Supplemental statistics

Correlations were calculated between the percentage of Foxp3$^+$ cells and the alternative variables of CD44 expression, IFNγ production, IL-4 production, IL-17 production, IL-10 production, Th1 fold-change, Th2 fold-change and Th2/Th1 ratio. The variables of CD44 expression within CD4$^+$ T cells, IFNγ expression within CD4$^+$CD44$^+$ T cells, IL-4 expression within CD4$^+$CD44$^+$ T cells, IL-17 expression within CD4$^+$CD44$^+$ T cells and IL-10 expression within CD4$^+$CD44$^+$ T cells were percentages as measured by flow cytometry. Fold-change of Th1 and Th2 responses were calculated by using a baseline of the average % IFNγ$^+$ (Th1) and % IL4$^+$ (Th2) within CD4$^+$ CD44$^+$ lymphocytes in control uninjected *Foxp3^{DTR}* mice. Each individual sample was then converted to a fold-change from this baseline. The Th2/Th1 ratio was calculated as % IL4$^+$ within CD4$^+$ CD44$^+$ lymphocytes divided by the % IFNγ$^+$ within CD4$^+$ CD44$^+$ lymphocytes for each single sample.

For each variable the relationship between the variable and the percentage of Foxp3$^+$ cells was tested by fitting to linear, exponential, quadratic and log models using JMP 7.0 software (SAS Institute). The optimal model was selected using the adjusted least-squares approach. Models were only considered significant if the correlations showed a significant non-zero relationship with a $p<0.0011$ (Bonferroni-corrected p-value for 9 variable analysis with an alpha value for significance set at 0.01). The significance of differences between the relationships between the percent of Foxp3$^+$ cells and Th1-fold change vs Th2-fold change was calculated by subtracting the value of Th1-fold change from the value of Th2-fold change for each mouse, and testing the resulting values for a relationship with Foxp3$^+$ cells, which gave a significant non-zero relationship ($p<0.0001$) in both lymph nodes and spleen.

Best-fit models for each variable against % Foxp3

T-cell activation in the lymph nodes:

Non-zero relationship with %Foxp3, $p < 0.0001$

$$T_{act} = \left[65 - 3.0 \times (\%Foxp3)\right]$$

Goodness of fit, $r^2 = 0.64$

IFNγ production in the lymph nodes:

Non-zero relationship with %Foxp3, $p < 0.0001$

$$\text{IFN}\gamma = \left[23 - 1.4 \times (\%Foxp3)\right]$$

Goodness of fit, $r^2 = 0.78$

IL-4 production in the lymph nodes:

Non-zero relationship with %Foxp3, $p < 0.0001$

$$\text{IL - 4} = \left[e^{2.9 - 0.24 \times (\%Foxp3)}\right]$$

Goodness of fit, $r^2 = 0.77$

IL-17 production in the lymph nodes:

Relationship with %Foxp3 not significant to $p<0.01$

IL-10 production in the lymph nodes:

Relationship with %Foxp3 not significant to p<0.01

Th1-fold change in the lymph nodes:

Non-zero relationship with %Foxp3, p < 0.0001

$$\Delta Th1 = \left[5.2 - 0.3 \times (\%Foxp3)\right]$$

Goodness of fit, r^2 = 0.78

Th2-fold change in the lymph nodes:

Non-zero relationship with %Foxp3, p < 0.0001

$$\Delta Th2 = \left[e^{3.1 - 0.24 \times (\%Foxp3)}\right]$$

Goodness of fit, r^2 = 0.77

Th bias in the lymph nodes:

Non-zero relationship with %Foxp3, p < 0.0001

$$\frac{Th2}{Th1} = \left[0.75 - 0.22 \times \log_e (\%Foxp3)\right]$$

Goodness of fit, r^2 = 0.62

T-cell activation in the spleen:

Relationship with %Foxp3 not significant to p<0.01

IFNγ production in the spleen:

Non-zero relationship with %Foxp3, p < 0.001

$$\text{IFN}\gamma = \left[37 - 0.8 \times (\%Foxp3)\right]$$

Goodness of fit, r^2 = 0.28

IL-4 production in the spleen:

Non-zero relationship with %Foxp3, p < 0.0001

$$\text{IL - 4} = \left[15 - 4.9 \times \log_e (\%Foxp3)\right]$$

Goodness of fit, r^2 = 0.78

IL-17 production in the spleen:

Relationship with %Foxp3 not significant to p<0.01

IL-10 production in the spleen:

Relationship with %Foxp3 not significant to p<0.01

Th1-fold change in the spleen:

Non-zero relationship with %Foxp3, p < 0.001

$$\Delta Th1 = \left[1.8 - 0.04 \times (\%Foxp3)\right]$$

Goodness of fit, r^2 = 0.28

Th2-fold change in the spleen:

Non-zero relationship with %Foxp3, p < 0.0001

$$\Delta Th2 = \left[7.3 - 2.4 \times \log_e (\%Foxp3)\right]$$

Goodness of fit, $r^2 = 0.78$

Th bias in the spleen:

Non-zero relationship with %Foxp3, $p < 0.0001$

$$\frac{Th2}{Th1} = \left[0.45 - 0.14 \times \log_e (\%Foxp3)\right]$$

Goodness of fit, $r^2 = 0.64$

Figure S1. The relationship between induction of T-cell activation and Treg ablation across anatomical location
Tregs were ablated via the injection of limiting concentrations of DT (0, 2.5, 5.0, 7.5, 10 and 20μg/kg diphtheria toxin), with measurement 9 days after first treatment. (A) Representative flow cytometry profiles of a titration series of *Foxp3^{DTR}* mice injected with limiting DT, displaying the percentage of CD4^{+} T cells positive for both Foxp3 and the DTR-GFP fusion protein. (B) The correlation between T_{reg} cell frequency in the spleen and lymph nodes after diphtheria toxin treatment. (C) The relationship between T_{reg} cell frequency and upregulation of CD44 on CD4^{+}Foxp3^{-} T cells in the spleen. In both (B) and (C), diamonds represent individual mice and the trend line represents the optimal model for relationship fitting using the least-squares approach. *r2* values are the goodness of fit and p values show significance of a non-zero relationship.

