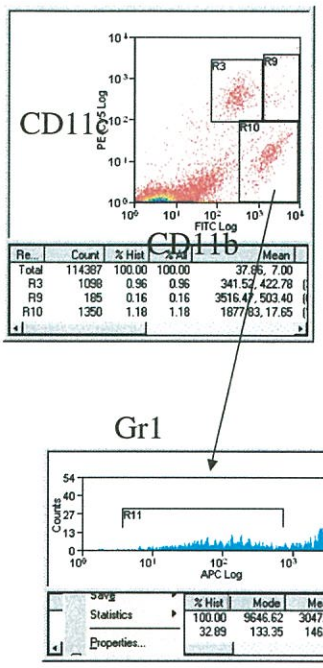
B O C E D/F

CD4+ T	CD8+ T	CD19+ B	NK/NK T	Monocyte/DC	Color
CD3	CD3	CD3 (-)	CD3		APC CY7
CD4	CD8	CD19	CD8	CD11c -biotin	PE CY5
CD44	CD44	CD5	γδ T cell	CD115 – PE	FITC
CD62L	CD62L	CD62L		CD11b FITC	APC
				GR1 APC	PE Cy7
FoxP3			DX5 (CD49b)		PE
		IgD PE		CD206-biotin	PE FITC
			CD1d	PE-Cy7	APC
					PE Cy7

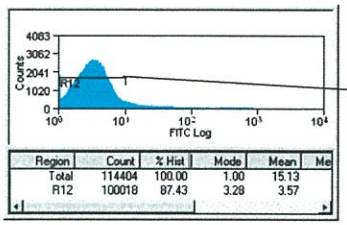
Intracellular staining for ~~BrdU~~ and FoxP3

Cells were surface stained as described, washed, and then fixed for 5 min at 37° C in 4% paraformaldehyde. Cell suspensions were then permeabilized by incubating in PBS containing 0.1% Triton X-100 for 1 h on ice. For detection of BrdU incorporation, permeabilized cells were incubated for 10 min at 37°C in RPMI 1640 containing recombinant DNase. Afterward, suspensions were washed in incubated on ice for 40 min with anti-BrdU Abs following the manufacturer's protocol. Suspensions were washed and then incubated overnight with anti-FoxP3 Abs in PBS containing 0.5% BSA and analyzed the following day by flow cytometry. For FoxP3 staining only, the BrdU relevant steps were omitted.

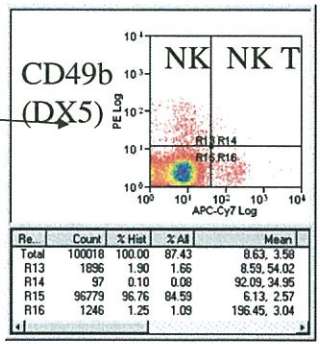


R3 = Inflammatory Macrophages (iMacs)
R9 = dendritic cells
R10 two poulations subdivide by Gr1
Gr1 low = resident macrophage
Gr1 high = granulocyte

Start with ungated population



F4/80 a scavenger receptor on macrophages
Use F4/80 negative population since NKs are big and granulated like macrophages



