Supplemental information to the manuscript by Wang et al.

"TGF-β-dependent suppressive function of regulatory T cells requires CD18 wild-type levels in a psoriasis murine model"

SUPPLEMENTAL RESULTS

Depletion of $CD25^+$ T cells results in alleviation of the psoriasiform skin disease in affected $CD18^{hypo}$ PL/J mice

To investigate the function of $CD25^+$ T cells *in vivo*, we intraperitoneally injected 500 μg anti-CD25 mAb (clone: PC61) every second day up to twenty-one days to deplete $CD25^+$ T cells. Depletion of $CD25^+$ T cells in $CD18^{hypo}$ mice with a severe inflammatory phenotype (**Fig. S1A left**) led to a remarkable improvement of the psoriasiform skin inflammation after 21 days of treatment (**Fig. S1A right**). To evaluate the severity of the psoriasiform phenotype, an adapted PASI score (1) was used for affected $CD18^{hypo}$ mice before and after treatment with PC61 or IgG. The reduction in severity and extent of erythema, plaque formation and scaling after treatment with PC61 was highly significant (adapted PASI score, 5.86 ± 1.68 versus 2.29 ± 1.11; $p = 0.0006$) (**Fig. S1B**). In contrast, no significant changes in adapted PASI score were observed in four mice treated with IgG control ($p = 0.4827$) (**Fig. S1C**). FACS analyses using anti-CD25 mAb derived from another clone (7D4) showed that in periphery blood the $CD25^+$ T cells are mostly removed in affected $CD18^{hypo}$ mice that had been injected with PC61 mAb for 21 days compared with affected $CD18^{hypo}$ mice treated with IgG (**Fig. S1D, E**). To examine

whether the PC61 mAb also depletes $CD25^+$ T cells in skin of affected $CD18^{hypo}$ mice, immunohistochemical staining of 7D4 mAb for $CD25^+$ T cells was performed. In contrast to affected $CD18^{hypo}$ mice treated with IgG (**Fig. S1F**), cryosections of skin from affected $CD18^{hypo}$ mice treated with PC61 mAb (**Fig. S1G**) $CD25^+$ T cells was virtually undetectable. To better quantify $CD25^+$ T cells in skin, 12 skin cryosections taken from $CD18^{hypo}$ mice (n =4) treated with IgG or anti-CD25 mAb were counted for $CD25^+$ T cells. The depletion of $CD25^+$ T cells in the skin was highly effective (**Fig. S1H**).

Expression of Foxp3 by $CD4^+CD25^+CD127^-$ Tregs in $CD18^{hypo}$ PL/J mice

To study Foxp3 expression by $CD4^+CD25^+CD127^-$ T cells, FACS analysis was performed using anti-CD4, anti-CD25, anti-CD127 and anti-Foxp3 mAbs. We first gated cells of $CD18^{wt}$ or healthy $CD18^{hypo}$ or affected $CD18^{hypo}$ mice for CD4 and CD25 expression (**gated as in Fig. S2A)**, and thereafter analyzed this fraction for CD127 and Foxp3 expression (**Fig. S2B**). In spleen, 93.6% of $CD4^+CD25^+CD127^-$ Tregs derived from $CD18^{wt}$ mice (**Fig. S2B**), while 78.6% of $CD4^+CD25^+CD127^-$ Tregs derived from healthy $CD18^{hypo}$ mice and 74.5% of $CD4^+CD25^+CD127^-$ Tregs derived from affected $CD18^{hypo}$ mice fell within the $Foxp3^+$ gate (**Fig. S2B**). In contrast to $CD18^{wt}$ mice the numbers of $CD4^+CD25^+CD127^-Foxp3^+$ Tregs is significantly decreased in healthy $CD18^{hypo}$ mice, and even more pronounced in affected $CD18^{hypo}$ mice (**Fig. S2C**).

Neutralizing antibody against CD18 decreases cluster formation of $CD18^{wt}$ Tregs in MLRs

We studied whether neutralizing mAb against CD18 impairs the DC- Treg contact.

MLRs were performed with Tregs from PL/J CD18^{wt} (H-2^{u}) mice together with allogeneic DCs from C57BL/6J (H-2^{b}) mice in the presence of 500 units/ml recombinant murine IL-2, with or without neutralizing mAb against CD18. Diminished cell-cell contacts between allogeneic DCs and CD18^{wt} Tregs and cluster formation dose-dependently occurred in the presence of anti-CD18 mAb at a concentration of 10μg/ml and more obvious at a concentration of 20μg/ml compared to IgG control antibodies (**Fig. S3A-D**).