Figure S2. IL-17 and IL-10 production show low sensitivity to titrated Treg depletion
CD4^{+}CD44^{+} T-cell subsets were assessed for production of IL-17 and IL-10 after injection of limiting concentrations of DT in *Foxp3^{DTR}* mice. Diamonds represent individual mice. All data is presented from pooled lymph nodes. (A) The relationship between Treg frequency and IL-17 production by CD4^{+}CD44^{hi}Foxp3^{-} T cells. (B) The relationship between Treg frequency and IL-10 production by CD4^{+}CD44^{hi}Foxp3^{-} T cells.

Figure S3. Strong correlation between cytokine responses in the lymph nodes and spleen
Cytokine production by CD4^{+} T-cell subsets were assessed after injection of limiting concentrations of DT in *Foxp3^{DTR}* mice. The relationship between spleen and lymph node values was assessed for (A) IFNγ production within CD4^{+}CD44^{hi}Foxp3^{-} cells, (B) IL-4 production within CD4^{+}CD44^{hi}Foxp3^{-} cells, (C) IL-17 production within CD4^{+}CD44^{hi}Foxp3^{-} cells, (D) IL-10 production within CD4^{+}CD44^{hi}Foxp3^{-} cells, and (E) Th2/Th1 ratio.

Figure S4. The relationship between effector T-cell subsets and Treg ablation in the spleen
Cytokine production in CD4^{+}CD44^{+} T cells after injection of limiting concentrations of DT in *Foxp3^{DTR}* mice. (A) Representative flow cytometry profiles showing staining for CD44 and IFNγ (*upper*), and CD44 and IL-4 (*lower*), by CD4^{+} Foxp3^{-} T cells. (B,C) The relationship between Treg frequency and (B) IFNγ or (C) IL-4 production by CD4^{+}CD44^{hi}Foxp3^{-} T cells. (D) The relationship between Treg frequency and fold-change in IL-4 (empty diamonds) and IFNγ (filled diamonds) production by CD4^{+}CD44^{hi}Foxp3^{-} T cells. (E) The relationship between Treg frequency and the IL-4^{+}(Th2):IFNγ^{+}(Th1) ratio. (F,G) The relationship between Treg frequency and (F) IL-17 and (G) IL-10 production by CD4^{+}CD44^{hi}Foxp3^{-} T cells. (B–G) Diamonds represent individual mice and the trend line represents the optimal model for relationship fitting using the least-squares approach. *r2* values are the goodness of fit and p values show significance of a non-zero relationship.

Figure S5. Rapid restoration of lymph node Th2 suppression by Treg transfer
The magnitude and Th bias was compared for wildtype mice ('WT'), *Foxp3^{DTR}* mice injected with DT ('DTR'), and *Foxp3^{DTR} Ly5.1* mice injected with DT (on day 0) and then injected with at 5×10^{6} *Ly5.2 Foxp3^{GFP}* CD4 T cells 7 days later ('i.v.Treg'). (A) Cellularity of pooled lymph nodes. (B) Proportion of T-cell activation, as measured by CD44 expression on CD4^{+} T cells. (C) Frequency of CD4^{+}CD44^{hi}Foxp3^{-} T cells producing IL-4 (empty bars) and IFNγ (filled bars).

Within the i.v.Treg group, only the responses within the host Ly5.1^{+} cells were measured. n=3/group.

Figure S6. Asymmetric splenic induction of Th1 vs Th2 apoptosis by Tregs
The effect of Tregs on the proliferation and apoptosis of Th1 and Th2 cells was determined by measurement of BrdU incorporation, Ki67 expression and activated Caspase 3 expression within IL-4– and IFNγ-producing cells in the spleen of wildtype and DT-treated *Foxp3*^{DTR} mice. (A) Wildtype and DT-treated *Foxp3*^{DTR} mice were treated daily with BrdU from the time of DT administration, and incorporation was measured in IFNγ-expressing Th1 cells and IL-4–expressing Th2 cells (n=5/group). (B) Ki67 expression in Th1 and Th2 subsets of wildtype and DT-treated *Foxp3*^{DTR} mice (n=9,11). (C) Activated Caspase 3 expression in Th1 and Th2 subsets of wildtype and DT-treated *Foxp3*^{DTR} mice (n=9,11). (D) The effect of Tregs on Th1 and Th2 subsets, measured by calculating the percentage change in Ki67 and activated caspase 3 expression in wildtype versus DT-treated *Foxp3*^{DTR} mice. Each diamond represents an individual wildtype mouse (n=9), normalized to the average value of DT-treated *Foxp3*^{DTR} mice (n=11).

