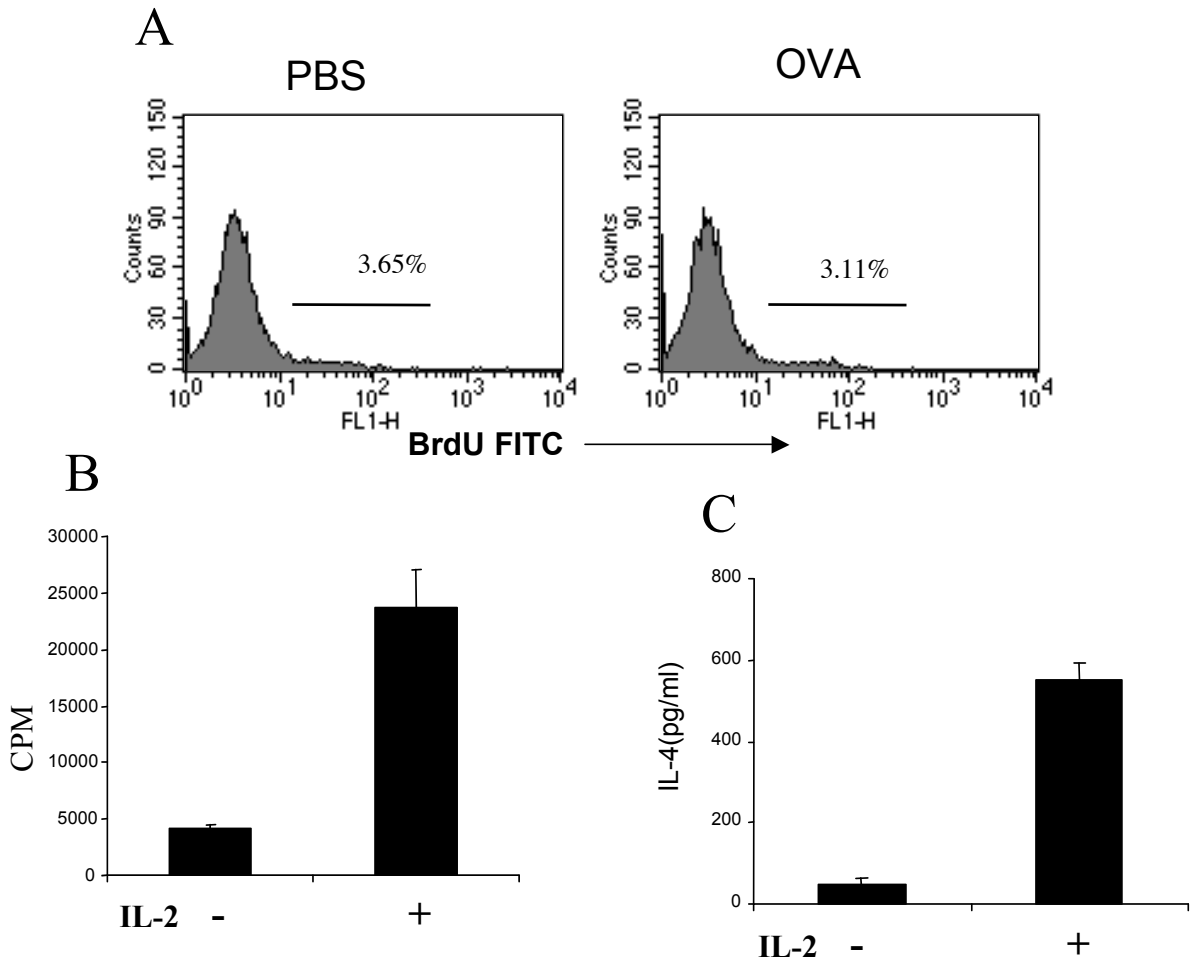

Figure S1

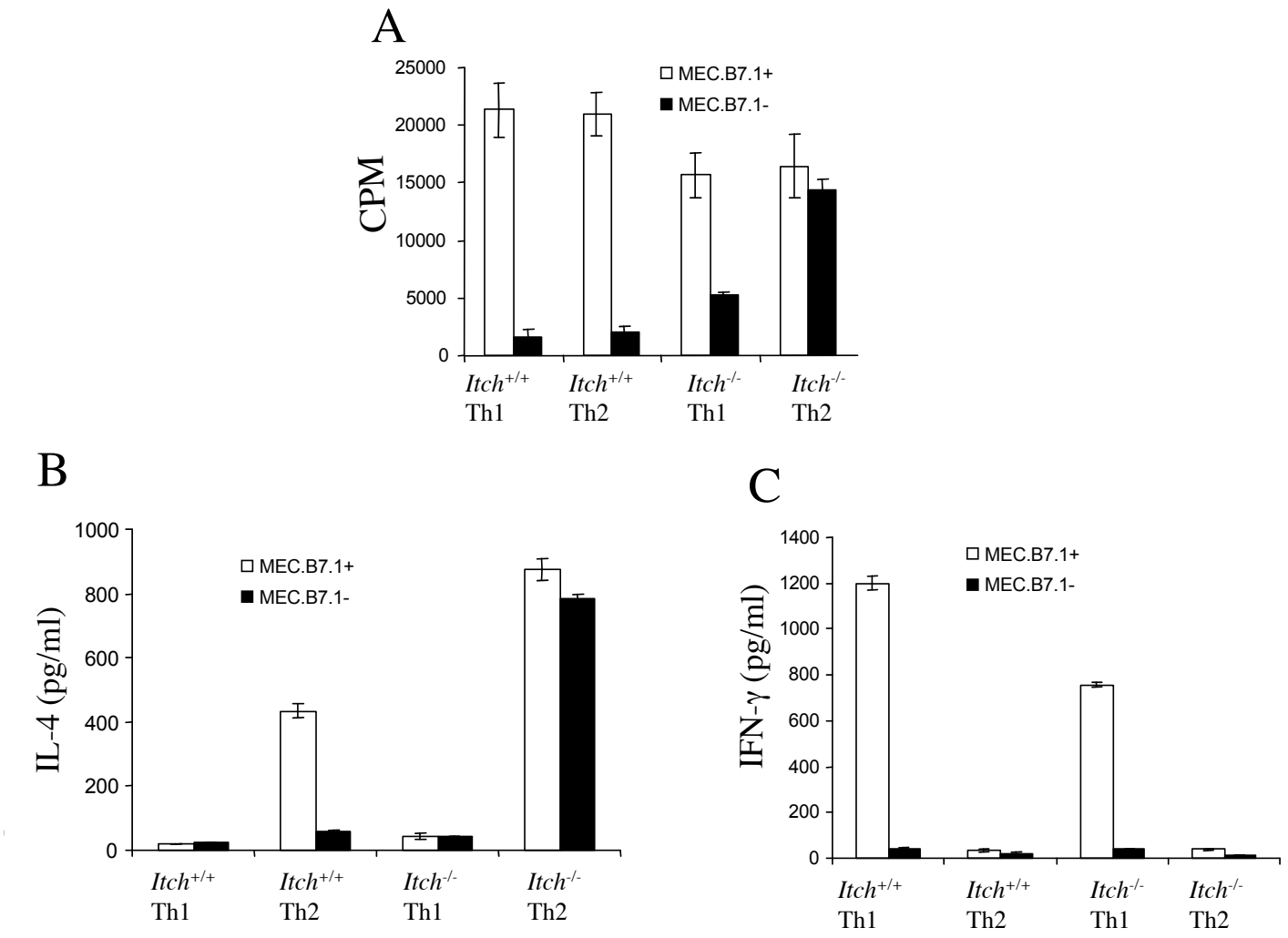
A
CPM
25000
20000
15000
10000
5000
0
MEC.B7.1+
MEC.B7.1-
Itch+/+ Th1
Itch+/+ Th2
Itch-/- Th1
Itch-/- Th2
B
IL-4 (pg/ml)
1000
800
600
400
200
0
MEC.B7.1+
MEC.B7.1-
Itch+/+ Th1
Itch+/+ Th2
Itch-/- Th1
Itch-/- Th2
C
IFN-γ (pg/ml)
1400
1200
1000
800
600
400
200
0
MEC.B7.1+
MEC.B7.1-
Itch+/+ Th1
Itch+/+ Th2
Itch-/- Th1
Itch-/- Th2

