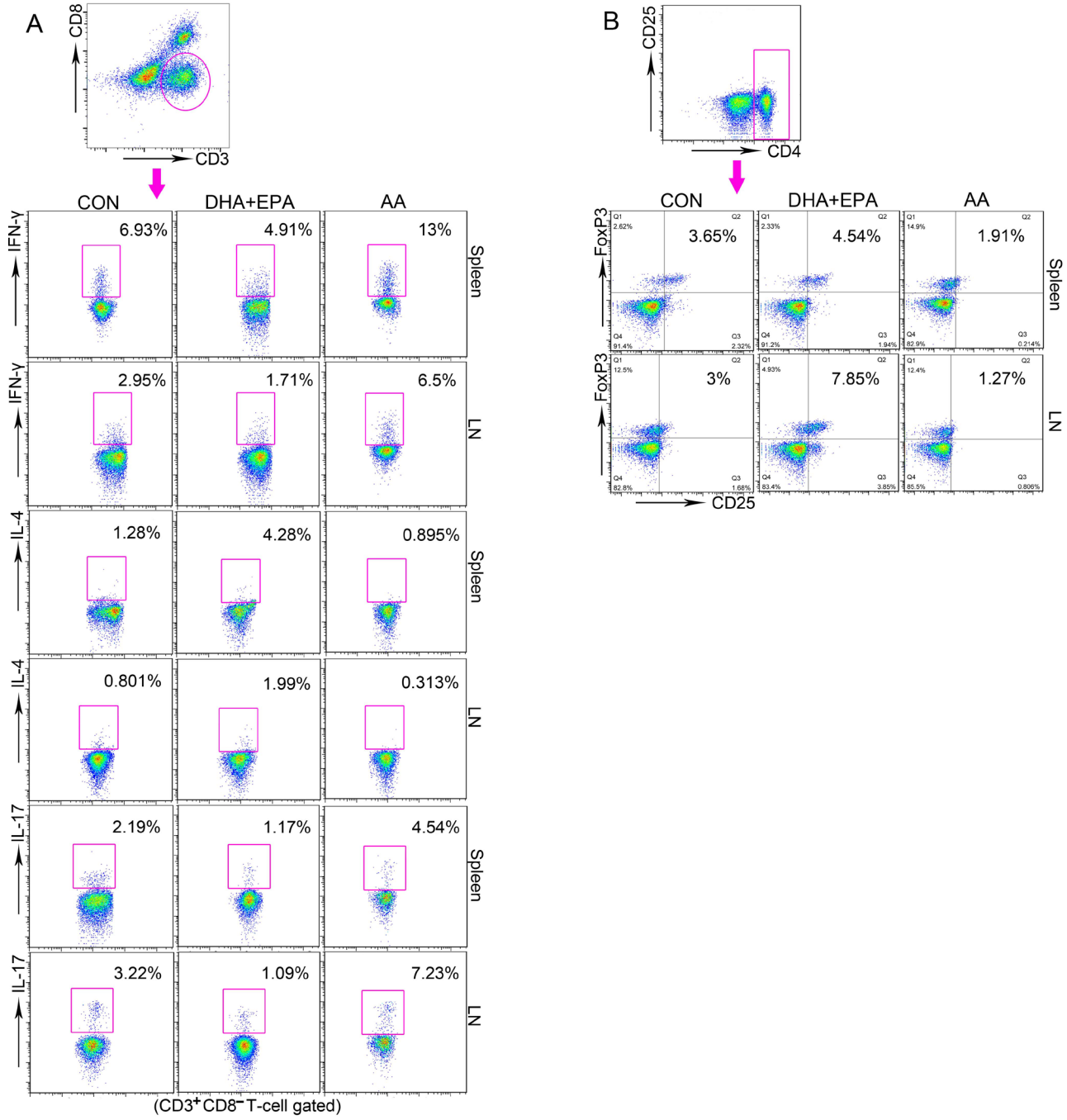
Supplemental Figure 1. ω-3 PUFAs blocked the progression of immune infiltration in NOD mice, related to Figure 1A and 6D. Representative hematoxylin/eosin images of four categories based on the relative degree of islets immune infiltration. Islets were characterized by the relative degree of leukocyte infiltration in non-diabetic mice. The graph showed the fraction of islets with no insulitis(0), periinsulitis, in which leukocytes remained largely in the periphery of the islet(1),invasive insulitis, corresponding to 25–50% coverage of the islet(2), and severe insulitis, in which greater than 50% of the islet was covered with infiltrate(3). Scale bars = 100 μm.