Figure S7. CTLA4-Ig can substitute for Tregs in restoring effector T-cell bias
Wildtype and DT-treated *Foxp3*^{DTR} mice were compared to DT-treated *Foxp3*^{DTR} mice treated with CTLA4-Ig on day 0, 5 or 7. (A) Frequency of IL-4– and IFNγ-producers among CD4^{+}CD44^{hi}Foxp3^{-} T cells and (B) Th2:Th1 ratio in wildtype mice, DT-treated *Foxp3*^{DTR} mice and DT- and CTLA4-Ig–treated *Foxp3*^{DTR} mice and DT- and anti–IL-2–treated *Foxp3*^{DTR} mice, after 9 days of treatment with DT. All data is from pooled lymph nodes (n=15, 14, 4, 12, 3).

Figure S1

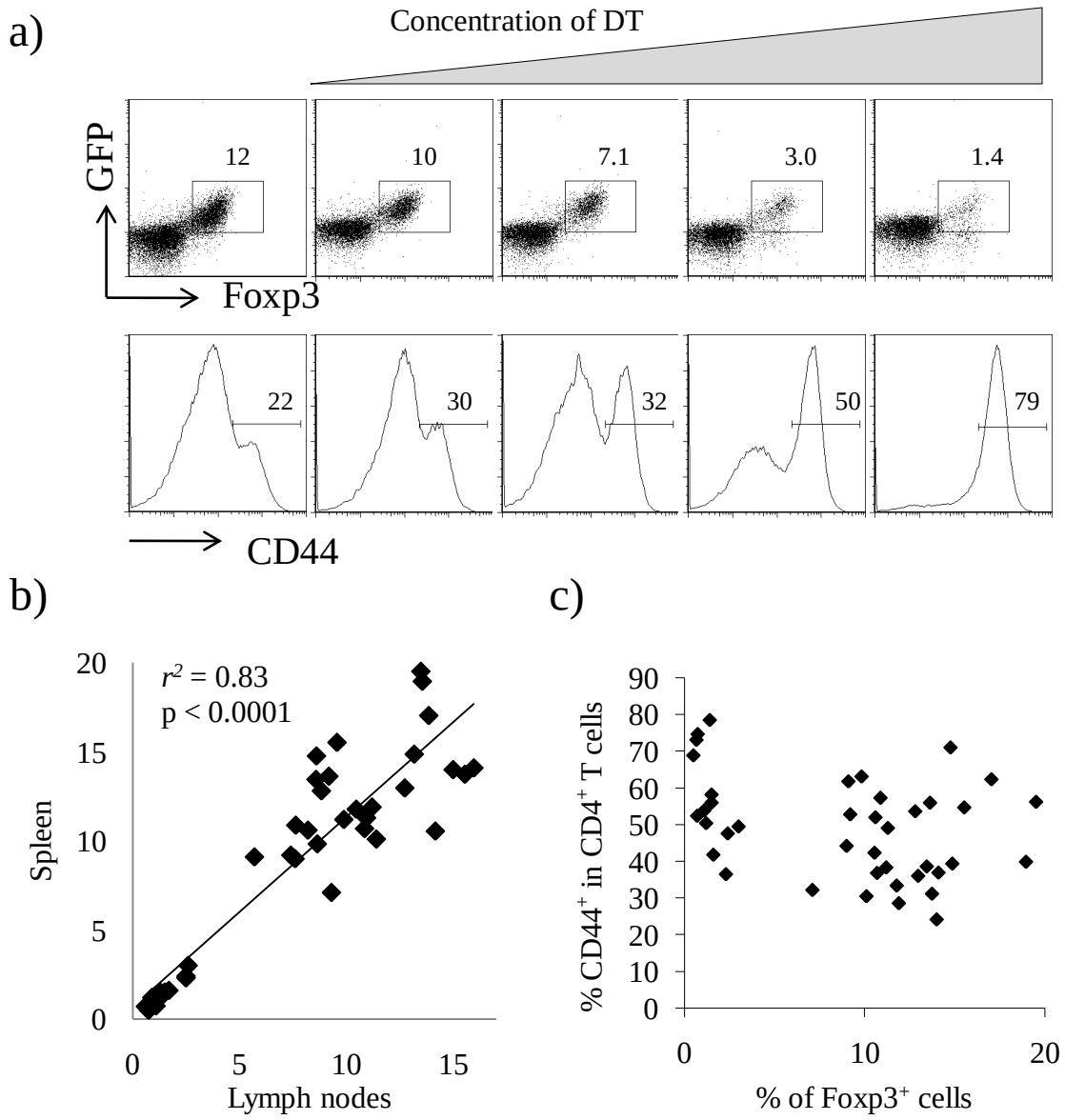

a)
Concentration of DT
GFP
Foxp3
12
10
7.1
3.0
1.4
22
30
32
50
79
CD44
b)
r^2 = 0.83
p < 0.0001
Spleen
Lymph nodes
0
5
10
15
20
c)
% CD44+ in CD4+ T cells
% of Foxp3+ cells
0
10
20
30
40
50
60
70
80
90