Figure S2

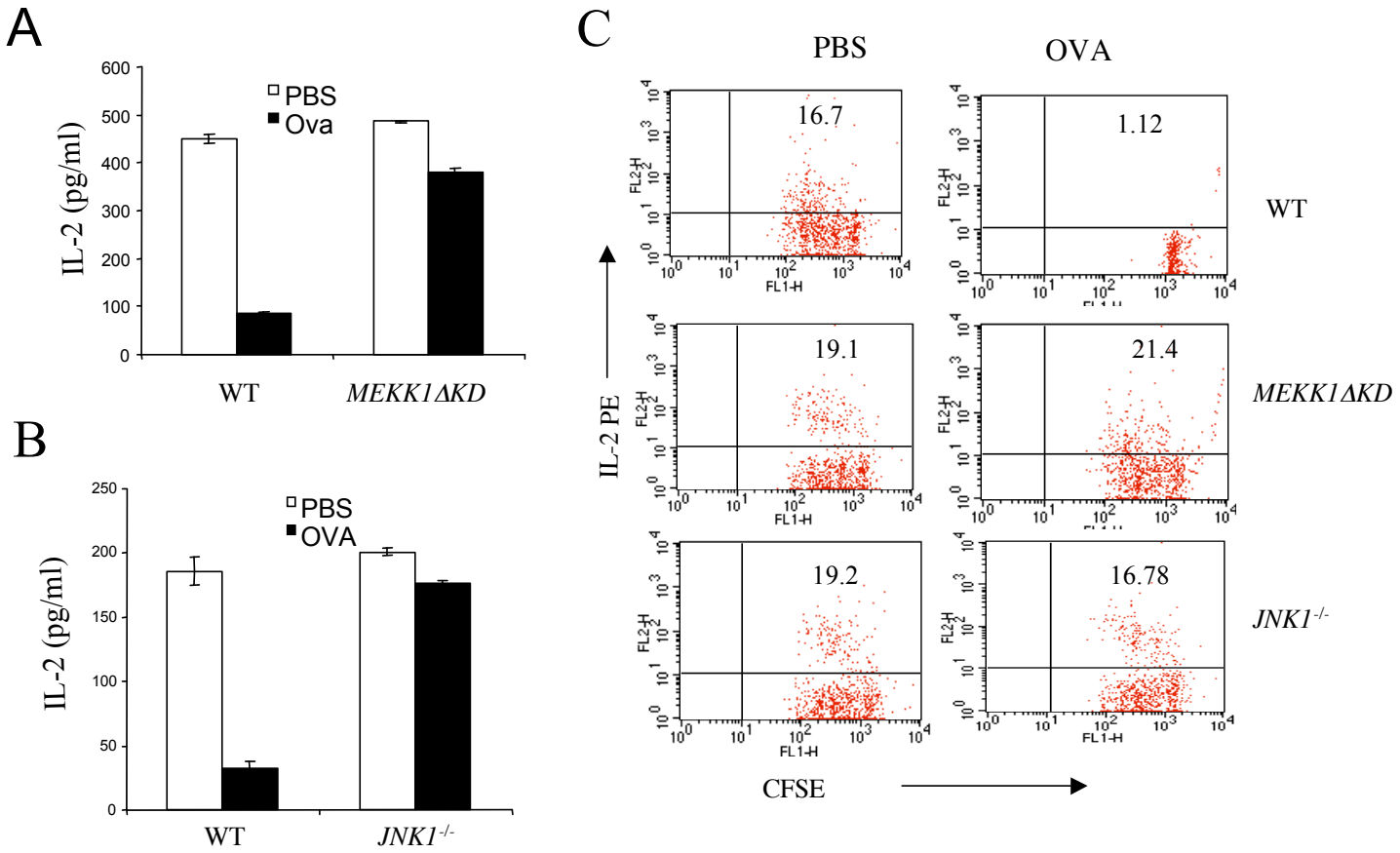
A
IL-2 (pg/ml)
PBS
Ova
WT
MEKK1ΔKD
B
IL-2 (pg/ml)
PBS
OVA
WT
JNK1-/-
C
PBS
OVA
16.7
1.12
19.1
21.4
19.2
16.78
WT
MEKK1ΔKD
JNK1-/-
IL-2 PE
CFSE
FL1-H
FL2-H