Supplemental Figure 2



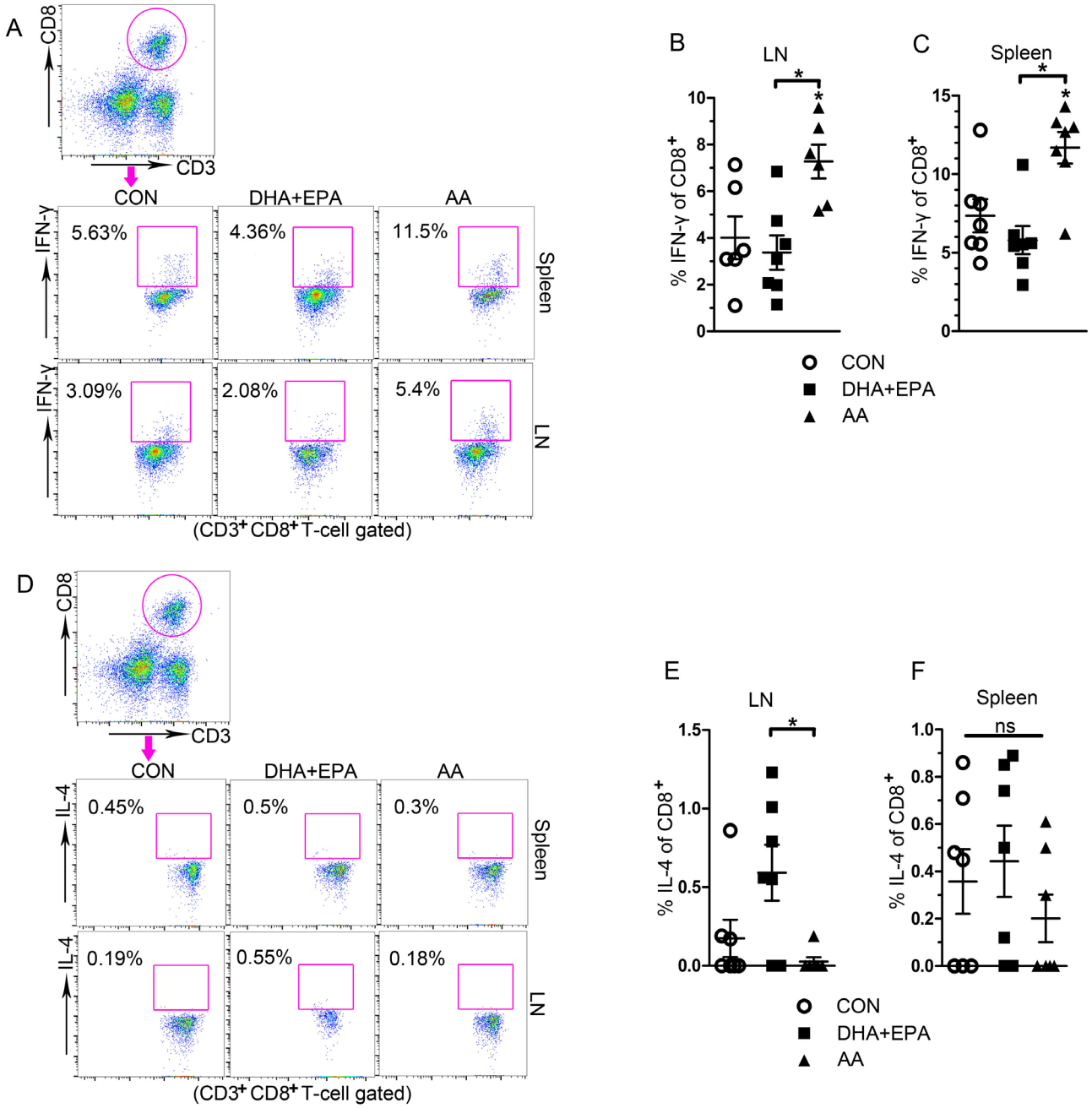
Supplemental Figure 2. The ratio of Th1/Th2 cells was polarized towards Th1 in diabetic NOD mice. IFN-γ^+ and IL-4^+ of Th cells in spleen of non-diabetic and diabetic NOD mice. (**A**)Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+ and IL-4^+ of Th cells (top right) and (**B-D**) quantification of n = 3 per group. Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$). *, P< 0.05; **, P< 0.01 when compared with non-diabetic group. Values are means ± SEM(Student *t* tests).

Supplemental Figure 3



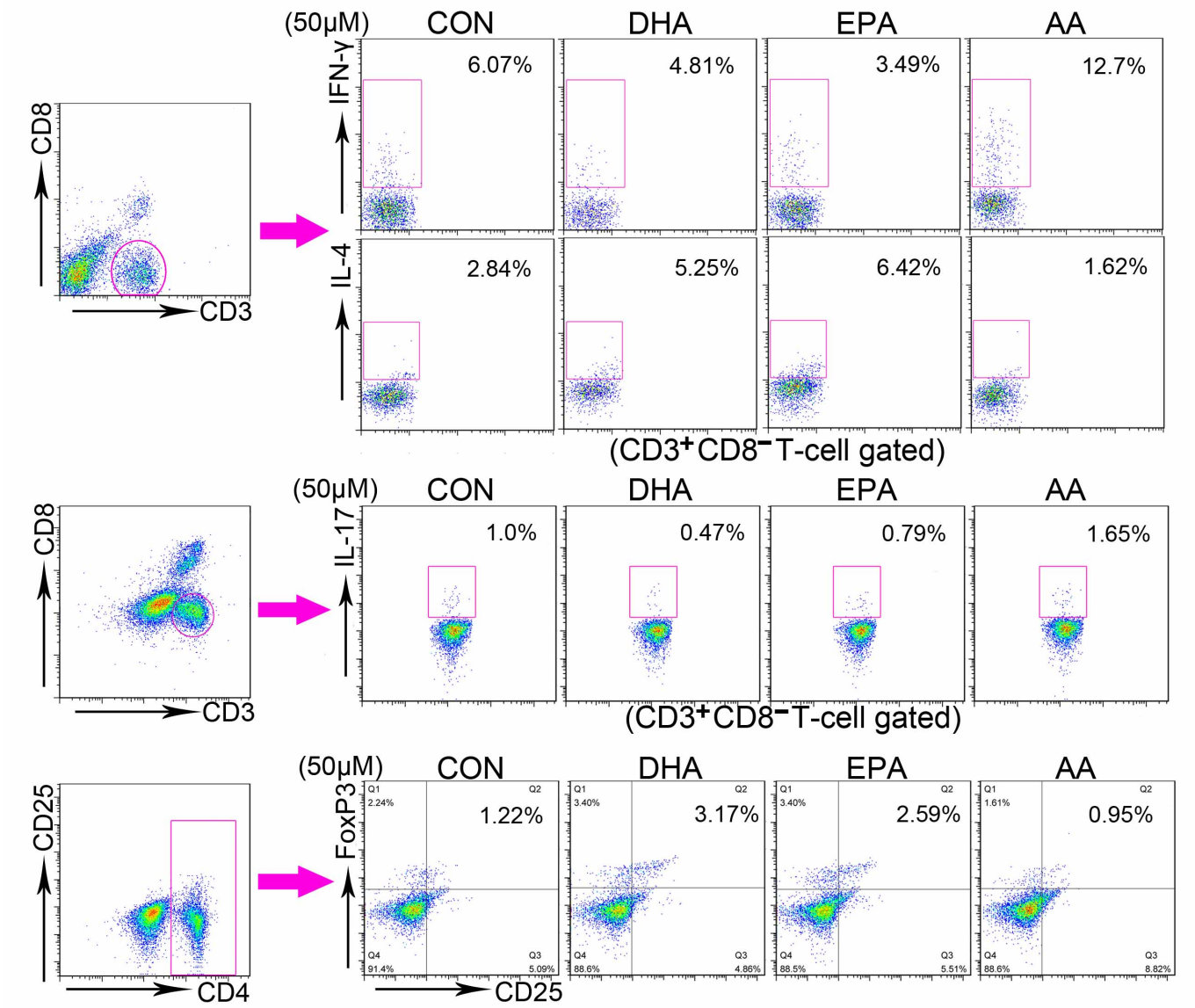
Supplemental Figure 3. Modulation of Th cells by ω-3 and ω-6 PUFAs *in vivo*, related to Figure 2. Percentage of Th1, Th2, Th17 and Treg cells in spleen and lymph node(LN) of non-diabetic NOD mice fed on control, DHA+EPA and AA diets. Spleen and lymph node were harvested when NOD mice were sacrificed at 20 weeks following dietary intervention(n = 6-8 per group). Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+, IL-4^+, IL-7^+ and $CD25^+$ $FoxP3^+$ of Th cells (top right). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$).