Anti-TGF-β antibody reverses suppressor function of CD18^{wt} Tregs expanded by allogeneic DCs

To study the role of TGF-β in the suppressor function of DC-expanded CD18^{wt} Tregs, these cells were co-cultured with allogeneic DCs in the presence of 500 units/ml recombinant murine IL-2 for 7 days. Thereafter Tregs were separated from allogeneic DCs using anti-CD11c beads, washed three times with PBS and mixed at a ratio of 1 to 4 with CFSE labeled CD18^{wt} Tresps in the presence of anti-TGF-β (clone: 1D11) or IgG control. In line with a previous report (2), anti-TGF-β mAb (clone: 1D11) at least partly reversed suppressor function of CD18^{wt} Tregs expanded by allogeneic DCs (**Fig. S4A-C**).

Neutralization of TGF-β does not result in apoptosis of CD18^{wt} Tregs after adoptive transfer into affected CD18^{hypo} mice

In order to exclude that the persistence of the psoriasiform skin disease upon combined treatment of adoptive transfer of CD18^{wt} Tregs and concomitant administration of mAb

against TGF-β into affected $CD18^{hypo}$ PL/J mice is simply due to apoptosis or reduced proliferation of the Tregs, we undertook the following set of experiments.

Firstly, we performed skin biopsies of affected skin of $CD18^{hypo}$ mice after adoptive transfer of $CD18^{wt}$ Tregs and injection of neutralizing anti-TGF-β mAb (**Fig. S5A, left panel**) or isotype control IgG (**Fig. S5A, right panel**). We did not find any difference in CD18 positive Treg numbers (**Fig. S5B**). Secondly, FACS analysis of skin DLNs for CD18 positive cells with an apoptotic markers was performed at day 9 after adoptive transfer of $CD18^{wt}$ Tregs into $CD18^{hypo}$ affected mice in the presence of neutralizing anti-TGF-β mAb or isotype control IgG. No difference in apoptotic Treg numbers was observed in the skin DLN in the case of coinjection with neutralizing anti-TGF-β mAb or isotype control IgG (**Fig. S5C**). Thirdly, no decrease in $CD18^{wt}$ Treg proliferation was found in MLRs in the presence of various neutralizing anti-TGF-β mAb concentrations (**Fig. S5D**).

Collectively, these control experiments strongly support the view that the persistence of the psoriasiform skin disease after adoptive transfer of $CD18^{wt}$ Tregs into $CD18^{hypo}$ affected mice in the presence of neutralizing anti-TGF-β mAb is not due to apoptosis of Tregs, but most likely occurs through the abrogation of their TGF-β mediated suppressor function on Tresps.

SUPPLEMENTAL SECTION - METHODS

$CD25^+$ T cell depletion

For depletion of $CD25^+$ T cells, affected $CD18^{hypo}$ mice were injected intraperitoneally with 500 μg/mouse anti-CD25 mAb (clone: PC61) every second day for 21 days. Control mice were treated similarly with the corresponding isotype Ab (Caltag Laboratories®, Rat IgG1 isotype controls). Depletion was confirmed by FACS analysis of tail blood or skin samples using anti-CD25 mAb with another clone (7D4) detecting an independent epitope by the end of treatment.

Apoptosis assessment

To study whether neutralizing mAbs against TGF-β induce apoptosis of $CD18^{wt}$ Tregs after adoptive transfer into affected $CD18^{hypo}$ mice, 250μg TGF-β neutralizing mAb or isotype matched IgG were injected intraperitoneally into recipient mice every second day after the transfer of 1×10^6 $CD18^{wt}$ Tregs. Subsequently, recipients were sacrificed for analysis at day 9. Apoptosis was detected by FACS using an Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen™, Cat.No.: 556547).

SUPPLEMENTAL SECTION - FIGURE LEGENDS

Figure S1. Improvement of the psoriasiform phenotype of affected CD18^{hypo} mice after depletion of CD25^{+} T cells after administration of PC61 mAb. Affected CD18^{hypo} mice were injected intraperitoneally with 500 μg/mouse anti-CD25 mAb (clone: PC61) at every second day for 21 days. (**A**) A representative clinical picture of a CD18^{hypo} mouse with severe psoriasiform skin disease before (**left**) and improve after 21 days of treatment with PC61 mAb (**right**). The severity of the psoriasiform phenotype as assessed by the adapted PASI score was significantly reduced after the depletion of CD25^{+} cells using PC61 mAb (**B**) but not after treatment with control IgG (**C**). Peripheral blood samples were analyzed for the presence of CD25^{+} T cells by flow cytometry using anti-CD4 and anti-CD25 mAb from different clone (7D4) 21 days after treatment with IgG control (**D**) or neutralizing PC61 mAb (**E**). To assess whether PC61 mAb depletes CD25^{+} T cells in skin of affected CD18^{hypo} mice, skin cryosections from affected CD18^{hypo} treated with IgG (**F**) or PC61 (**G**) were stained with CD25-Alexa488 (clone: 7D4). Cell nuclei (blue) were counterstained with DAPI (original magnification, ×40). e, epidermis; d, dermis; h, hair follicle. Dotted line indicates the border between epidermis and dermis. (**H**) To quantify CD25^{+} T cells in the skin of affected CD18^{hypo} treated with IgG or PC61 after 21 days, the positively stained cells were calculated. For all measurements, the median of CD25^{+} T cells counted in 12 high power fields (HPF) (n =4) is presented. (** p<0.01 by *Student's t test*).

