Supplement Material

Methods

Generation and characterization of sMICB-neutralization monoclonal antibody P10G5. Two balb/c mice were immunized with purified recombinant soluble MICB (rsMICB) three times at two weeks intervals. A 100μg weight of the conjugated KLH peptide in Freund's complete adjuvant was used for the first immunization. At the subsequent immunizations, 50 μg of the KLH peptide in incomplete Freund's adjuvant were administered. One week prior to the last immunization, blood was collected and antibody titers were determined by ELISA using rsMICB coated plates. Hybridomas were generated by fusing splenocytes from immunized mice with the murine myeloma cell line SP2/0 using polyethylene glycol. Fusion hybrids were selected using HAT medium and the reactivity of the secreted antibody was tested by ELISA and flow cytometry analysis against C1R and C1R-MICB cells. Antibody produced by one of the hybridoma B10G5 was purified by BioXcell (Labnon, NH).

Figure S1 Legend

Prostate-restricted expression of NKG2D ligands does not alter prostate physiology or systemic immunity. **A.** The prostate-specific transgene constructs. Expression of shedding-sensitive native MICB or shedding-resistant mutant MICB.A2 was under the direction of the prostate-specific rat probasin promoter (-426 to +28 bp). **B.** RT-PCR detection of prostate-specific expression of MICB or MICB.A2. PR, prostate. LV, liver. LU, lung. MU, muscle. SP, spleen. **C.** Prostate histology of transgenic mice (B6 background). H&E and basal cell cytokeratin p63 staining demonstrate normal structure of the prostates with expression of MICB or MICB.A2. Bar, 50μm. **D.** Expression of NKG2D ligands does not significantly influence prostate weight.