Supplemental Figure 4



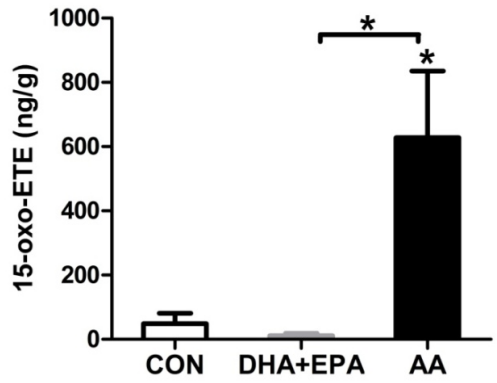
Supplemental Figure 4. Modulation of CD8$^+$ T-cells by ω-3 and ω-6 PUFAs *in vivo*, related to Figure 2. IFN-γ$^+$ and IL-4$^+$ of CD8$^+$ T-cells percentage in spleen and lymph node(LN) of non-diabetic NOD mice fed on control, DHA+EPA and AA diets. Spleen and lymph node were harvested when NOD mice were sacrificed at 20 weeks following dietary intervention. Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ$^+$(**A**) and IL-4$^+$(**D**) of CD8$^+$ T-cells (top right) and quantification(**B-C and E-F**) of n = 6-7 per group. Data shown are gated on CD8$^+$ T-cells. *, P< 0.05 when compared with control group. Values are means ± SEM(Student *t* tests).

Supplemental Figure 5



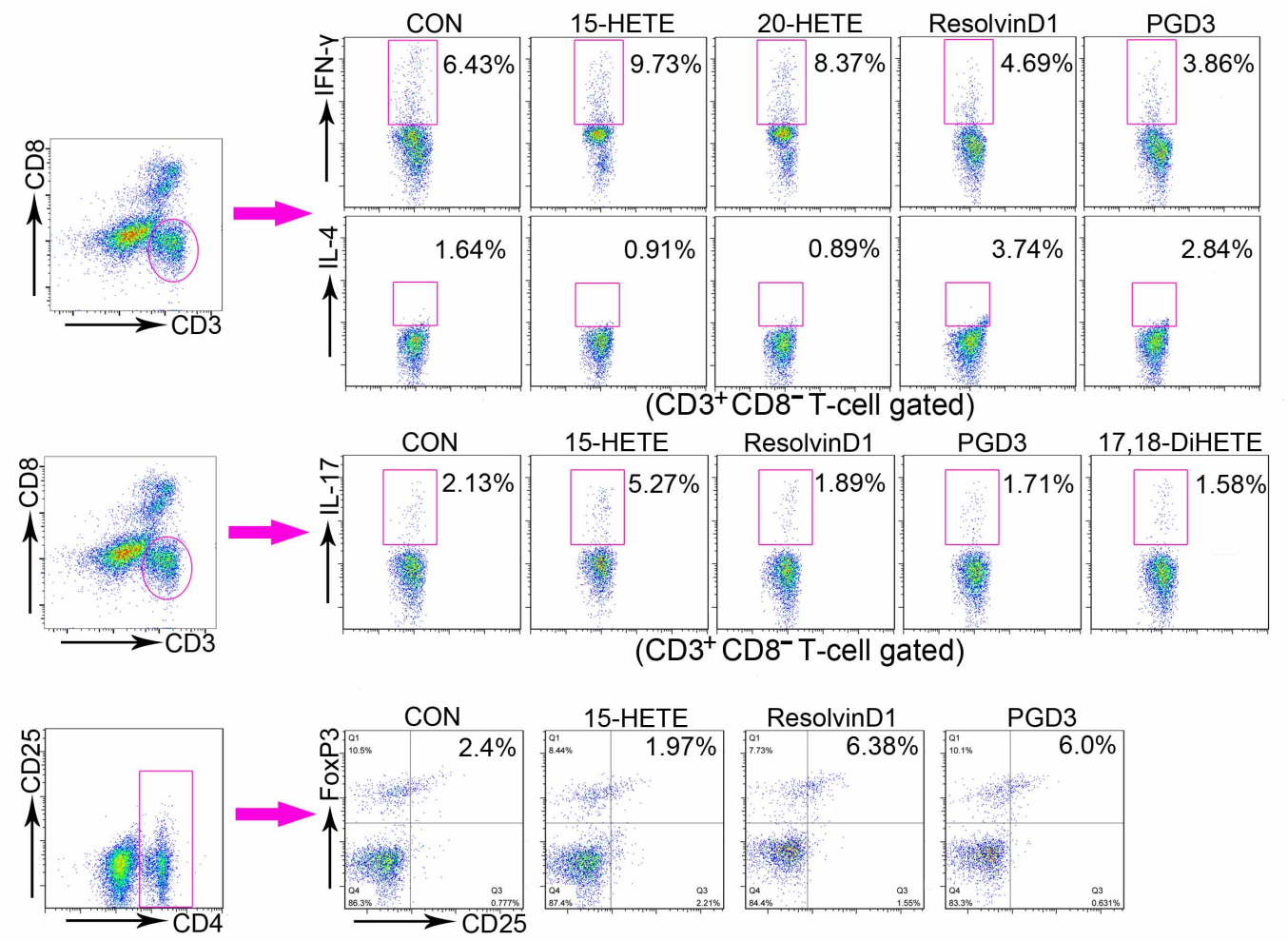
Supplemental Figure 5. ω-3 and ω-6 PUFAs changed Th cells polarization in cultured splenocytes, related to Figure 2. Intracellular staining of Th1, Th2, Th17 and Treg cells of 10-wk-old non-diabetic NOD mice cultured under PMA and ionomycin stimulation in the presence of DHA, EPA and AA(50μM) added at the time of activation for 24 h. Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+, IL-4^+, IL-7^+ and $CD25^+$ $FoxP3^+$ of Th cells (top right). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$).