Figure S2

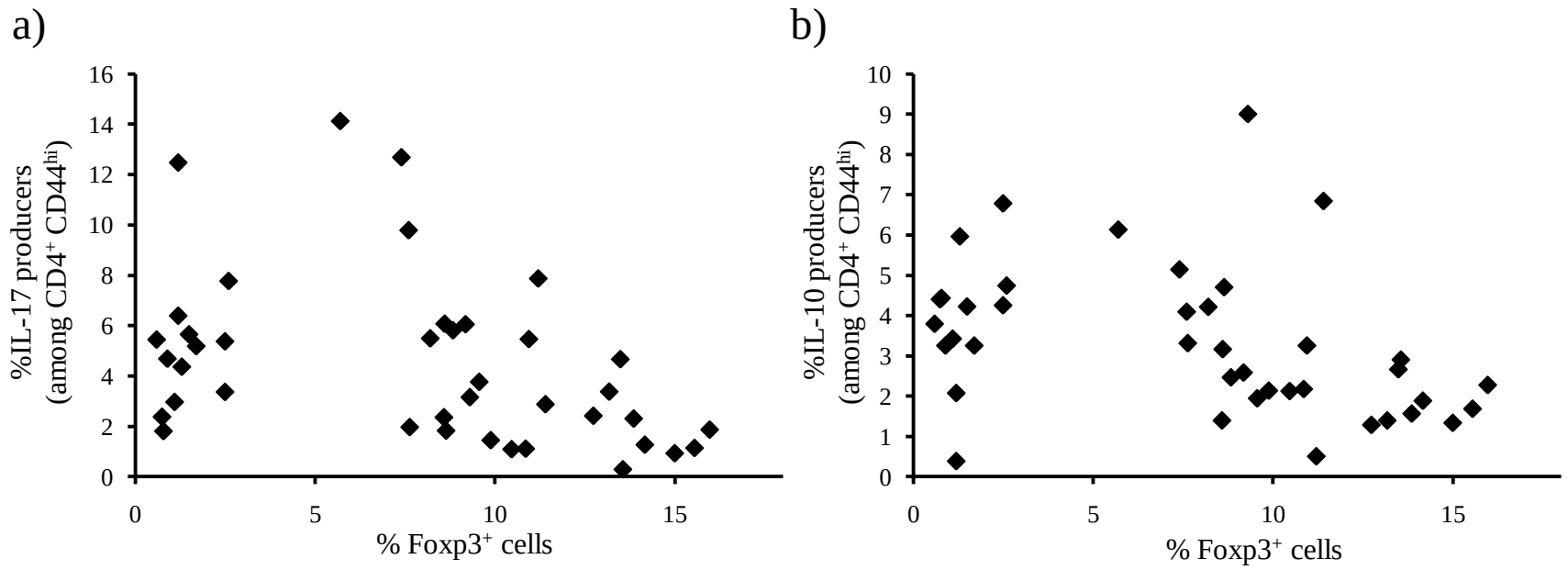

a)
%IL-17 producers
(among CD4+ CD44hi)
0
2
4
6
8
10
12
14
16
0
5
10
15
% Foxp3+ cells
b)
%IL-10 producers
(among CD4+ CD44hi)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
% Foxp3+ cells