Figure S2. Foxp3 expression in $CD4^+CD25^+CD127^-$ Tregs in $CD18^{hypo}$ mice. Lymphocytes from spleens of $CD18^{wt}$ or healthy $CD18^{hypo}$ mice or affected $CD18^{hypo}$ mice were analyzed by flow cytometry. (**A, B**) Foxp3 expression by $CD4^+CD25^+CD127^-$ Tregs derived from spleen of $CD18^{wt}$ or healthy $CD18^{hypo}$ or affected $CD18^{hypo}$ mice. One representative experiment out of 3 independent experiments is shown. (**C**) Percentage of $CD4^+CD25^+CD127^-Foxp3^+$ Tregs in spleens of $CD18^{wt}$ or healthy $CD18^{hypo}$ mice or affected $CD18^{hypo}$ mice. (*$p<0.05$, ** $p<0.01$, *Student's t test*).

Figure S3. Decreased cluster formation of $CD18^{wt}$ Tregs in presence of neutralizing mAb against CD18 in MLRs. $CD4^+CD25^+CD127^-$ Tregs were purified from spleens of $CD18^{wt}$ mice using MACS sorting, and co-cultureed with irradiated allogeneic DCs derived from C57BL/6J mice in presence of 500 units/ml recombinant murine IL-2. (**A-D**) Cluster formation between allogeneic DCs and $CD18^{wt}$ Tregs in the presence of different concentrations of anti-CD18 mAb or IgG control was assessed by counting aggregated clusters/HPF in 100 randomly selected HPFs (original magnification, ×40). Cluster formation with allogeneic DC was substantially reduced for Tregs derived from $CD18^{wt}$ mice in the presence of anti-CD18 mAb compared to IgG control (* $p<0.05$, ** $p<0.01$ by *Student's t test*). One representative experiment in triplicate out of 3 independent experiments is shown.

Figure S4. Anti-TGF-β diminishes the suppressor function of $CD18^{wt}$ Tregs expanded by allogeneic DCs. (**A, B**) $CD4^+CD25^+CD127^-$ Tregs were purified from 4 pooled spleens of $CD18^{wt}$ PL/J mice and co-cultured with irradiated allogeneic DCs for 7

days in the presence of 500 units/ml recombinant murine IL-2. Tregs were then separated from allogeneic DCs by CD11c MACS beads, washed three times with PBS and mixed at a ratio of 1 to 4 with CFSE labled $CD18^{wt}$ Tresps in presence of 20μg/ml anti-TGF-β or IgG control. After 3 days of culture cells were harvested, and analyzed by flow cytometry. (**C**) CFSE labeled $CD18^{wt}$ Tresps without stimulation served as negative control. One representative experiment out of 4 independent experiments is shown.

Figure S5. Neutralization of TGF-β does not result in apoptosis of $CD18^{wt}$ Tregs after adoptive transfer into affected $CD18^{wt}$ recipients. 1×10^6 $CD18^{wt}$ Tregs were transferred into syngeneic affected $CD18^{hypo}$ recipients. Following adoptive transfer of Tregs, 250μg TGF-β neutralizing mAb or isotype control IgG were injected intraperitoneally into recipient mouse at days 3, 5 and 7. These mice were sacrificed for analysis at day 9 after the first injection. (**A**) Anti-CD18 mAb (green) was used to detect $CD18^{wt}$ Tregs in lesional skin of affected recipients which were injected with neutralizing mAb against TGF-β (left) or isotype control IgG (right) (original magnification, ×40). e, epidermis; d, dermis. The dotted line indicates the border between epidermis and dermis. (**B**) To quantify $CD18^{wt}$ Tregs in the skin of affected $CD18^{hypo}$ and $CD18^{wt}$ mice, the positively stained cells with anti-CD18 mAb were counted. For all measurements, the median of cells counted in 20 HPFs (n =4) is presented. (**C**) Lymphocytes derived from skin DLNs of affected recipients treated with neutralizing mAb against TGF-β or isotype control IgG were subjected to FACS analysis using the apoptotic marker Annexin V. $CD18^{+}$ Tregs were gated to analyze Annexin V expression. (**D**) $CD18^{wt}$ Tregs were labeled with CFSE and cultured with irradiated C57BL/6J DCs for 7 days in the presence

of various neutralizing anti-TGF-β mAb concentrations. Representative CFSE dilution profiles of gated $CFSE^{+}$ $CD18^{wt}$ Tregs from different treatments are shown.

SUPPLEMENTAL SECTION – REFERENCES

1. Kess, D., Peters, T., Zamek, J., Wickenhauser, C., Tawadros, S., Loser, K., Varga, G., Grabbe, S., Nischt, R., Sunderkotter, C., et al. 2003. CD4+ T cell-associated pathophysiology critically depends on CD18 gene dose effects in a murine model of psoriasis. *J Immunol* 171:5697-5706.
2. Nakamura, K., Kitani, A., Fuss, I., Pedersen, A., Harada, N., Nawata, H., and Strober, W. 2004. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol* 172:834-842.