Supplemental Figure 6



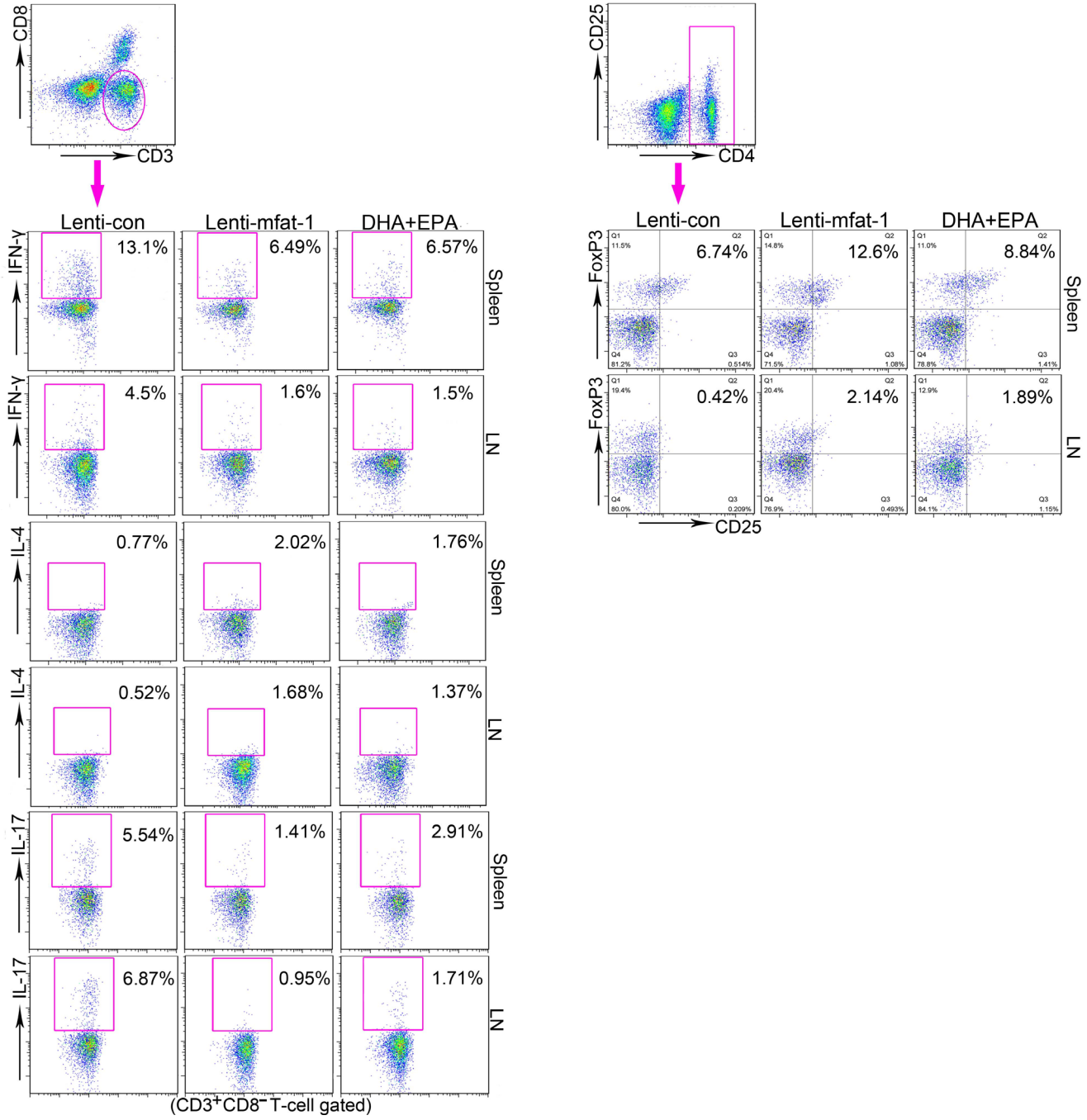
Supplemental Figure 6. AA-derived epoxyeicosatrienoic acids (EETs) increased in pancreas of AA-fed NOD mice, related to Figure 3. Presence of eicosanoids from ω-3 or ω-6 PUFAs in pancreas samples of NOD mice fed on control, DHA+EPA and AA diets(n = 6).*, P< 0.05 when compared to control group. Values are means ± SEM(Student *t* tests).

Supplemental Figure 7



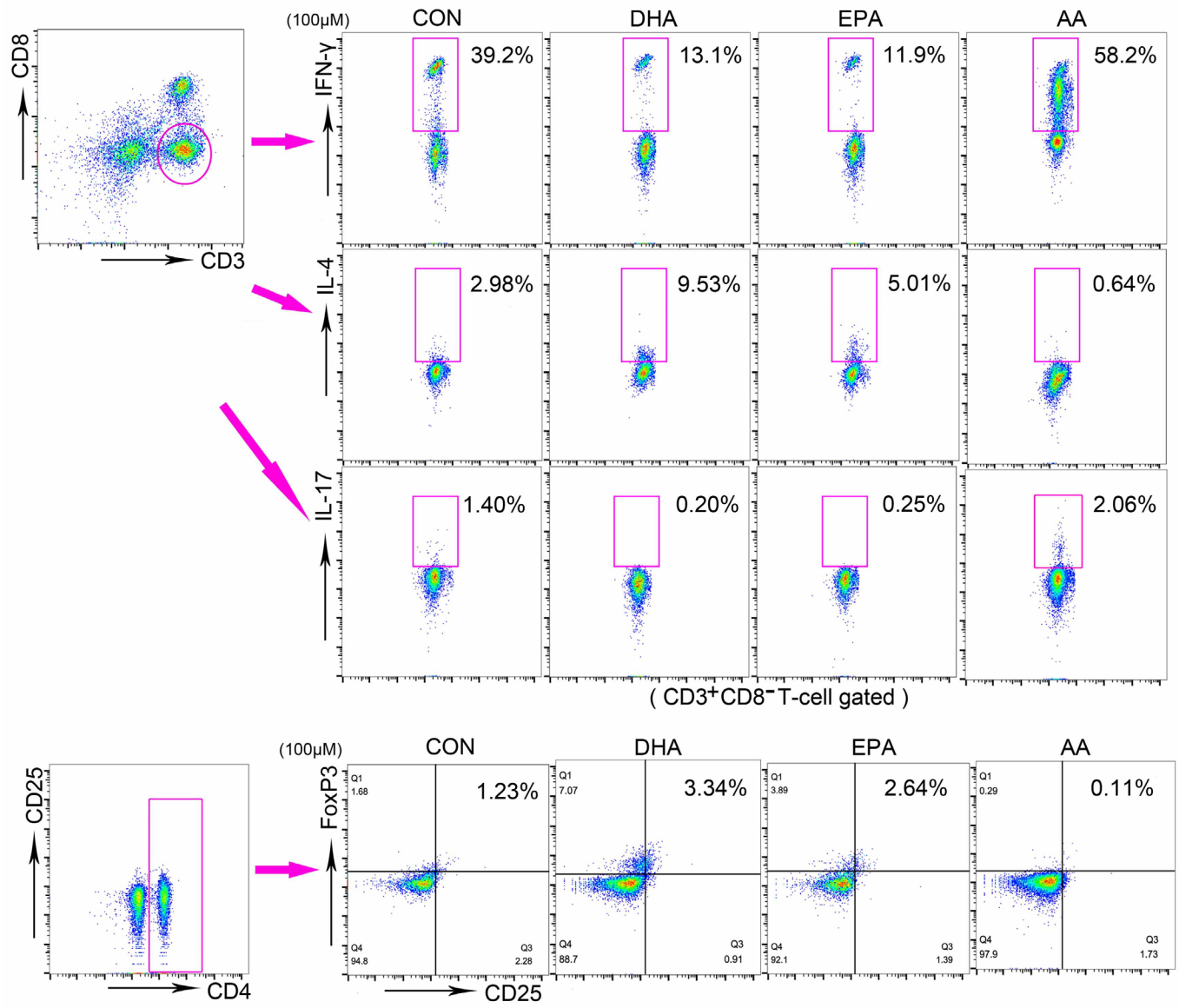
Supplemental Figure 7. ω-3 PUFAs regulates the differentiation of Th cells through its diverse metabolic production, related to Figure 3. Intracellular staining of Th1, Th2, Th17 and Treg cells of splenocytes in 10-wk-old non-diabetic NOD mice cultured under PMA and ionomycin stimulation in the presence of distinctive PUFAs metabolites added at the time of activation for 24 h. Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+, IL-4^+, IL-7^+ and $CD25^+$ $FoxP3^+$ of Th cells (top right). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$).