Figure S3

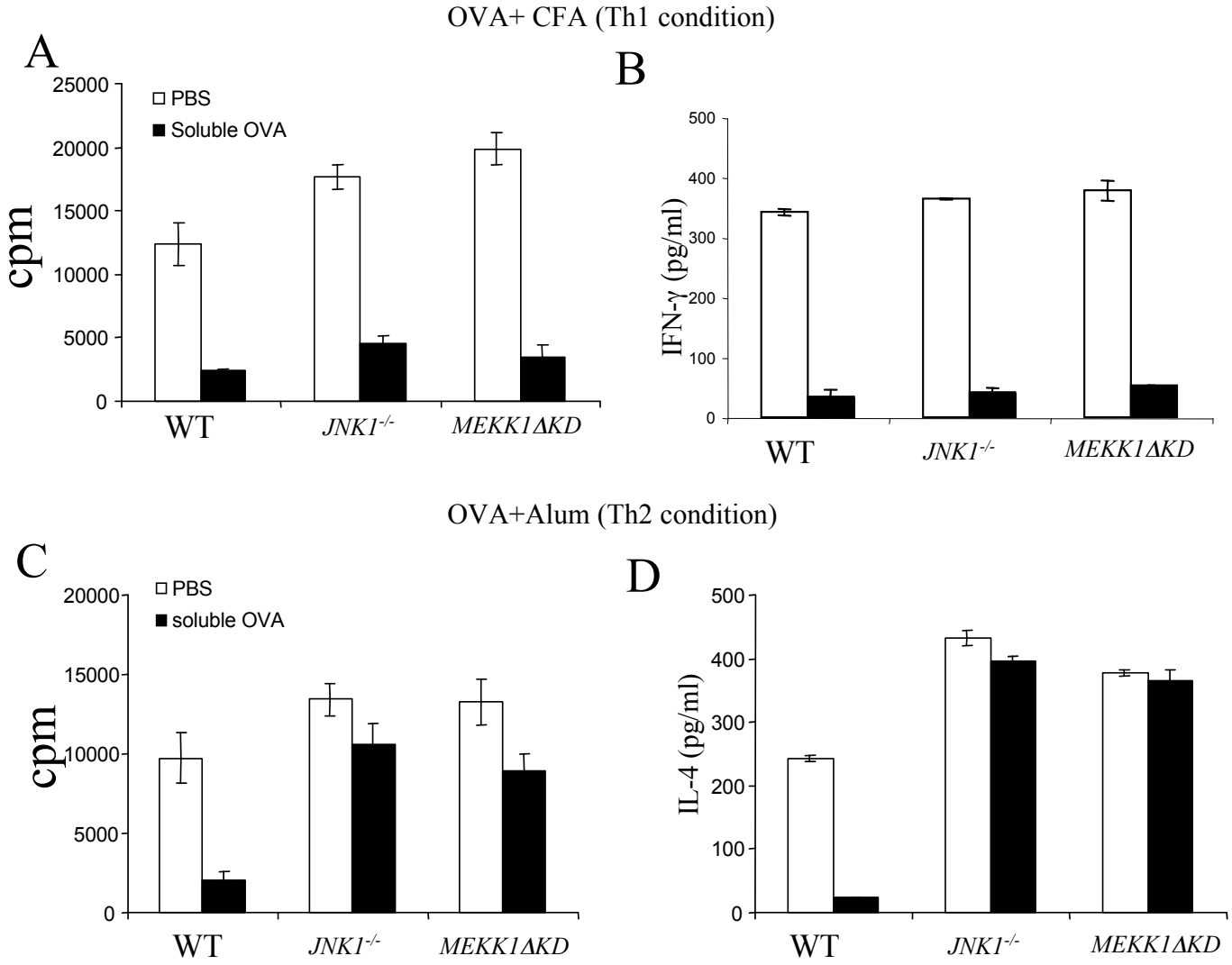
OVA+ CFA (Th1 condition)
A
B
PBS
Soluble OVA
cpm
25000
20000
15000
10000
5000
0
WT
JNK1-/-
MEKK1ΔKD
IFN-γ (pg/ml)
500
400
300
200
100
0
OVA+Alum (Th2 condition)
C
D
PBS
soluble OVA
cpm
20000
15000
10000
5000
0
WT
JNK1-/-
MEKK1ΔKD
IL-4 (pg/ml)
500
400
300
200
100
0