Fig. S1

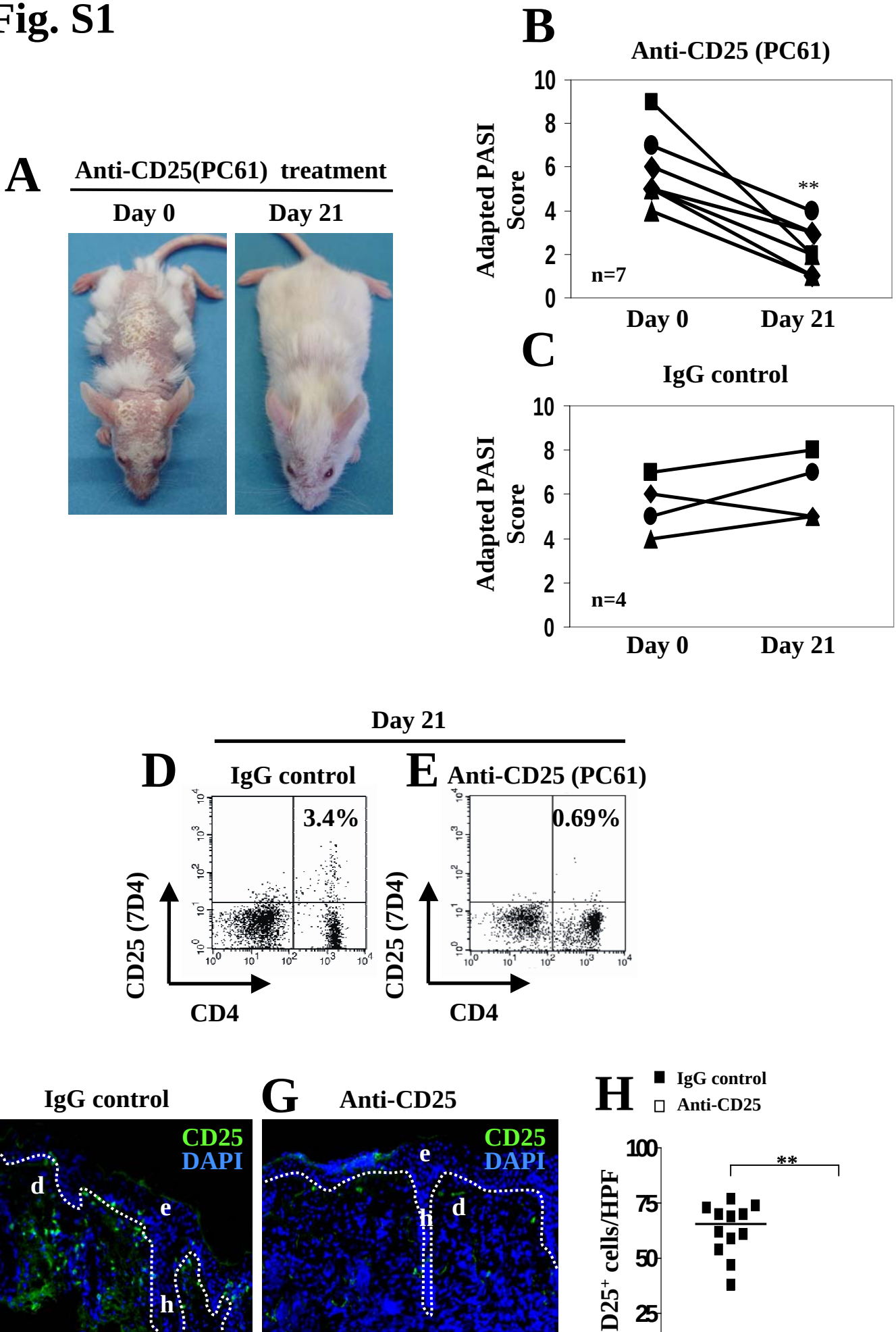
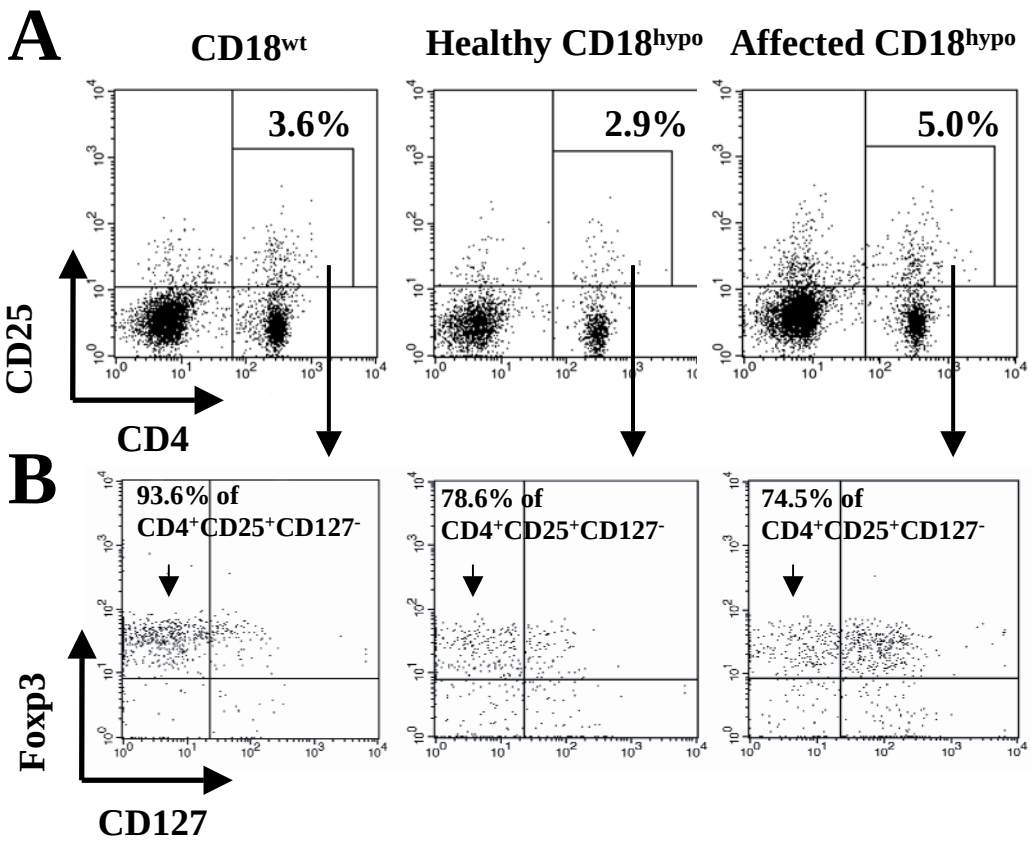