Figure S3

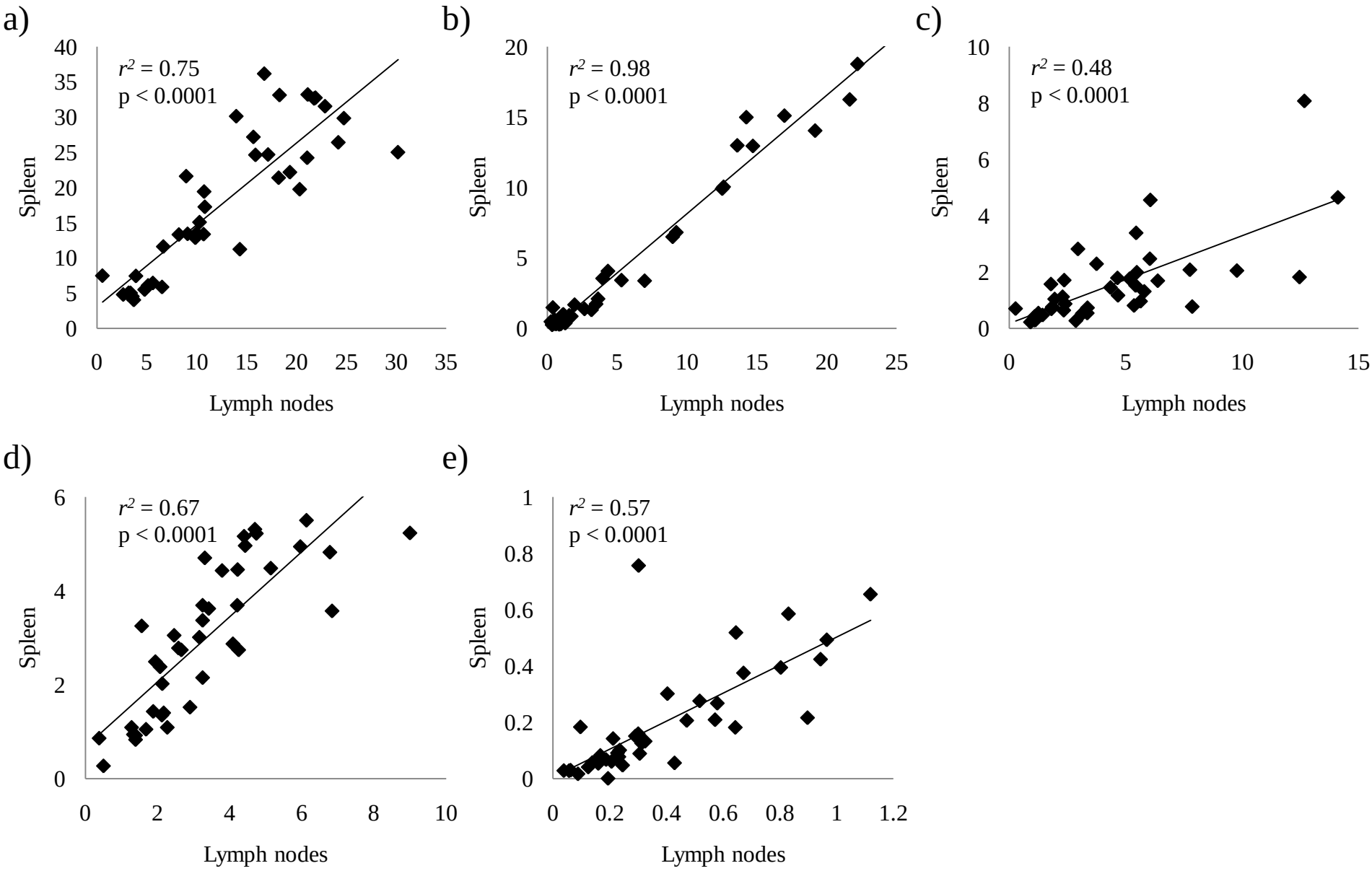
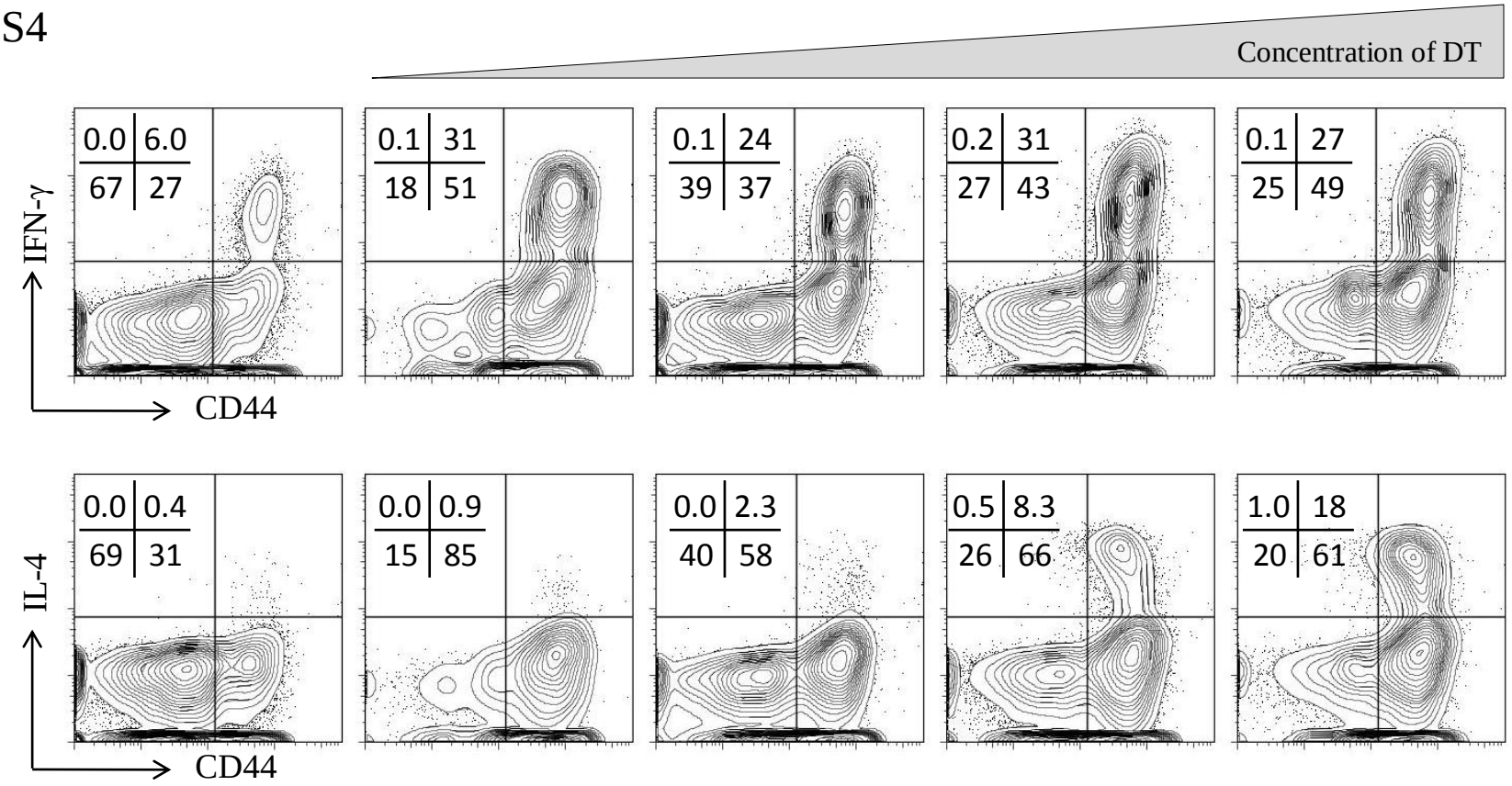

a)
r^2 = 0.75
p < 0.0001
Spleen
Lymph nodes
b)
r^2 = 0.98
p < 0.0001
Spleen
Lymph nodes
c)
r^2 = 0.48
p < 0.0001
Spleen
Lymph nodes
d)
r^2 = 0.67
p < 0.0001
Spleen
Lymph nodes
e)
r^2 = 0.57
p < 0.0001
Spleen
Lymph nodes