Figure s1

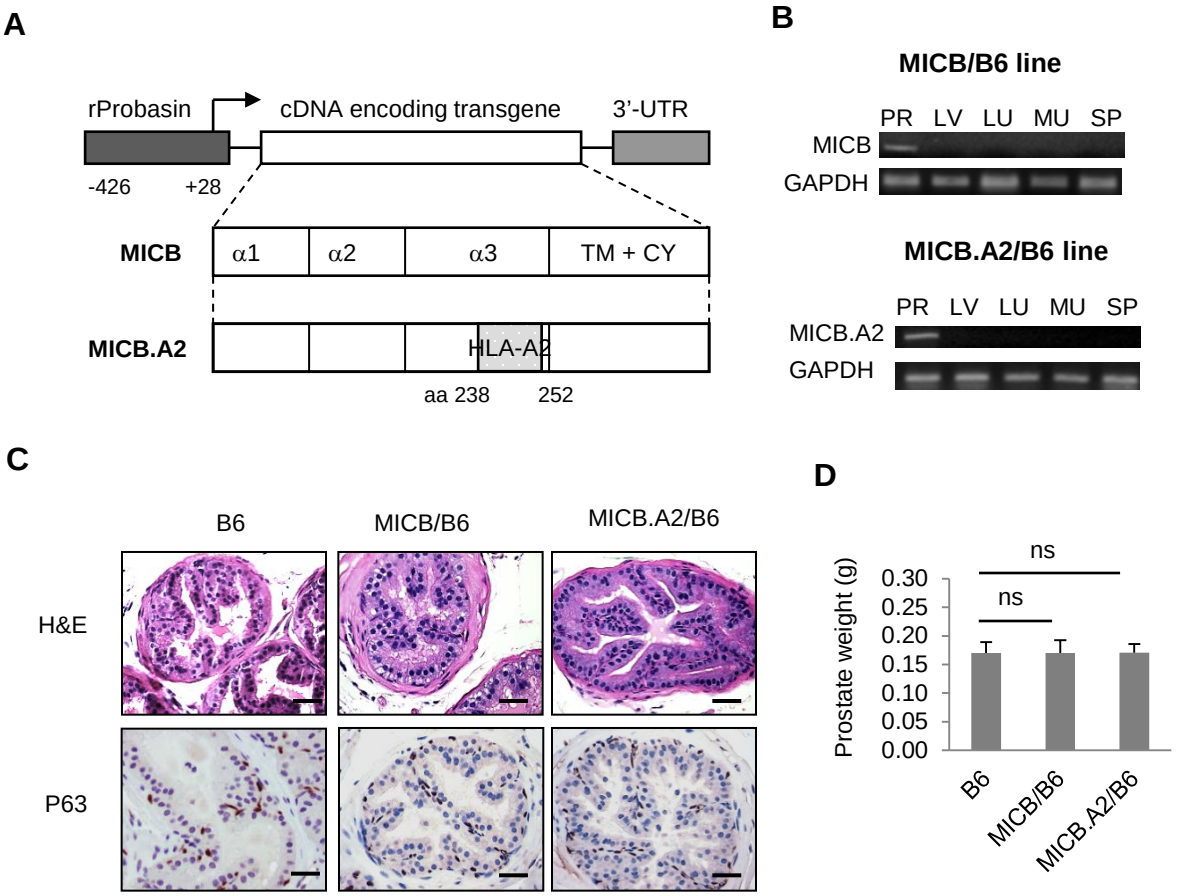

A
rProbasin
cDNA encoding transgene
3'-UTR
-426
+28
MICB
α1
α2
α3
TM + CY
MICB.A2
HLA-A2
aa 238
252
B
MICB/B6 line
PR LV LU MU SP
MICB
GAPDH
MICB.A2/B6 line
PR LV LU MU SP
MICB.A2
GAPDH
C
B6
MICB/B6
MICB.A2/B6
H&E
P63
D
Prostate weight (g)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
ns
ns
B6
MICB/B6
MICB.A2/B6

Figure S2 legend

Prostate-specific expression of MICB or MICB.A2 has no impact on the number of systemic NK cells and CD8 T cells. **A**. Representative flow cytometry analyses (left panel) and quantification (right panel) of pooled data of NK1.1 population ($CD3^{-}NK1.1^{+}$) of each genotypes. **B**. Representative histogram of NKG2D expression on NK cells. **C**. Representative dot plots of flow cytometry analyses of CD8 T cell population. All data represent at least 6 animals per genotype.