Supplemental Figure 8



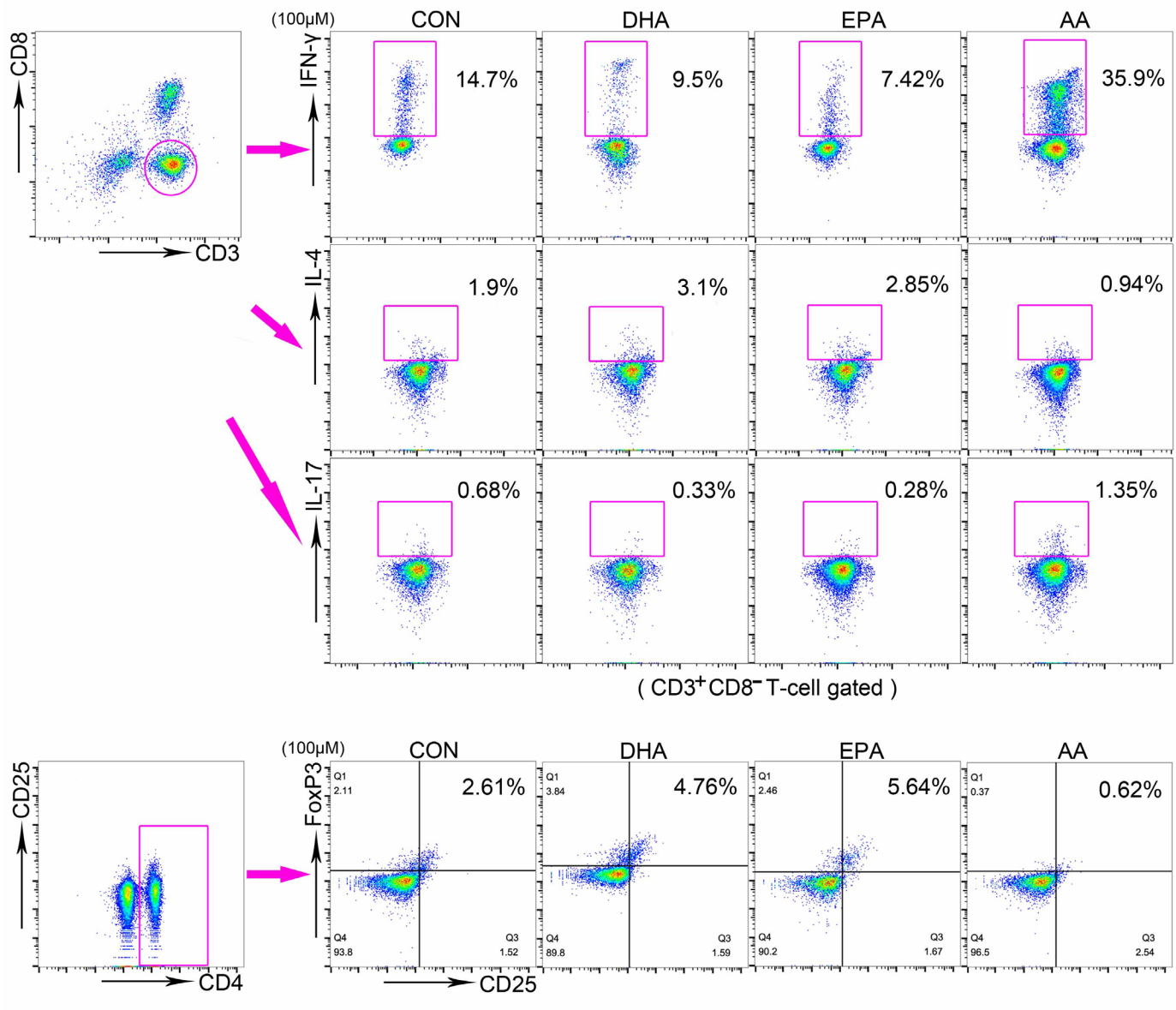
Supplemental Figure 8. Therapeutic ω-3 PUFAs improved Th cell differentiation in diabetic NOD mice, related to Figure 9. Intracellular staining of Th1, Th2, Th17 and Treg cells in spleen and lymph node (LN) of diabetic NOD mice treated with lenti-con, lenti-mfat-1 and fed on DHA+EPA diet. Mice were sacrificed and spleen and lymph node harvested at 9 weeks following lentivirus treatment and DHA+EPA dietary intervention. Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+, IL-4^+, IL-7^+ and $CD25^+$ $FoxP3^+$ of Th cells (top right). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$).