Figure S4

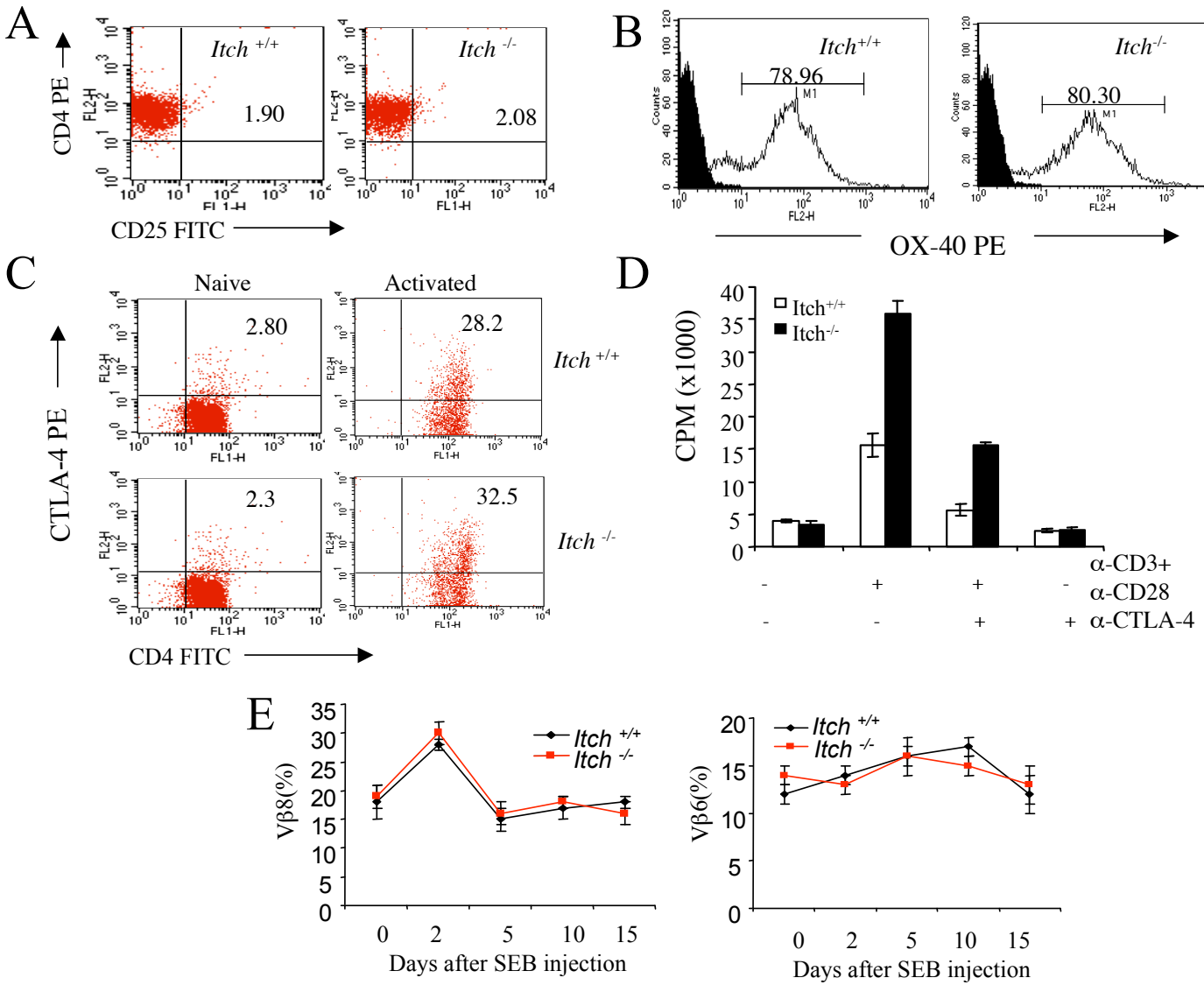
A
CD4 PE
CD25 FITC
Itch +/+
1.90
Itch -/-
2.08
B
Itch+/+
78.96
M1
Itch-/-
80.30
M1
Counts
FL2-H
OX-40 PE
C
Naive
Activated
2.80
28.2
Itch +/+
2.3
32.5
Itch -/-
CTLA-4 PE
FL1-H
CD4 FITC
D
CPM (x1000)
Itch+/+
Itch-/-
α-CD3+
α-CD28
α-CTLA-4
E
Vβ8(%)
Itch +/+
Itch -/-
Days after SEB injection
Vβ6(%)
Itch +/+
Itch -/-
Days after SEB injection