CD3 a T cell marker

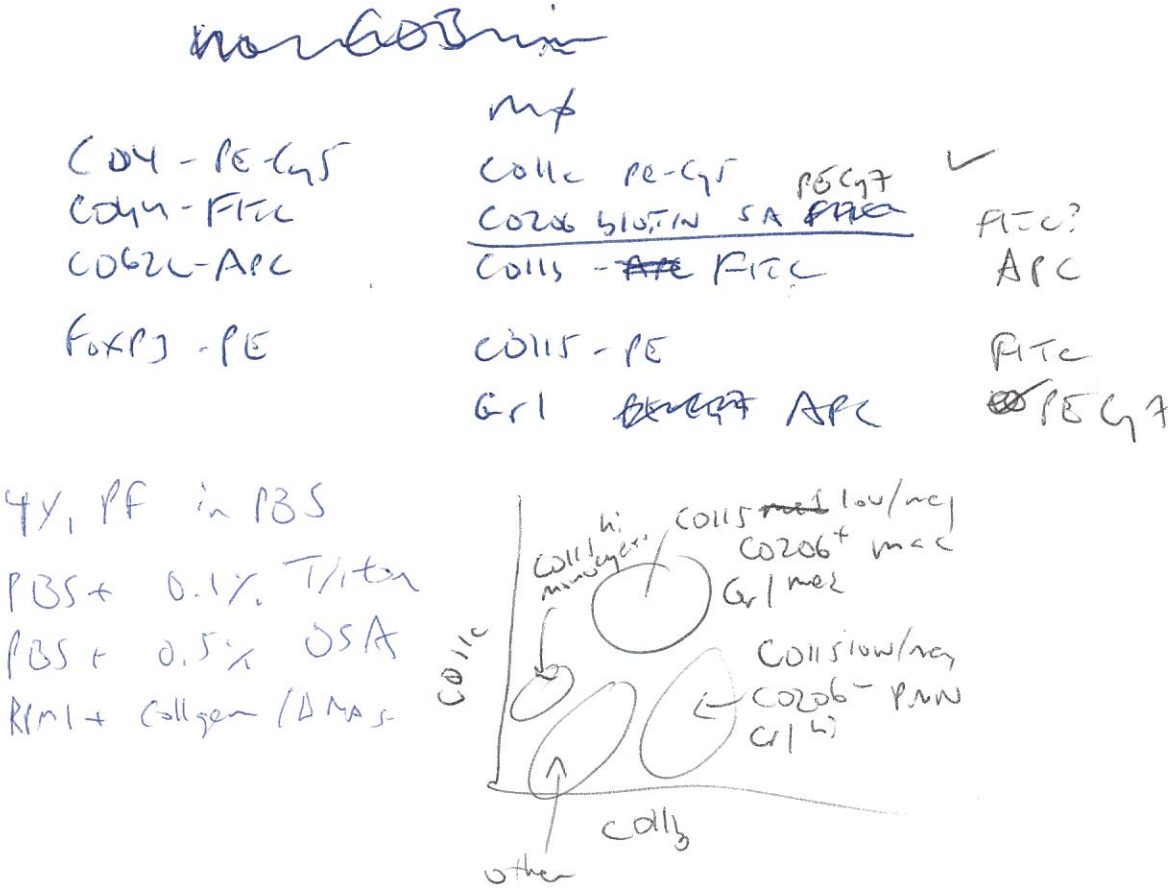
Mice were euthanized and the pulmonary cavities were opened. After severing the descending aorta, the blood in the lungs was cleared by perfusion through the right heart with 5 ml of PBS containing 50 U of heparin (Sigma- Aldrich, St. Louis, MO) per ml until the lungs and liver became pale. Using an 18-gauge gavage needle, the trachea was cannulated, and 1 ml of PBS was slowly injected into the lungs and then withdrawn.

To obtain lung cell populations, the lungs were aseptically removed and minced. One lobes was saved and fixed in 1% paraformaldehyde/PBS for histology and immunohistochemistry. The remaining lobes were incubated in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 mg/ml; Sigma-Aldrich) during 30–45 min at 37°C.

The action of the enzymes was stopped by adding 10 ml of RPMI medium containing 10% FBS, and digested lungs were further disrupted by gently pushing the tissue through a nylon screen (70 mm). The single-cell suspension was then washed and centrifuged at 200*g*. To lyse contaminating RBCs, the cell pellet was incubated during 30 seconds room temperature with 1.8 ml ddH2O, then mixed with 200ul 10X PBS.

Intracellular staining for FoxP3

Cells were surface stained as described, washed, and then fixed for 5 min at 37° C in 4% paraformaldehyde. Cell suspensions were then permeabilized by incubating in PBS containing 0.1% Triton X-100 for 1 h on ice. Suspensions were washed and then incubated overnight with anti-FoxP3 Abs in PBS containing 0.5% BSA and analyzed the following day by flow cytometry.