Figure S2

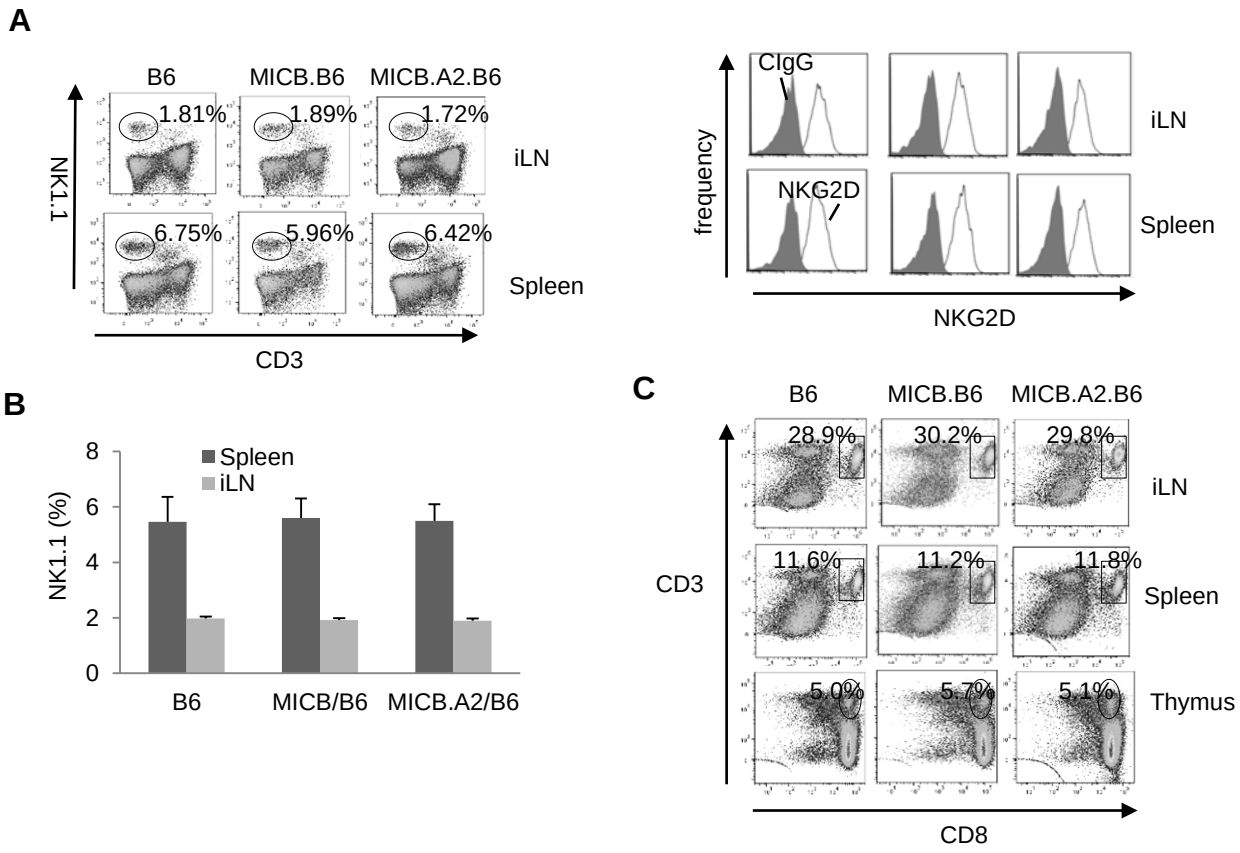

A
B6
MICB.B6
MICB.A2.B6
1.81%
1.89%
1.72%
iLN
6.75%
5.96%
6.42%
Spleen
NK1.1
CD3
CIgG
NKG2D
frequency
iLN
Spleen
NKG2D
B
Spleen
iLN
NK1.1 (%)
8
6
4
2
0
B6
MICB/B6
MICB.A2/B6
C
B6
MICB.B6
MICB.A2.B6
28.9%
30.2%
29.8%
iLN
11.6%
11.2%
11.8%
Spleen
5.0%
5.7%
5.1%
Thymus
CD3
CD8

Figure S3 legend

A and **B**, absolute number of NK cells (**A**) and CD8 T cells (**B**) in the spleens and lymph nodes of TRAMP and TRAMP/MICB mice. Data show significant loss of NK cell number, not CD8 T cell number with progression to PD tumors. **C**, Representative dot plots and pooled statistical data showing total CD8 T cells and $NKG2D^{+}$CD8 T cells infiltrated in tumor parenchyma of TRAMP and TRAMP/MICB mice. Data show no significant difference in total CD8 T infiltrates, but a significant reduction of $NKG2D^{+}$CD8 T cells. TIL, tumor infiltrated lymphocytes.