A
Anti-CD25(PC61) treatment
Day 0
Day 21
B
Anti-CD25 (PC61)
Adapted PASI Score
10
8
6
4
2
0
**
n=7
Day 0
Day 21
C
IgG control
Adapted PASI Score
10
8
6
4
2
0
n=4
Day 0
Day 21
Day 21
D
IgG control
3.4%
CD25 (7D4)
CD4
E
Anti-CD25 (PC61)
0.69%
CD25 (7D4)
CD4
F
IgG control
CD25
DAPI
d
e
h
G
Anti-CD25
CD25
DAPI
e
h
d
H
IgG control
Anti-CD25
CD25+ cells/HPF
100
75
50
25
0
**

Fig. S2


A
CD18wt
Healthy CD18hypo
Affected CD18hypo
3.6%
2.9%
5.0%
CD25
CD4
B
93.6% of CD4+CD25+CD127-
78.6% of CD4+CD25+CD127-
74.5% of CD4+CD25+CD127-
Foxp3
CD127

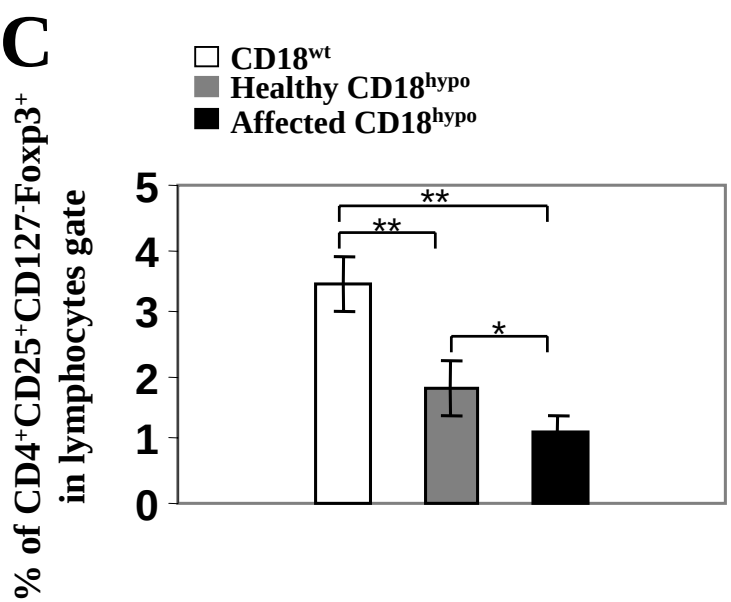

C
CD18wt
Healthy CD18hypo
Affected CD18hypo
% of CD4+CD25+CD127-Foxp3+ in lymphocytes gate
0
1
2
3
4
5
**
**
*