Figure S5

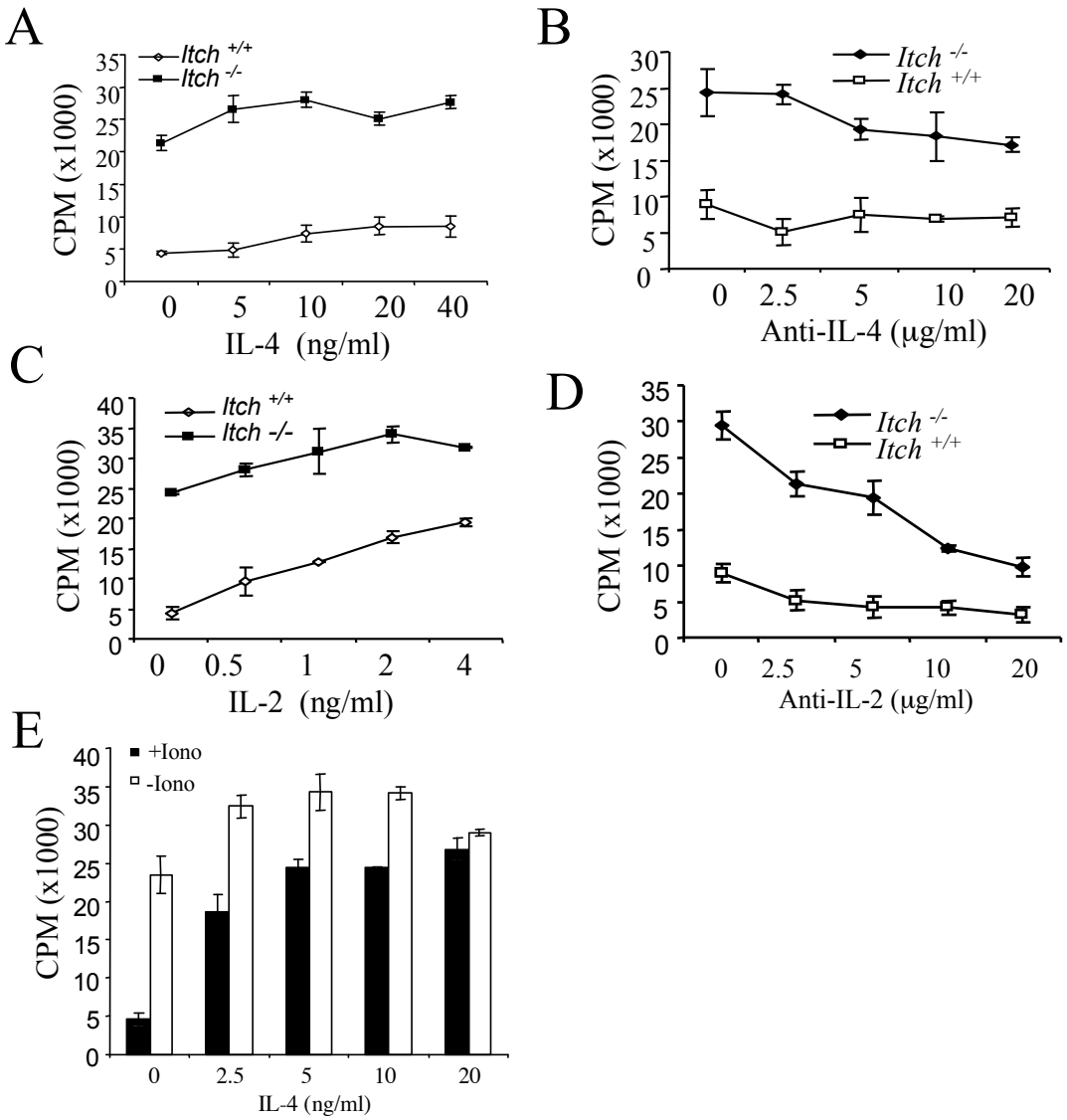
A
Itch +/+
Itch -/-
CPM (x1000)
IL-4 (ng/ml)
0 5 10 20 40
B
Itch -/-
Itch +/+
CPM (x1000)
Anti-IL-4 (μg/ml)
0 2.5 5 10 20
C
Itch +/+
Itch -/-
CPM (x1000)
IL-2 (ng/ml)
0 0.5 1 2 4
D
Itch -/-
Itch +/+
CPM (x1000)
Anti-IL-2 (μg/ml)
0 2.5 5 10 20
E
+Iono
-Iono
CPM (x1000)
IL-4 (ng/ml)
0 2.5 5 10 20