Stick reanalysis 3/2-4-01

	CO3+, CO8+, CO88	CO8- other CO3+	CO11+
1	.03		
2	.5		
3	.4		
4	.64		
5	.73		
6	.46		
7	.65		
8	.49		
9	.27		
10	.17		

3/20/08 Reanalysis

	Autofluorescence Gate	resident macs $11c^{hi}/11b^{lo}$	PMN ? monocyte/mac $11c^{lo}/11b^{hi}$ (Colli*)	OC $11b^{eff}/11c^{hi}$	CD206
WT1 66549 #1	1.1	~~46.5~~ 34	~~34~~ 46.5	2.5	0.5
WT2 " #2	0.6	~~35~~ 36	~~36~~ 35	1.5	0.2
WT3 " #3	7.6	~~35~~ 50	~~50~~ 35	4	5.3
WT4 " #4	(30 ??) ?	~~57~~ 27	~~27.3~~ 57	3.2	13
WT5 54548M tumor	30 ??	~~34~~ 56	~~56~~ 24	2.17	20
KO1 58848F wild	5.6	~~16~~ 60.2	~~60.2~~ 16	5	4.5
KO2 66546M wild	1.0	32	37	1.8	0.16
KO3 70087 MF (moderate disease)	0.9	80	3.5	2.3	0.75
KO4 70087 FM	0.6	75	10	0.9	1.1
KO5 58805 wild	0.5	56	25	3.6	0.4

histology → relatively little to be seen
70087 M + F have a few early lesions
other three are healthy

7/20/08 Sk
APC+
PE+/−

other cry
pos for everything

	APC++, PE++	γδ−, Oδ5−	APC-Cy7+, PECy5− CD3+
1	0	0.17	.12
2	0.17	1.8	2.4
3	.13	0.8	1.7
4	0.14	0.7	2.1
5	0.21	1	4
6	.07	0.5	2.9
7	.014	1.2	4.9
8	.09	0.75	2.08
9	0.04	0.42	2.1
6	.02	0.32	1.15

NK skin re-analysis 3/24/08

- Cd1 tetramer - APC
- γδ FITC
- CD3 APC-Cy7
- CD8 PECy5
- DX5 - PE

what do we see?

① a population that is bright on all channels

FITC	γδ
PECy5	CD8
APC	CD3+ or Cd1d+?
PE	DX5

w/ lung

② 2 purple populations

CD8 - PE-Cy5 (-)

Cd1d+ or - APC (+) + (-)

DX5 PE (-)

γδ (-)

③ DX5+, Cd1d-

CD3-, CD8-

γδ + + (-)

2 populations?

Cluster Analysis — Macs/DCs

LUNG Gate first on auto fluorescent cells

	Gate	$11c^{hi}/11b^{lo}$	$11c^{lo/neg}/11b^{hi}$	[illegible]+	$11b^{+}/11c^{+}$	$CD206^{+}$
WT1	1.3	52	34	100%	4	1
WT2	1.4	62	23	100%	4	1
WT3	1.6	55	30	"	3.4	0.3
WT4	2.6	64	19	"	2.8	1
WT5	2.7	60	23	"	4.2	2
KO1	17	87	1.4	"	2.8	10.4
KO2	2	83	8.4	"	2.4	2
KO3	2	48	30	"	3.4	5.5
KO4	6.6	87	3		4	3.2
KO5	9.6	93	0.7		0.7	5.3

Higher in skin

SKIN UNGATED DUE TO KERATINOCYTE AUTO FLUORESCENCE

↓ very prominent

	$11c^{hi}/11b^{lo}$	$11c^{lo/neg}/11b^{hi}$	$11b^{+}/11c^{+}$	$CD206^{+}$
WT1	3.6	1.6	3.5	N/A
WT2	2.2	0.8	1.9	↓
WT3	0.5	0.05	0.4	
WT4	2.3	0.25	0.6	
WT4	2.2	0.24	3.9	
KO1	0.3	0.07	0.3	
KO2	1	0.07	0.2	
KO3	0.7	0.07	0.3	
KO4	0.9	0.09	0.13	
KO5	0.8	0.05	0.2	