Fig. S3

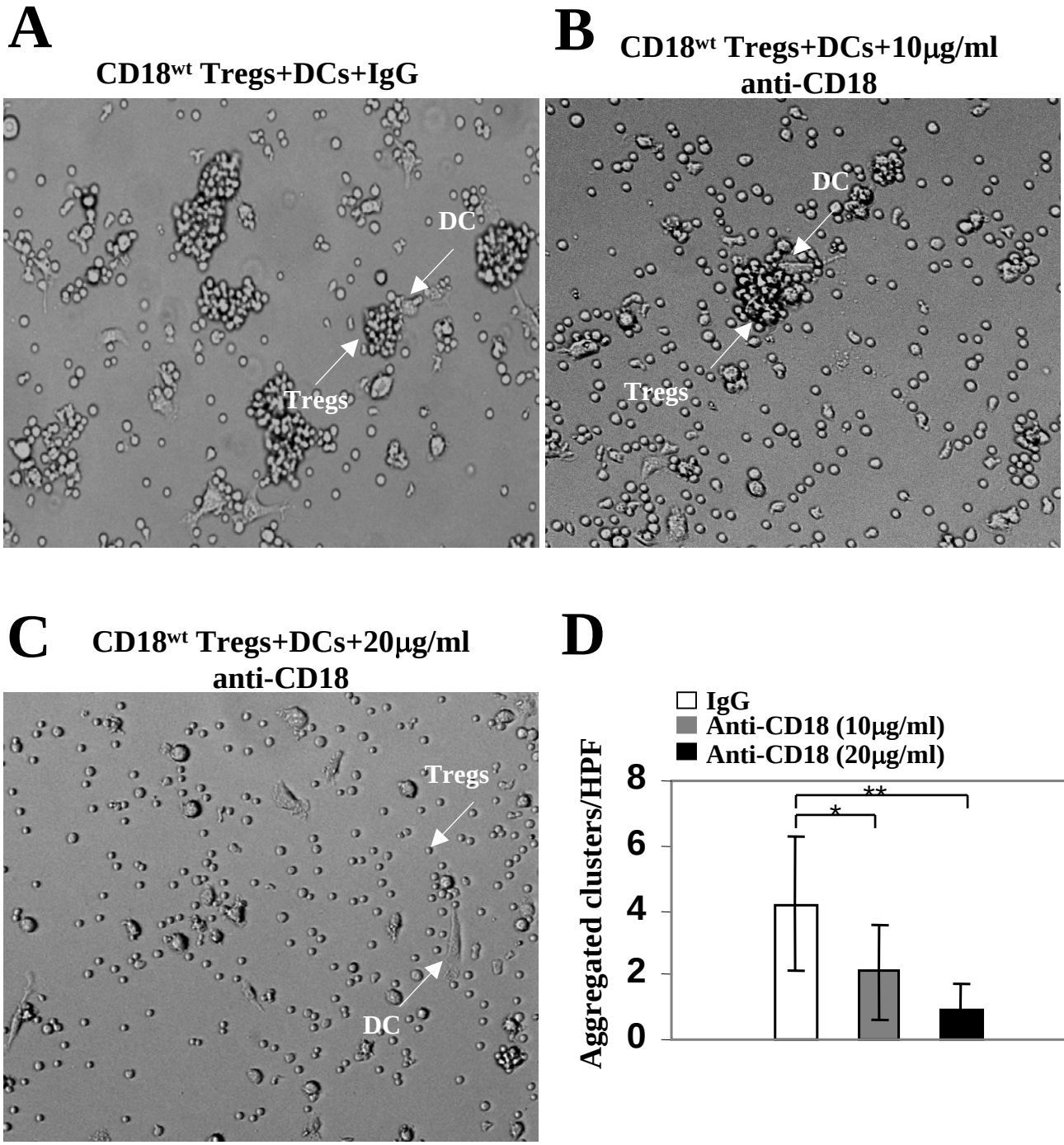

A
CD18wt Tregs+DCs+IgG
DC
Tregs
B
CD18wt Tregs+DCs+10μg/ml anti-CD18
DC
Tregs
C
CD18wt Tregs+DCs+20μg/ml anti-CD18
Tregs
DC
D
IgG
Anti-CD18 (10μg/ml)
Anti-CD18 (20μg/ml)
Aggregated clusters/HPF
0
2
4
6
8
*
**