Figure S6

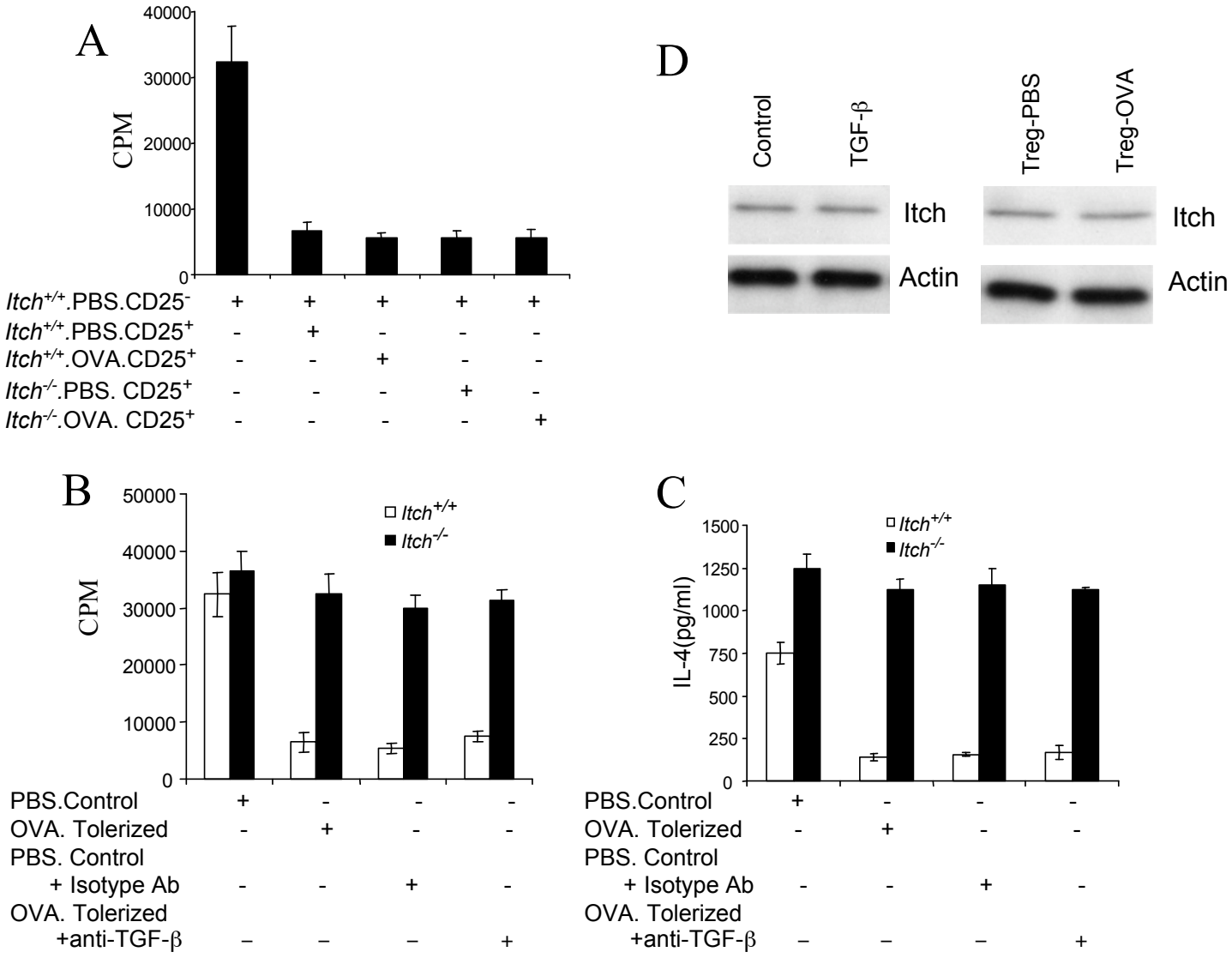
A
CPM
40000
30000
20000
10000
0
Itch+/+.PBS.CD25- + + + + +
Itch+/+.PBS.CD25+ - + - - -
Itch+/+.OVA.CD25+ - - + - -
Itch-/-.PBS. CD25+ - - - + -
Itch-/-.OVA. CD25+ - - - - +
D
Control
TGF-β
Treg-PBS
Treg-OVA
Itch
Actin
Itch
Actin
B
CPM
50000
40000
30000
20000
10000
0
Itch+/+
Itch-/-
PBS.Control + - - -
OVA. Tolerized - + - -
PBS. Control
+ Isotype Ab - - + -
OVA. Tolerized
+anti-TGF-β – – – +
C
IL-4(pg/ml)
1500
1250
1000
750
500
250
0
Itch+/+
Itch-/-
PBS.Control + - - -
OVA. Tolerized - + - -
PBS. Control
+ Isotype Ab - - + -
OVA. Tolerized
+anti-TGF-β – – – +

Figure S7

Supplementary Figures

Figure S1

Reversal of in vivo tolerized T cells by the addition of IL-2. (**A**) Soluble Ova injection does not induce cell death. Wild-type mice immunized with OVA+ Alum and simultaneously injected with high dose soluble OVA or PBS as control. On day 10 $CD4^+$ T cells were isolated and the cell survival was examined by using BrdU-FITC labeling. (**B** and **C**) OVA tolarized wild-type $CD4^+$ T cells from the above experiment were restimulated with OVA + irradiated APCs either with or without recombinant IL-2. The cell proliferation (**B**) and IL-4 production (**C**) were measured.

Figure S2

T cell anergy induction by the absence of costimuation. (**A**) $CD4^+$ T cells from WT and *Itch*$^{-/-}$ OTII mice were differentiated into Th1 and Th2 cells. T cell tolerance was induced by culturing these cells with irradiated microvascular endothelial cells (MECs) in which B7.1 expression was stably expressed (MEC.B7.1+) or MEC control cells (MEC.B7.1-) in the presence of anti-CD3 (1 μg/ml) for 48 hr. The live cells were rested overnight and re-stimulated with anti-CD3 plus anti-CD28. Cell proliferation was measured by thymidine incorporation. (**B** and **C**) The culture supernatants collected after 48 hours were analyzed for IL-4 (**B**) and IFN-γ (**C**) secretion.

Figure S3

Role of JNK1 signaling in Th2 tolerance in vivo. WT, *MEKK1ΔKD*, or *JNK1*$^{-/-}$ mice were tolerized with soluble OVA or treated with PBS as control, as described in Figure 5. The cytokine production of IL-2 (**A**, **B**) from in vitro re-stimulated cultures were measured. T cells from those mice were adoptively transferred and the IL-2 production

and CFSE dilution were determined by FACS analysis (**C**).