6/14/08

Skin DNFB day 3

	γδ+, CD1-	γδ+ CD1+	DX5+, CD1-	DX5+, CD1+
WT1	10	1	13	15
WT2	10	3	14	10
WT3	6	1	7	2
WT4	7	1	6	14
WT5	9.5	3	11	12
KO1	2	0	2	1
KO2	1	0.5	6.4	3.7
KO3	2	1	3	3
KO4	2	1	2	8
KO5	1.5	1.5	1.6	1

δγ analysis | used skin pt 1.plo 3/20/08 + 6/19/08 | used skin pt 2.plo

3/20/08	con?	δγ	INKT	CD8	NK	~~NK~~ ~~CD8~~ to
WT 1	n/d	3.7	0.02	0.13	0.19	
WT 2		5.7	0.26	2.8	2	
WT 3		4.3	0.06	0.85	1.4	
WT 4		6.3	0.04	0.5	1.1	
WT 5		10.6	0.1	1.1	2.3	
KO 1		4	0.05	0.8	1.35	
KO 2		5.3	0.14	1.75	3.5	
KO 3		4.9	0.09	0.6	2	
KO 4		2.5	0.05	0.6	1.1	
KO 5		2.2	0.04	0.25	1.1	

6/19/08

WT 1	~~n/d~~ 0.01	7	0.17	~~0.3~~ 1.6	~~0.3~~ 1.0	~~1.0~~
WT 2	~~0.8~~ 0.3	13 ~~1.2~~	0.10	~~0.30~~ 0.57	~~0.6~~ 1.5	0.9
WT 3	0.07	~~1.5~~ 4	0.05	~~0.4~~ 0.2	0.9	~~0.4~~ 0.
WT 4	0.3	~~5.3~~ 5.3	0.27	0.35	~~0.6~~ 1.5	0.4
WT 5	~~0.08~~ 0.3	8.5	0.10	~~0.2~~ 0.3	~~0.3~~ 1.4	0.2
KO 1	0.1	2.6	0.01	0.03	0.2	
KO 2	0.2	1.3	0.03	0.16	0.2	
KO 3	0.1	2.5	0.05	0.08	0.5	
KO 4	0.4	1.5	0.05	0.14	0.16	
KO 5	n/d	2.9	~~0.06~~ n/d	~~0.07~~ n/d	0.6	

6/20/08 CD4 | FoxP3 Analysis

→ APC @ 900V but still too low

Generally, it appears FoxP3+ are also CD4+

	CD4+ (of total)	CD4+, FoxP3+ (of total)	% of CD4+
WT 1	~~18~~ 15	~~1.8~~ 2.5	17
WT 2	18	1.8	10.4
WT 3	14.3	1.2	8.5
WT 4	20.3	1.4	6.9
WT 5	12.1	1.6	12.8
KO 1	6	1.8	31
KO 2	13	1.3	10
KO 3	9.3	0.7	7.4
KO 4	6.1	1.6	27
KO 5	5.6	0.7	13.2

8/14/08 digest 0.7mg/ml Collagenase #1 + 0.7mg Type 2

	×5	×1 ml
W1L	2e4	1e5
W1R	10e4	
W2L	2e4	
W2R	4e4	
W3L	4e4	
W3R	10e4	
W4L	1e4	
W4R	4e4	
W5L	6e4	
W5R	12e4	

K1L	1e4
K1R	2e4
K2L	5e4
K2R	7e4
K3L	2e4
K3R	4e4
K4L	2e4
K4R	8e4
K5L	1e4
K5R	2e4

L → NK/γδ only

R → hope we have enough to see 1e5/tube

A - CD4 Pacific Blue/CD45 FITC/CD62L APC

B CD8 " "/" "/" "