Figure S4


Concentration of DT
a)
IFN-γ
CD44
0.0 6.0 67 27
0.1 31 18 51
0.1 24 39 37
0.2 31 27 43
0.1 27 25 49
IL-4
CD44
0.0 0.4 69 31
0.0 0.9 15 85
0.0 2.3 40 58
0.5 8.3 26 66
1.0 18 20 61
b)
%IFNγ producers (among CD4+ CD44hi)
% Foxp3+ cells
r2 = 0.28
p < 0.001
c)
%IL-4 producers (among CD4+ CD44hi)
% Foxp3+ cells
r2 = 0.78
p < 0.0001

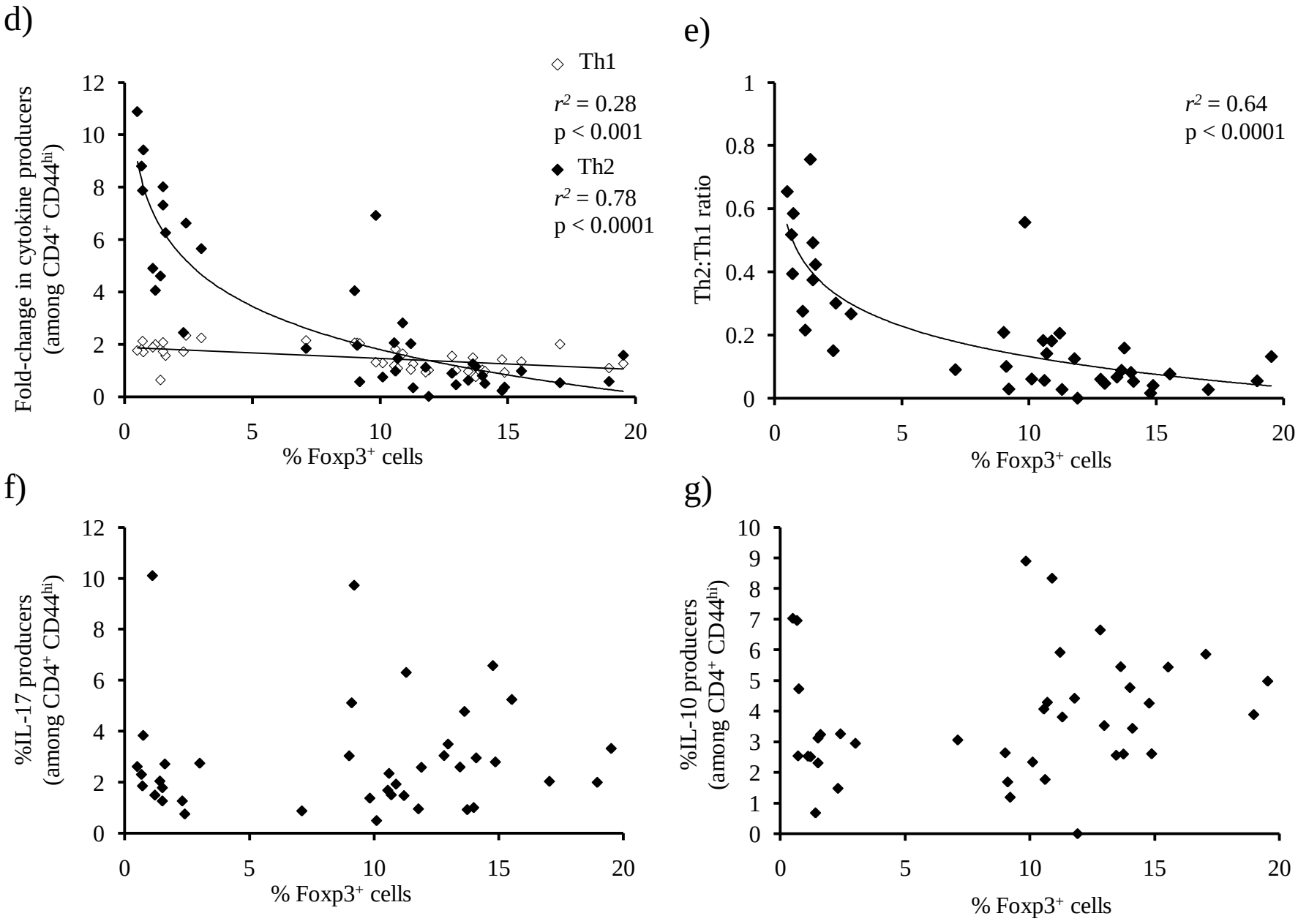

d)
Th1
r^2 = 0.28
p < 0.001
Th2
r^2 = 0.78
p < 0.0001
Fold-change in cytokine producers (among CD4+ CD44hi)
% Foxp3+ cells
e)
r^2 = 0.64
p < 0.0001
Th2:Th1 ratio
% Foxp3+ cells
f)
%IL-17 producers (among CD4+ CD44hi)
% Foxp3+ cells
g)
%IL-10 producers (among CD4+ CD44hi)
% Foxp3+ cells