Figure S4

Loss of JNK1 signaling does not affect Th1 tolerance. Mice containing *MEKK1* mutation or *JNK1*$^{-/-}$ mice were treated with PBS or high dose OVA peptide, and immunized under either Th1 or Th2 inducing conditions, as described in Fig. 1. T cells were restimulated in vitro and the cell proliferation (**A** and **C**) and cytokine production (**B** and **D**) were analyzed.

Figure S5

Other tolerance-inducing mechanisms are Intact in *Itch*$^{-/-}$ mice. (**A**) CD4^{+} T cells isolated from *Itch*$^{+/+}$ and *Itch*$^{-/-}$ mice were stained with anti-CD4-PE and anti-CD25-FITC and the percentage of CD4^{+}CD25^{+} Tregs was analyzed using flow cytometry. (**B**) CD4^{+} T cells were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (2 μg/ml) for 48 h and the cell surface expression of OX-40 was measured by staining the cells with PE-labeled anti-OX-40 antibody followed by flow cytometry. (**C**) CD4^{+} T cells were stimulated with anti-CD3 plus anti-CD28 for 48 h and the surface expression of CTLA-4 was examined by staining the cells with PE-labeled anti-CTLA-4, followed by flow cytometry. (**D**) CD4^{+} T cells (2X10^{5}) were stimulated with anti-CD3 plus anti-CD28 for 48 h, rested for 6 h, and then restimulated with either anti-CD3 plus anti-CD28 in the presence or the absence of the immobilized anti-CTLA-4 antibody (5 μg/ml). The cell proliferation was measured by ^{3}H-thymidine incorporation. (**E**) Peripheral deletion of autoreactive T cells is intact in *Itch*$^{-/-}$ mice. SEB (100 μg) was injected intraperitonially into either *Itch*$^{+/+}$ or *Itch*$^{-/-}$ mice. Peripheral blood was taken on indicated days from the tail vein and the cells were stained with Vβ8.1/2-FITC (left panel) or Vβ6-FITC (right panel) and anti-CD4-PE, followed by FACS analysis. The percentage of Vβ8- or Vβ6-positive cells was shown.

Figure S6

Cytokine rescue of anergized Th2 cells. (**A**-**D**) Naïve CD4 T cells were first polarized into Th2 cells, and anergized by ionomycin pretreatment. The cells were then stimulated with anti-CD3 plus anti-CD28 in the presence of indicated concentrations of recombinant IL-4 (**A**), anti-IL-4 blocking antibody (**B**), recombinant IL-2 (**C**), or anti-IL-2 blocking antibody (**D**). Cells were cultured for 48 h, of which the last 12 h was pulsed with ^{3}H-thymidine. Thymidine incorporation was measured using scintillation counter. (**E**) Differentiated Th2 cells were cultured without or with ionomycin (50 ng/ml) in the presence of indicated concentrations of IL-4 for 16 h. The cells were then washed, stimulated with anti-CD3 plus anti-CD28, and measured for cell proliferation.

Figure S7

Involvement of Tregs and TGF-β in Itch-medaited T cell anergy induction. (**A**) Wild-type and Itch$^{-/-}$ mice were tolarized with high dose sobluble OVA or PBS as control. On day 10, CD4^{+}CD25^{+} cells were isolated from those mice and were co-cultured CD4^{+}CD25^{-} responder T cells isolated from wild-type mice (ratio 1:1), and irradiated APC plus anti-CD3 (1 μg/ml). The cell proliferation was measured by ^{3}H-thymidine incorporation. (**B** and **C**). Wild-type and Itch$^{-/-}$ mice were tolarized as described using high dose soluble OVA, and the CD4^{+} T cells isolated on day 10 were stimulated in vitro with OVA peptide + irradiated APCs. Either anti-TGF-β or isotype matched control antibody was added to the cultures. The cell proliferation was examined by ^{3}H-thymidine incorporation (**B**) and the IL-4 concentration in the supernatants was measured by using ELISA. (**D**) Itch expression in responder T cells is not modulated by Tregs or TGF-β. CD4^{+}CD25^{-} responder cells from wild-type OT-II mice were left untreated or stimulated with TGF-β (2.5 ng/ml, left panel) or co-cultured with Tregs from either PBS-treated or

soluble OVA-tolerized mice in the presence of APCs plus OVA peptide (right panel). Twelve hr later, Itch expression was examined by immununoblotting with anti-Itch antibody. The same membrane was reprobed with anti-Actin as a control for equivalent protein loading.