Figure S3

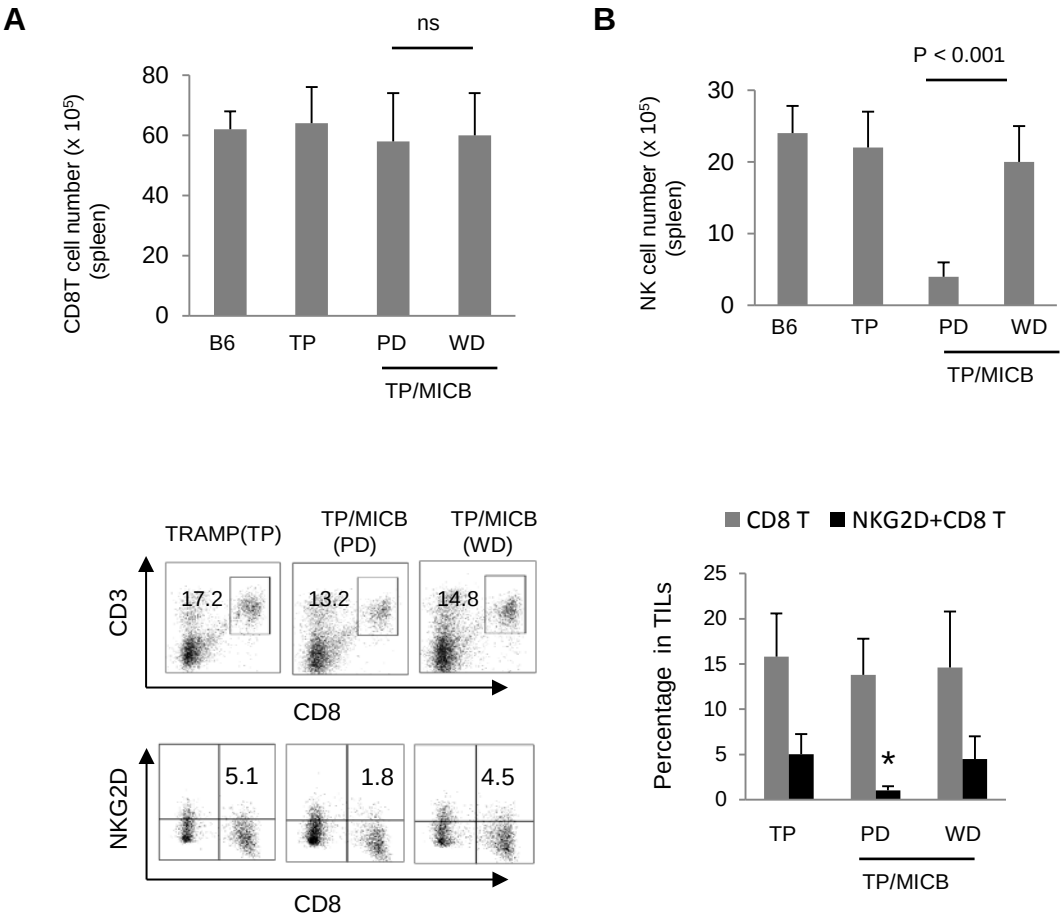
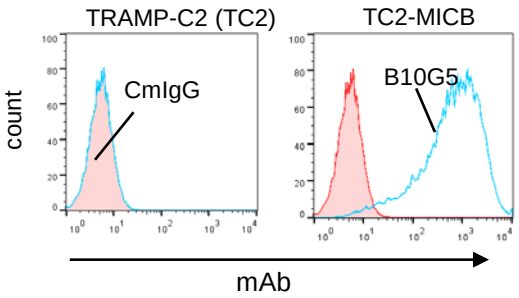

A
ns
CD8T cell number (x 10⁵) (spleen)
80
60
40
20
0
B6
TP
PD
WD
TP/MICB
B
P < 0.001
NK cell number (x 10⁵) (spleen)
30
20
10
0
B6
TP
PD
WD
TP/MICB
C
TRAMP(TP)
TP/MICB (PD)
TP/MICB (WD)
CD3
17.2
13.2
14.8
CD8
NKG2D
5.1
1.8
4.5
CD8
CD8 T
NKG2D+CD8 T
Percentage in TILs
25
20
15
10
5
0
*
TP
PD
WD
TP/MICB

Figure S4 Legend.