C γδ FITC/CD3 APC/DX5 PE/NK1.2 PECy7

D CD44 Pacific Blue/CD3 FITC/K1 APC/CD11b PE

10 tubes except C = 20

1mg 2mg

12/22/08

CD4+ T	CD8+ T	CD19+ B	NK/NK T	Monocyte/DC	Color
CD3	CD3	CD3 (-)	CD3		APC CY7
					PE CY5
CD4	CD8	CD19	CD8	CD11c	Pacific Blue
CD44	CD44	CD5	γδ T cell	CD11b	FITC
CD62L	CD62L	CD62L	CD1d	GR1	APC
				CD206-biotin	PE Cy7
FoxP3		IgD	DX5 (CD49b)	CD115	PE

NKG2D biotin next time

Intracellular staining for FoxP3

Cells were surface stained as described, washed, and then fixed for 5 min at 37° C in 4% paraformaldehyde. Cell suspensions were then permeabilized by incubating in PBS containing 0.1% Triton X-100 for 1 h on ice. Suspensions were washed and then incubated overnight with anti-FoxP3 Abs in PBS containing 0.5% BSA and analyzed the following day by flow cytometry.

[illegible]

16 samples

spin @ 1800 rpm

- 1% PF in PBS 15' RT
- WASH 1X PBS + BSA
- Permeabilize in 0.1% Saponin in PBS 30' RT
- O/N in 0.1% Saponin/PBS/BSA @ 4°C FoxP3

12/22/08

p02

One lobe

		Skin	Lung		
		5 x 10^4	10 x 10^4		
WT1		4	30	2e6	300
WT2		14	200	2e7	50
WT3		6	100	1e7	100
WT4		4	~~100~~ ~~100~~ 50	8e6	125
WT5		12	~~200~~ 50	5e6	200
Ko1	70087	97.7%	3	80	8e6	125
Ko2	78640	90.1%	6	80		
Ko3	72790	85%	5	120		
Ko4	75875	~~97%~~ 70 %	3	100		
Ko5	74890	~~90%~~ ~~80%~~ 77%	10	60		

50 mL

10 petri dishes ✓

20 tubes for flow 10 ear, 10 lung
+ ~~10~~ 6 spleen
+ ~~10~~ 4 blood
---
30 total ✓

30 flow Tubes

		10 x 10^4	
Spleen	WT1	30	2e6
	WT2	90	9e6
	WT3	70	9e6
	Ko1	70	9e6
	Ko2	120	1e7
	Ko3	40	7e6

- for P3 protocol from Tokuo
- Collagenase for Lungs + Skin

15 tubes for Ko mice

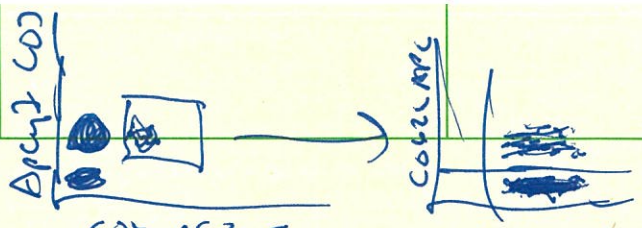
① BAL
② IHC
③ RNA

} mouse ID #, date, FKO

15 ml conical

(20) 50 mL conicals

ONFB consists for each site II



	COH	A	COMPLEX B	C	COHESIVE D
W1	5.4	3	50	0	47
W2	11.1	1	50	0.3	49
W3	7.8	0.4	50	0	50
W4	16.4	0.6	42	0.3	57
W5	9.6	0.9	34	0.8	64
K1	9	0.7	31	1.3	67
K2	4.9	1.4	42	1.5	55
K3	10.1	0.4	36	0.8	63
K4	11	0.5	21	2.5	76
W1	23	0	3	3	94
W2	34	0.3	4	3	93
W3	11	0.8	5	4	91
W4	21	1.6	14	7	77
W5	7.5	3.3	12	7.2	78
W6	26.8	0	16	0.4	84
K1	14	0.3	19	1.6	80
K2	15	0.2	9	0.8	90
K3	7.5	0.8	12	10	77
K4	8.8	0.1	14	1.9	85
K5	5.2	0.4	12	1.2	87