Supplemental Figure 9



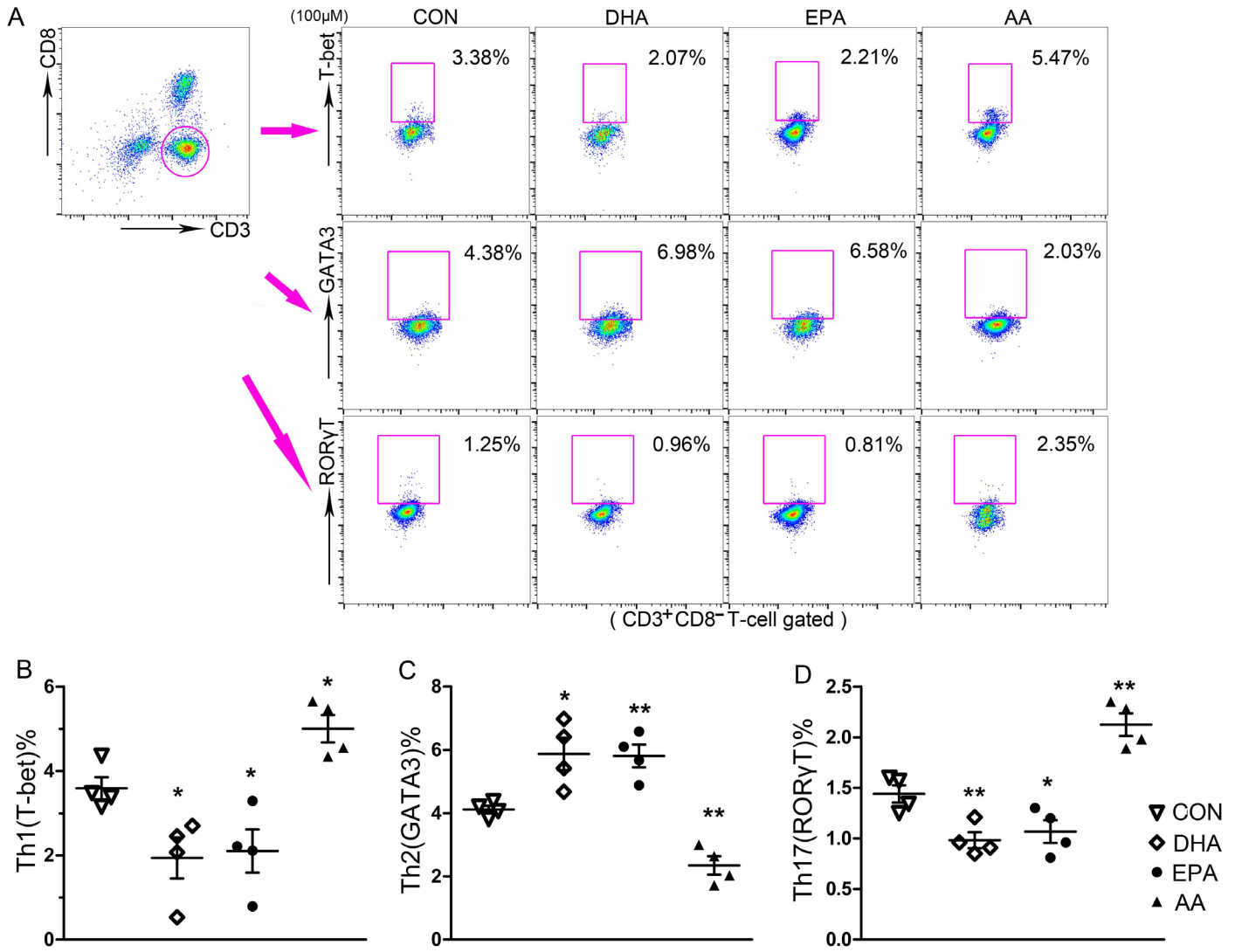
Supplemental Figure 9. ω-3 and ω-6 PUFAs changed Th cells polarization of PBMCs from T1D patients *in vitro*, related to Figure 10. Intracellular staining of Th1, Th2, Th17 and Treg cells of PBMCs from T1D patients cultured under PMA and ionomycin stimulation in the presence of DHA, EPA and AA(100μM) added at the time of activation for 24 h. Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+, IL-4^+, IL-7^+ and $CD25^+$ $FoxP3^+$ of Th cells (top right). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$).

Supplemental Figure 10



Supplemental Figure 10. Modulation of Th cells by ω-3 and ω-6 PUFAs in PBMCs from non-diabetic donors *in vitro*, related to Figure 10. Intracellular staining of Th1, Th2, Th17 and Treg cells of PBMCs from healthy donors cultured under PMA and ionomycin stimulation in the presence of DHA, EPA and AA(100μM) added at the time of activation for 24 h. Representative flow cytometry images with numbers in quadrants indicating percent IFN-γ^+, IL-4^+, IL-7^+ and $CD25^+$ $FoxP3^+$ of Th cells (top right). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$).

Supplemental Figure 11



Supplemental Figure 11. Expression of transcription factors of Th cells modulated by ω-3 and ω-6 PUFAs in PBMCs from non-diabetic donors *in vitro*. Representative flow cytometry images(**A**) and the quantification (**B-D**)of T-bet- ,GATA3- and RORγT- staining Th cells of PBMCs from non-diabetic donors cultured in the presence of PMA and ionomycin as well as DHA, EPA and AA(100μM) for 24 h(n = 4 per group). Data shown are gated on $CD4^+$ T-cells($CD3^+CD8^-$). *, P< 0.05; **, P< 0.01 when compared to control group. Values are means ±SEM.(Student *t* tests).

Supplemental Table 1. Distribution of ω-3 and ω-6 PUFA Species in Diets.