Fig. S4

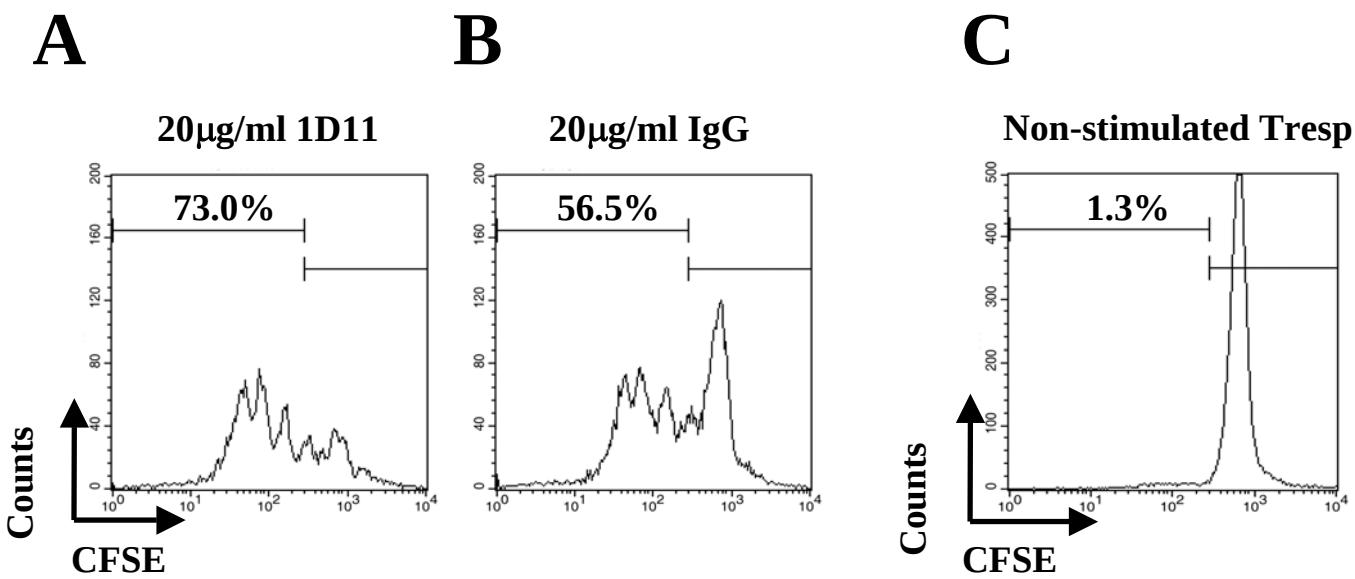
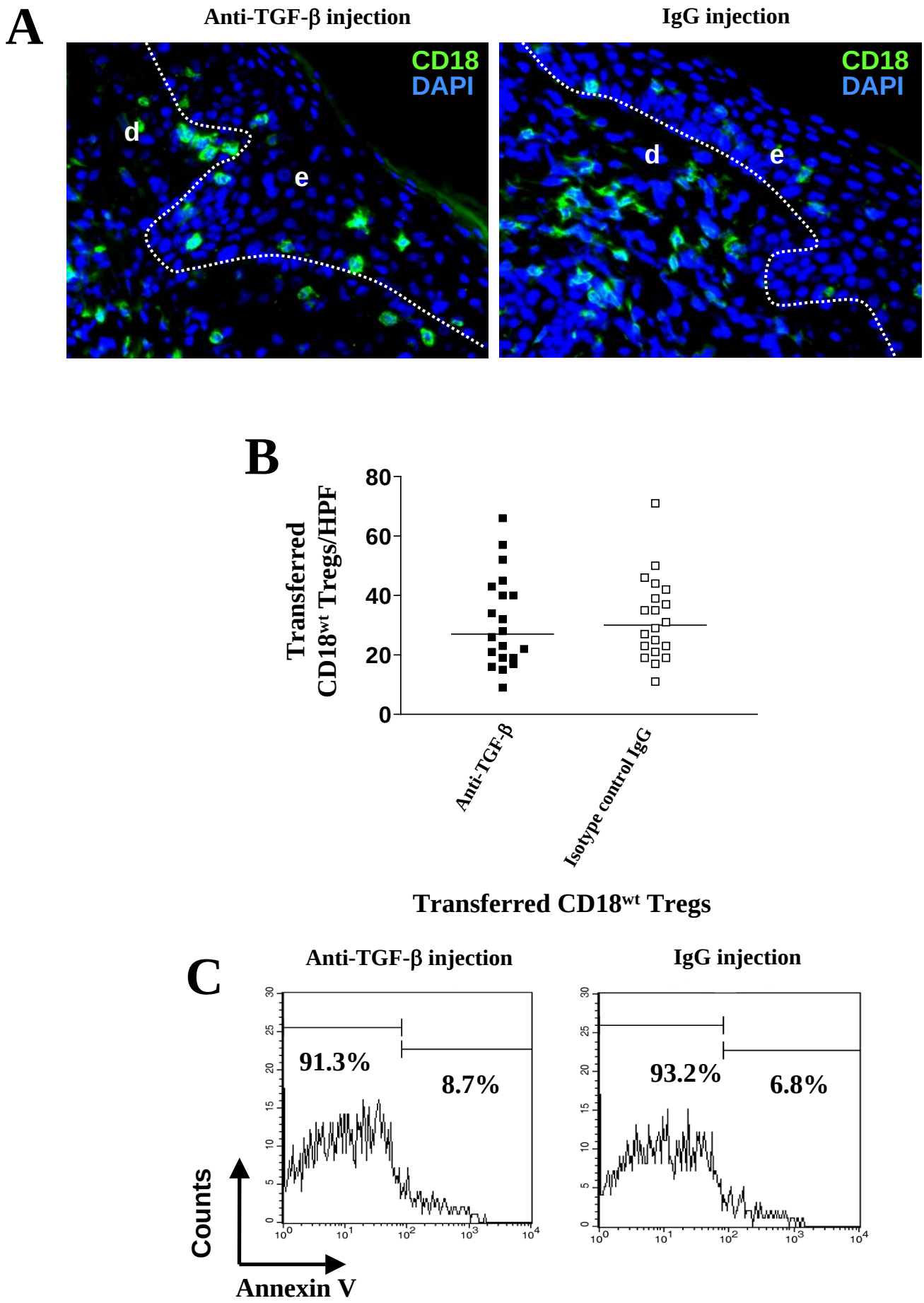
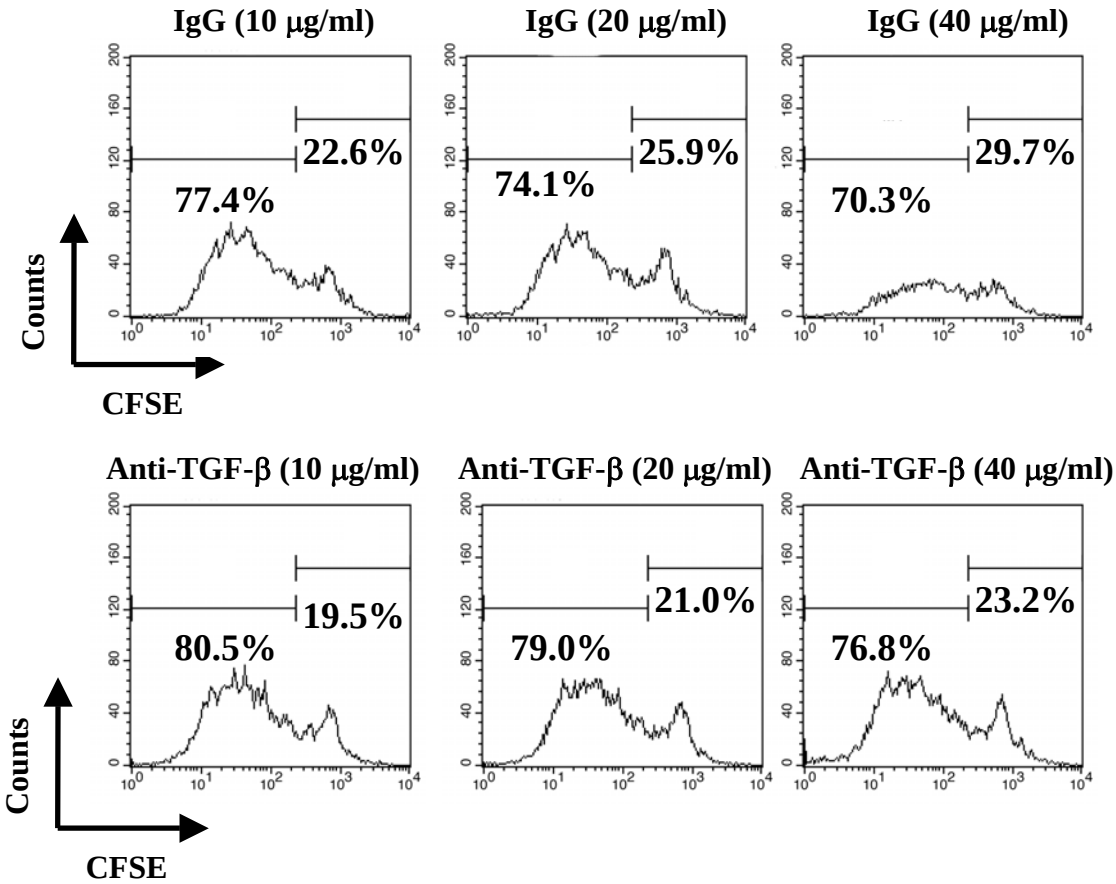

A
B
C
20μg/ml 1D11
20μg/ml IgG
Non-stimulated Tresp
73.0%
56.5%
1.3%
Counts
CFSE
Counts
CFSE

Fig. S5


A
Anti-TGF-β injection
IgG injection
CD18
DAPI
d
e
B
Transferred CD18wt Tregs/HPF
80
60
40
20
0
Anti-TGF-β
Isotype control IgG
Transferred CD18wt Tregs
C
Anti-TGF-β injection
IgG injection
91.3%
8.7%
93.2%
6.8%
Counts
Annexin V

D


IgG (10 μg/ml)
IgG (20 μg/ml)
IgG (40 μg/ml)
22.6%
77.4%
25.9%
74.1%
29.7%
70.3%
Counts
CFSE
Anti-TGF-β (10 μg/ml)
Anti-TGF-β (20 μg/ml)
Anti-TGF-β (40 μg/ml)
19.5%
80.5%
21.0%
79.0%
23.2%
76.8%
Counts
CFSE