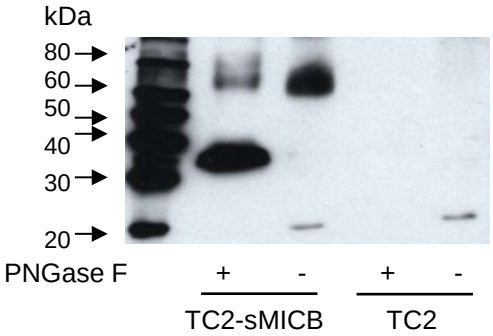
Characterization of sMICB specificity of the antibody. A. Representative histogram from flow cytometry analysis showing that purified B10G5 mAb is MICB-specific. Mouse prostate tumor cell line TRAMP-C2 (TC2) and its derivative TC2-MICB were incubated with CD16/32 to block FcRγ before incubating with 2μg/ml of B10G5 or control mouse IgG (cIgG) and PE-conjugated goat anti-mouse IgG. **B.** Unedited immunoblot showing B10G5 is sMICB-specific. 2 ml Culture supernatant of TC2-sMICB or TC2 cells were incubated with B10G5 and protein A-beads. Half of the immune complexes were treated with PNGaseF to deglycosylate. The immune complex were dissolved in SDS-sample buffer, loaded onto 7.5% SDS/PAGE gel, transferred into nitrocellulose membrane, and blotted with goat anti-MICB polyclonal antibody AF1599 (R&D Systems). Lane 1, deglycosylated sMICB. Lane 2, native sMICB. **C**. flow cytometry assay demonstrating that B10G5 inhibits sMICB binding to NKG2D on mouse NK cells. Purified mouse splenic NK cells were incubated with biotinylated rsMICB (5ug/ml) and various concentrations of B10G5 followed by PE-streptavidin. Cells were analyzed with BD FACSscan. Data were analyzed with CellQuest software. Data clearly demonstrate that B10G5 inhibits the binding of sMICB to NKG2D.

Figure S4

A



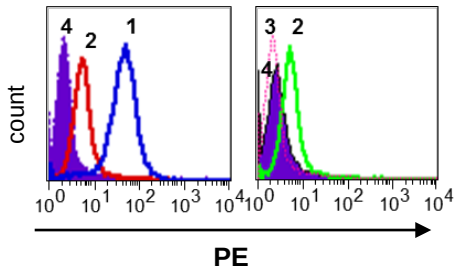
B



IP: B10G5 mAb

IB: goat anti-MICB
(R&D systems, AF1599, goat IgG)

C



1. sMICB only (5 ug / ml)