(%)	CON Diet	DHA+EPA Diet	AA Diet
ω-3 PUFA species			
C18:3 (α-LA)	1.83 ± 0.09	0.78 ± 0.004***	0.81 ± 0.004***
C20:5 (EPA)	1.17 ± 0.03	29.86 ± 0.15***	0.03 ± 0.001***
C22:5 (DPA)	0.21 ± 0.02	3.40 ± 0.01***	0.11 ± 0.001*
C22:6 (DHA)	1.96 ± 0.07	23.00 ± 0.06***	0.87 ± 0.01***
ω-6 PUFA species			
C18:2	29.93 ± 1.44	8.68 ± 0.02***	20.87 ± 0.13*
C18:3 (γ-LA)	0.07 ± 0.01	0.36 ± 0.03*	2.22 ± 0.01***
C20:4 (AA)	0.56 ± 0.03	3.62 ± 0.01***	31.92 ± 0.13***
C22:4	0.12 ± 0.001	0.18 ± 0.05***	0.29 ± 0.001***
ω-6/ω-3 ratio	5.94 ± 0.28	0.23 ± 0.002***	30.49 ± 0.24***

The compositions of ω-3 or ω-6 PUFAs were expressed using relative percentages, i.e., the distribution areas of ω-3 or ω-6 PUFAs peaks divided by the total peak areas of all detectable saturated and unsaturated free fatty acids (from the same sample) resolved from the gas chromatography column. Data were means ± SD; n=3. *, P< 0.05; **, P< 0.01; ***, P < 0.0001 when compared with the control diet group. AA: Arachidonic acid; ALA: α-lipoic acid; DHA: Docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: Eicosapentaenoic acid; LA: linoleic acid.

Supplemental Table 2. Analysis of ω-3 and ω-6 PUFA Species in the Whole Blood Samples of NOD Mice with Dietary Intervention

(%)	CON Diet	DHA+EPA Diet	AA Diet
ω-3 PUFA species			
C18:3 (α-LA)	0.16 ± 0.03	0.07 ± 0.06	NP
C20:5 (EPA)	0.70 ± 0.07	6.54 ± 0.57***	0.03 ± 0.04
C22:5	0.66 ± 0.08	1.82 ± 0.12***	0.29 ± 0.02***
C22:6 (DHA)	6.73 ± 0.62	11.18 ± 0.62***	3.52 ± 0.26***
ω-6 PUFA species			
C18:2	16.97 ± 0.42	9.59 ± 1.26***	3.52 ± 0.61***
C18:3 (γ-LA)	0.2 ± 0.01	0.16 ± 0.03	0.32 ± 0.07*
C20:4 (AA)	13.29 ± 1.16	10.14 ± 1.41	36.94 ± 2.34***
C22:4	0.86 ± 0.10	0.29 ± 0.05***	2.55 ± 0.08***
ω-6/ω-3 ratio	3.82 ± 0.40	1.03 ± 0.11***	11.32 ± 0.26***

Compositions of ω-3 or ω-6 PUFAs were expressed using the relative distribution area of the peaks in a given run of a gas chromatography assay. Data were means ± SD; n=3. *, P< 0.05; **, P< 0.01; ***, P < 0.0001 when compared with the control diet group. AA: Arachidonic acid; ALA: α-lipoic acid; DHA: Docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: Eicosapentaenoic acid; LA: linoleic acid; NP: no peak.

Supplemental Table 3. Analysis of ω-3 and ω-6 PUFA Species in the Tail Samples of NOD Mice with Dietary Intervention

(%)	CON Diet	DHA+EPA Diet	AA Diet
ω-3 PUFA species			
C18:3 (α-LA)	0.22 ± 0.08	0.32 ± 0.06	0.20 ± 0.01
C20:5 (EPA)	NP	1.37 ± 0.27***	NP
C22:5	0.10	1.00 ± 0.03***	NP
C22:6 (DHA)	1.78 ± 0.31	3.67 ± 0.45***	0.68 ± 0.03**
ω-6 PUFA species			
C18:2	14.10 ± 1.99	11.86 ± 2.76	11.03 ± 0.59
C18:3 (γ-LA)	NP	0.18 ± 0.11	1.06 ± 0.13***
C20:4 (AA)	5.57 ± 0.38	4.71 ± 1.121	19.96 ± 1.59***
C22:4	0.65 ± 0.09	0.06	1.27 ± 0.12*
ω-6/ω-3 ratio	9.93 ± 2.41	2.64 ± 0.20**	37.75 ± 1.78***

Each species of ω-3 and ω-6 PUFAs were expressed using relative percentages, ie, the distribution areas of ω-3 or ω-6 PUFA peaks divided by the total peak areas of all detectable saturated and unsaturated resolved from the gas chromatography column. Data presented were means ± SD; n=3. *, P< 0.05; **, P< 0.01; ***, P < 0.0001 when compared with the control diet group. AA: Arachidonic acid; ALA: α-lipoic acid; DHA: Docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: Eicosapentaenoic acid; LA: linoleic acid; NP: no peak.

Supplemental Table 4. Analysis of Cytokines Secretion in Blood Serum of Non-diabetic NOD Mice with Dietary Intervention

(pg/ml)	CON	DHA+EPA	AA
IFN-γ	0.78 ± 0.19	0.19 ± 0.05*	21.98 ± 10.34*
IL-4	0.53 ± 0.23	18.46 ± 6.33*	0.35 ± 0.19
IL-17A	0.60 ± 0.12	0.84 ± 0.59	15.90 ± 6.51*
IL-10	0.96 ± 0.21	27.81 ± 11.12*	0.89 ± 0.12
IL-6	10.82 ± 5.16	1.29 ± 0.40*	50.38 ± 9.56**
TNF-α	2.73 ± 0.20	1.16 ± 0.24	26.47 ± 8.77*

Cytokines secretion in blood serum of 20-wk-old non-diabetic NOD mice fed on control, DHA+EPA and AA diets. Data presented were means ± SEM; n=7. *, $P< 0.05$; **, $P< 0.01$ when compared with the control group.

Supplemental Table 5. Analysis of Cytokines Induced in NOD Splenocytes Activated with DHA , EPA and AA in Serum-containing Medium.

(pg/ml)	CON	DHA	EPA	AA
IFN-γ	123 ± 2.47	88.16 ± 3.91*	144.97 ± 22.86	153.79 ± 9.67*
IL-4	18.65 ± 0.19	17.50 ± 1.35	22.61 ± 0.75*	11.95 ± 0.24*
IL-17A	122.13 ± 4.94	92.81 ± 1.89*	102.71 ± 23.09	227.28 ± 1.15*
IL-10	105.37 ± 10.02	147.47 ± 7.35*	178.44 ± 3.54**	87.88 ± 8.77*
IL-6	122.07 ± 13.57	98.17 ± 20.32	125.60 ± 1.22	211.28 ± 13.62*

Cytokines induced in 10-wk-old non-diabetic NOD splenocytes (10^7 cells) with PMA and ionomycin plus 50μM of DHA, EPA and AA in serum-containing medium. Data presented were means ± SEM; n=3. *, $P< 0.05$; **, $P< 0.01$ when compared with the control group.