Figure S5

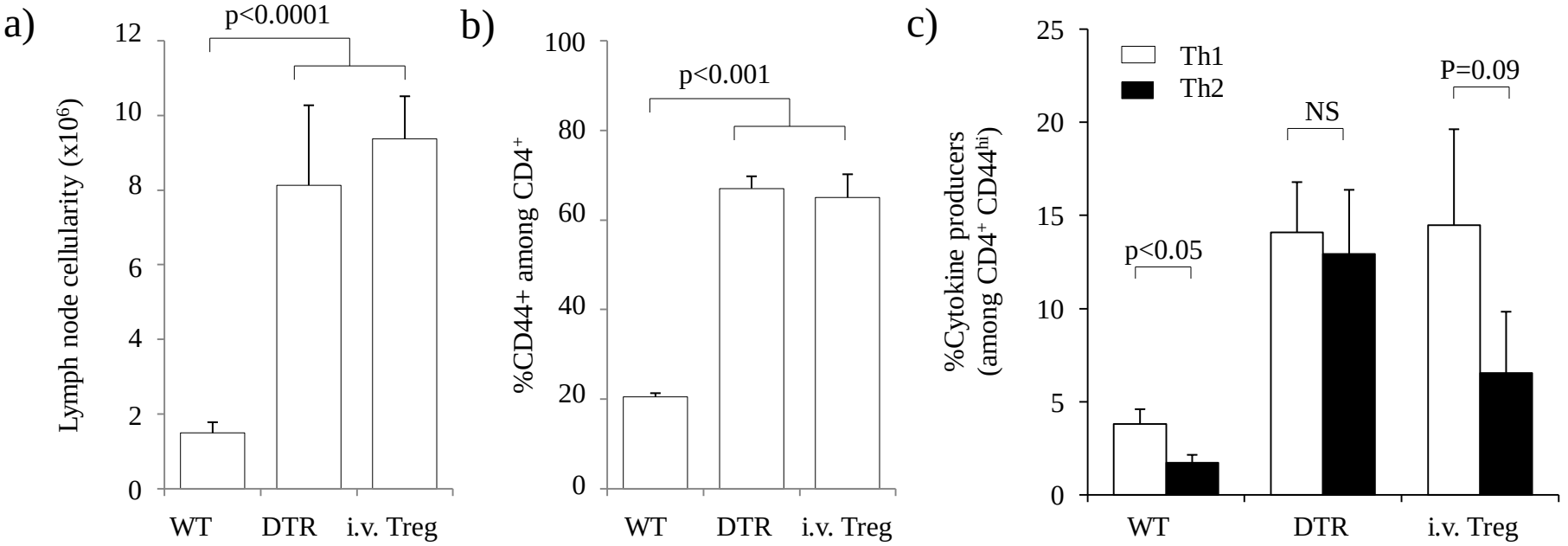

a)
p<0.0001
Lymph node cellularity (x10^6)
12
10
8
6
4
2
0
WT
DTR
i.v. Treg
b)
p<0.001
%CD44+ among CD4+
100
80
60
40
20
0
WT
DTR
i.v. Treg
c)
Th1
Th2
%Cytokine producers
(among CD4+ CD44hi)
25
20
15
10
5
0
p<0.05
NS
P=0.09
WT
DTR
i.v. Treg