Supplemental Table 6. Analysis of ω-3 and ω-6 PUFA Species in the Whole Blood Samples of Diabetic NOD Mice in ω-3 PUFAs Treatment.

(%)	Lenti-con	Lenti-mfat-1	DHA+EPA
ω-3 PUFA species			
C18:3 (α-LA)	0.34 ± 0.12	0.36 ± 0.48	0.08 ± 0.07*
C20:5 (EPA)	0.53 ± 0.09	5.75 ± 2.96*	10.5 ± 1.41**
C22:5	0.59 ± 0.04	1.82 ± 1.73	2.18 ± 0.56**
C22:6 (DHA)	6.26 ± 1.05	7.82 ± 5.22	13.5 ± 3.18*
ω-6 PUFA species			
C18:2	19.5 ± 1.67	16.7 ± 18.3	11.2 ± 0.52**
C18:3 (γ-LA)	NP	NP	NP
C20:4 (AA)	12.2 ± 3.18	8.01 ± 4.02	10.8 ± 1.66
C22:4	0.94 ± 0.19	NP	NP
ω-6/ω-3 ratio	4.24 ± 0.31	1.77 ± 1.71	0.84 ± 0.08***

Each species of ω-3 and ω-6 PUFAs were expressed using relative percentages, ie, the distribution areas of ω-3 or ω-6 PUFA peaks divided by the total peak areas of all detectable saturated and unsaturated resolved from the gas chromatography column. Data presented were means ± SD; n=3. *, $P< 0.05$; **, $P< 0.01$; ***, $P < 0.0001$ when compared with the control diet group. AA: Arachidonic acid; ALA: α-lipoic acid; DHA: Docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: Eicosapentaenoic acid; LA: linoleic acid; NP: no peak.

Supplemental Table 7. Characterization of T1D patients and non-diabetic donors

Case number	Age (years)	Sex	A1C	ICA	IAA	GAD-Ab	IA2A
Type 1 diabetic patients (*n* =4)							
P01	19	F	9.60%	—	—	+	—
P02	11	M	11.90%	—	—	+	+
P03	25	F	9.80%	+	—	+	+
P04	10	F	12.0%	+	—	+	—
Non-diabetic donors (*n* =5)							
C01	24	F	/	—	—	—	—
C02	28	M	/	—	—	—	—
C03	26	F	/	—	—	—	—
C04	27	F	/	—	—	—	—
C05	34	M	/	—	—	—	—

F, Female; M, Male; A1C, Glycated hemoglobin; ICA, Islet cell antibody; IAA, Autoantibodies against insulin; GAD-Ab, Glutamic acid decarboxylase antibody; IA2A, Protein tyrosine phosphatase antibody.

Supplemental Table 8. Distribution of Fatty Acid Species in Fish Oil and DHA+EPA Diets.

(mg/kg)	Fish oil	DHA+EPA Diet
Vitamin D3	NP	1.03
C14 (Myristic acid)	260.3	273.68
C15	10.71	28.48
C16 (Palmitic acid)	1182.01	10125.09
C16:1 (Palmitoleic Acid)	1090.31	643.41
C17	26.7	114.76
C18 (Stearic acid)	2616.58	3758.69
C20 (Arachidic acid)	1019.7	275.39
C20:1	2967.77	504.32
C21	160.58	30.42
C22 (Docosanoic acid)	411	167.81
C22:2	602.69	NP
C23	72.76	30.72
C24	101.3	97.71
ω-9 PUFA species		
C18:1 (Oleic acid)	5412.26	14612.32
C22:1 (Erucic acid)	7272.63	453.91
ω-3 PUFA species		
C18:3 (α-LA)	NP	NP
C20:3	35897.21	5247.04
C20:5 (EPA)	448607.21	70974.90
C22:6 (DHA)	394590.06	154902.34
ω-6 PUFA species		
C18:2	2010.77	41255.36
C18:3 (γ-LA)	199.53	NP
C20:4 (AA)	42977.95	5643.95

Vitamin D3 was tested according to standard procedure of determination of vitamin D3 in feed-High-performance liquid chromatography of China(GB/T17818-2010). The compositions of fatty acids were detected by GC-MS(gas chromatography-mass spectrometry). AA: Arachidonic acid; ALA: α-lipoic acid; DHA: Docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: Eicosapentaenoic acid; LA: linoleic acid; NP: No peak. The detection limit was 0.10 mg/kg.

Supplemental Table 9. Compositions of Natural-Ingredient Diet used for SPF Mouse Breeding Colonies

Basic Diet,g/kg diet	Conventional
Fish meal(60% protein)	100
Soybean meal(48% protein)	170
Alfalfa meal,dehydrated(17% protein)	40
Corn gluten meal(60% protein)	30
Ground corn	245
Ground wheat	230
Wheat middlings	100
Dried yeast	20
Maltose	15
Soybean oil	25
Salt	5
Dicalcium phosphate	12.5
Ground limestone	5
Mineral premix	1.2
Vitamin premix	1.3
Mineral premix,mg/kg diet	
Cobalt(as cobalt carbonate)	0.44
Copper(as copper sulfate)	4.4
Iron(as iron sulfate)	132.3
Manganese(as manganous oxide)	66.2
Zinc(as zinc oxide)	17.6
Iodine(as calcium iodate)	1.54
Vitamin premix,per kg diet	
Stabilized vitamin A palmitate or stearate	6060IU
Vitamin D3(D-activated animal sterol)	5040IU
Vitamin K(menadione activity)	3.09mg
Vitamin E	22.1mg
Choline chloride	617mg
Folic acid	5.44mg
Niacin	116.7mg
Ca-d-pantothenate	19.8mg
Vitamin B6	1.87mg
Riboflavin supplement	3.75mg
Thiamin monomitrate	11mg
D-Biotin	0.15mg
Vitamin B12 supplement	0.004mg

Amounts listed for mineral and vitamin premixes represent the mass or IU of the specific mineral element or vitamin rather than added compounds.

Supplemental Table 10. Real-time RT-PCR primers used for mRNA expression analysis.

Gene	Forward	Reverse
Pdx1	TGAAATCCACCAAAGCTCAC	TAGGCAGTACGGGTCCTCTT
Arx	CCACGTTCACCAGTTACCAG	TCAGGGTAGTGCGTCTTCTG
Pax4	TCCCAGGCCTATCTCCAAC	TATGAGGAGGAAGCCACAGG
β-actin	GCTCTGGCTCCTAGCACCAT	GGGCCGGACTCATCGTACT