Figure S6

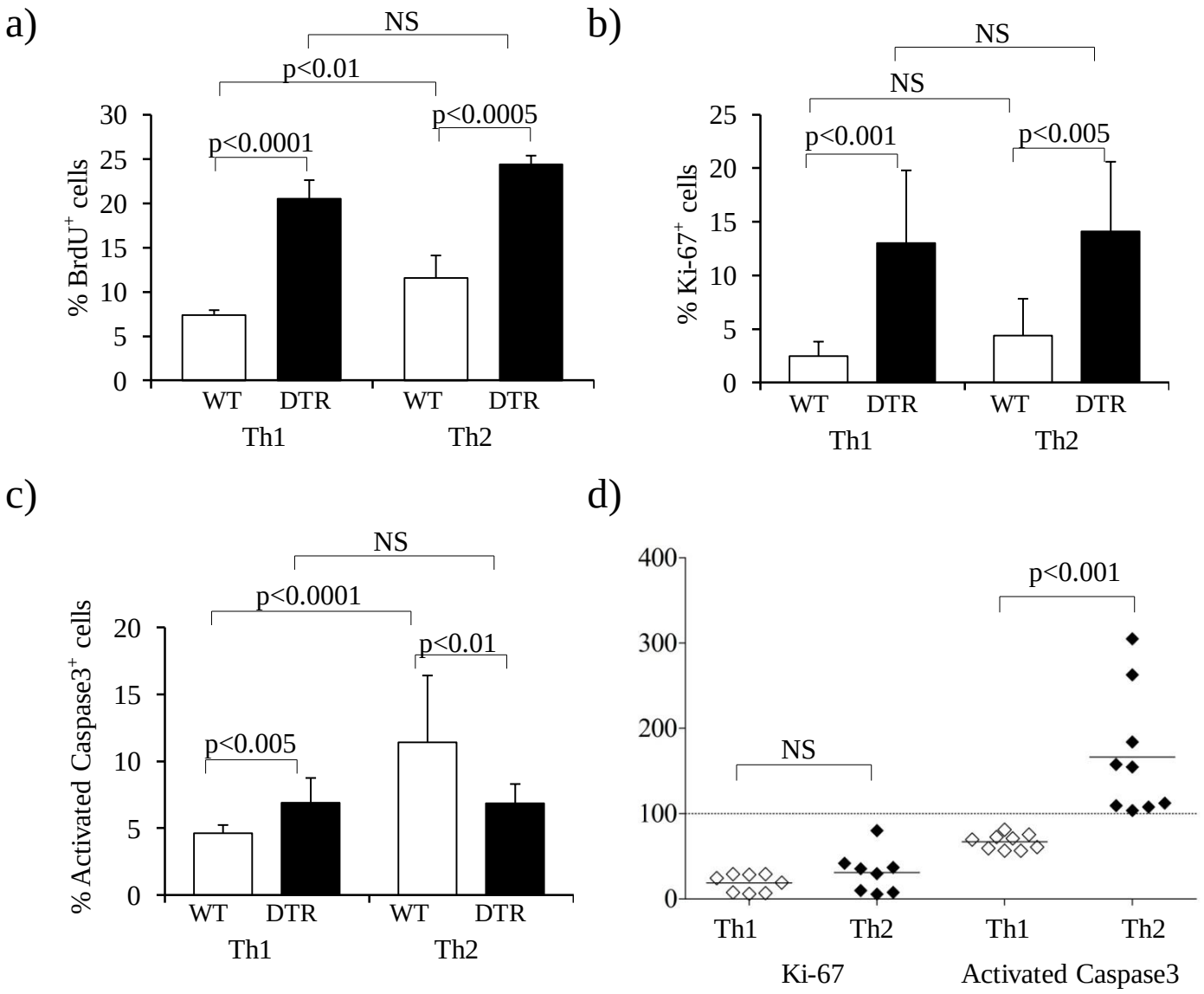
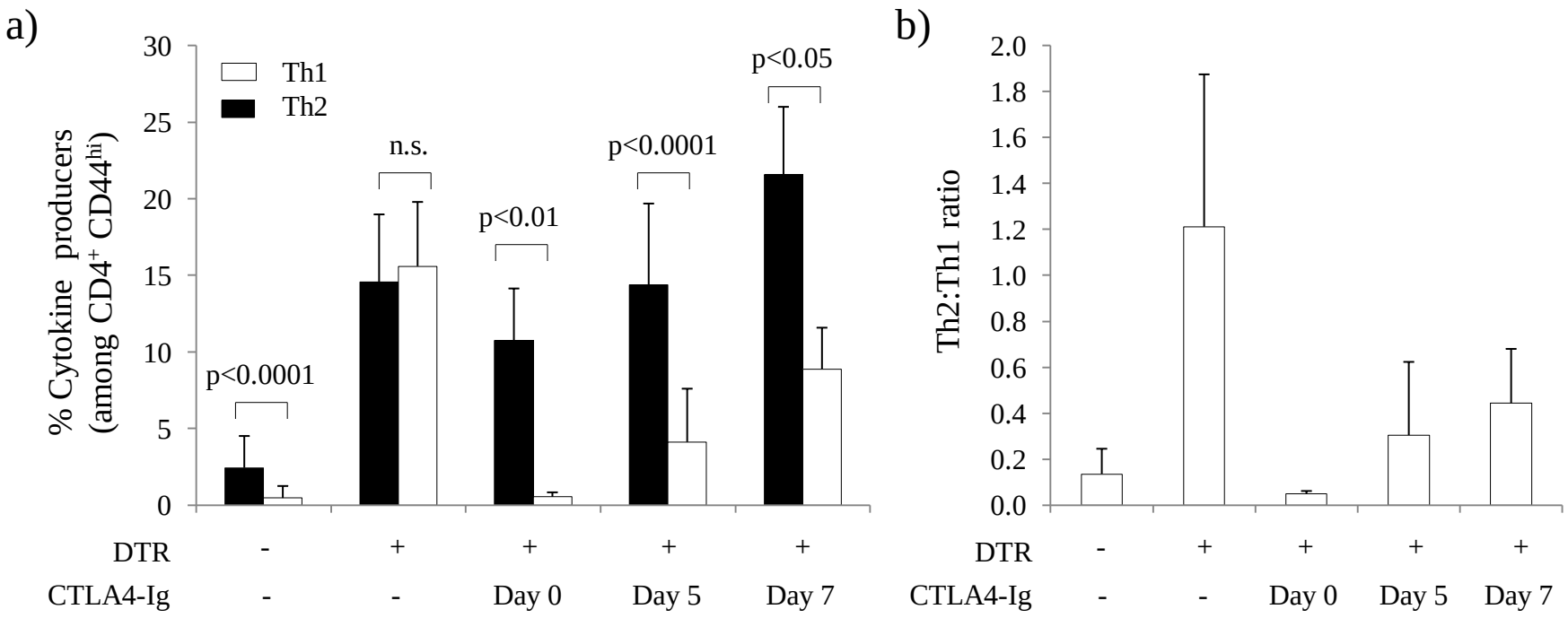

a)
NS
p<0.01
p<0.0005
p<0.0001
% BrdU+ cells
30
25
20
15
10
5
0
WT
DTR
WT
DTR
Th1
Th2
b)
NS
NS
p<0.001
p<0.005
% Ki-67+ cells
25
20
15
10
5
0
WT
DTR
WT
DTR
Th1
Th2
c)
NS
p<0.0001
p<0.01
p<0.005
% Activated Caspase3+ cells
20
15
10
5
0
WT
DTR
WT
DTR
Th1
Th2
d)
p<0.001
NS
400
300
200
100
0
Th1
Th2
Th1
Th2
Ki-67
Activated Caspase3

Figure S7


a)
% Cytokine producers (among CD4+ CD44hi)
Th1
Th2
p<0.0001
n.s.
p<0.01
p<0.0001
p<0.05
DTR
CTLA4-Ig
Day 0
Day 5
Day 7
b)
Th2:Th1 ratio