2. sMICB + 10 ug / ml B10G5

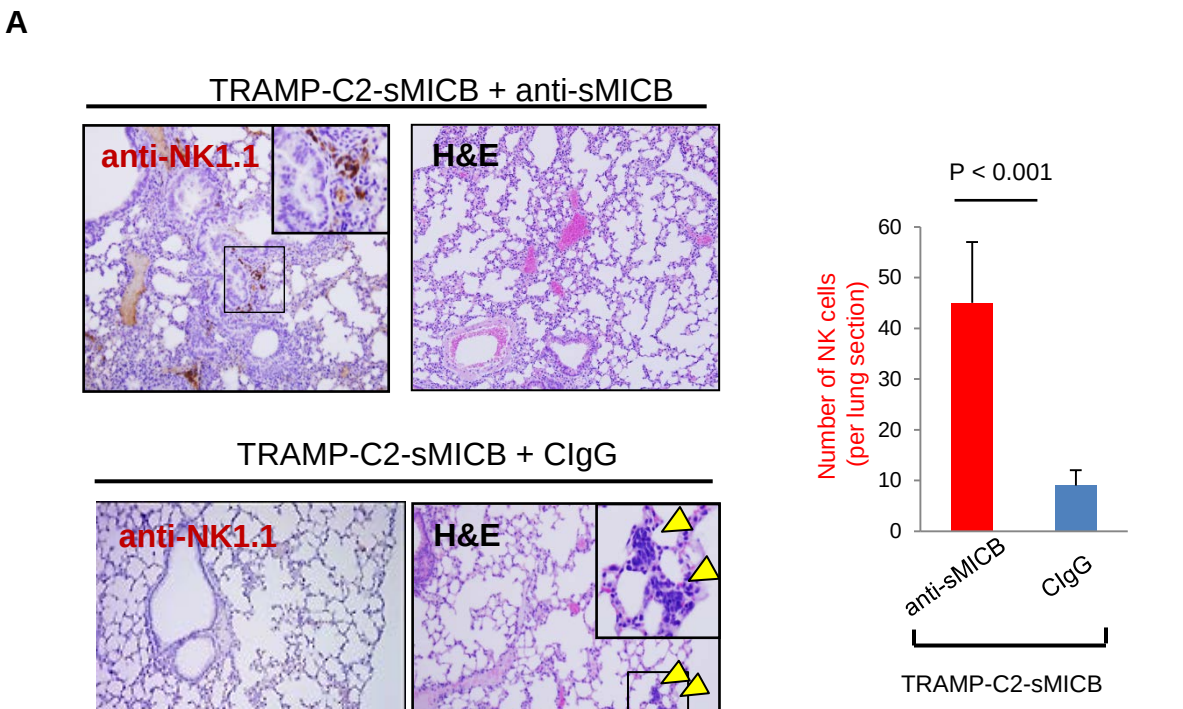
3. sMICB + 20 ug / ml B10G5

4. sMICB + anti-mNKG2D antibody C7 (20 ug/ml)

Figure S5 Legend

Loss of NK cells in tissues with tumor metastasis. **A**, Representative anti-NK1.1 staining of lung sections from mice implanted with TC2-sMICB mice that received sMICB-neutralizing antibody or control IgG treatment. Bar graph shows the summary data of NK cells counted from one lung section. Five lung sections were counted from each animal to obtain the mean data of an individual. Data show loss of NK cells in lung parenchyma with tumor micrometastases (arrows in H&E staining). **B**. Representative micrograph of anti-NK1.1 staining of lymph nodes from TRAMP (no metastasis) and TRAMP/MICB mice (with metastasis). Data show loss of NK cells in the lymph node with tumor metastases (SV40T-positive). Inserts show enlarged area.

Figure S5


A
TRAMP-C2-sMICB + anti-sMICB
anti-NK1.1
H&E
TRAMP-C2-sMICB + CIgG
anti-NK1.1
H&E
P < 0.001
Number of NK cells
(per lung section)
60
50
40
30
20
10
0
anti-sMICB
CIgG
TRAMP-C2-sMICB

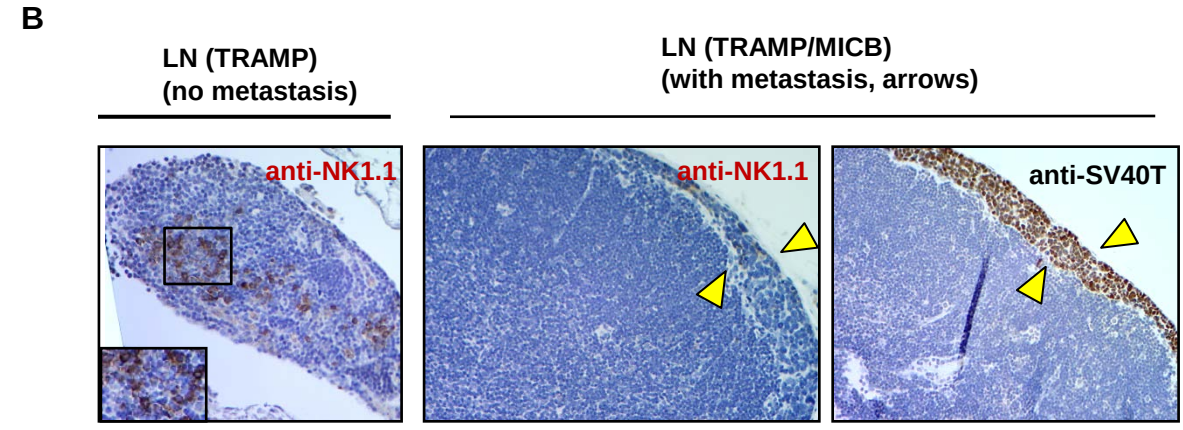
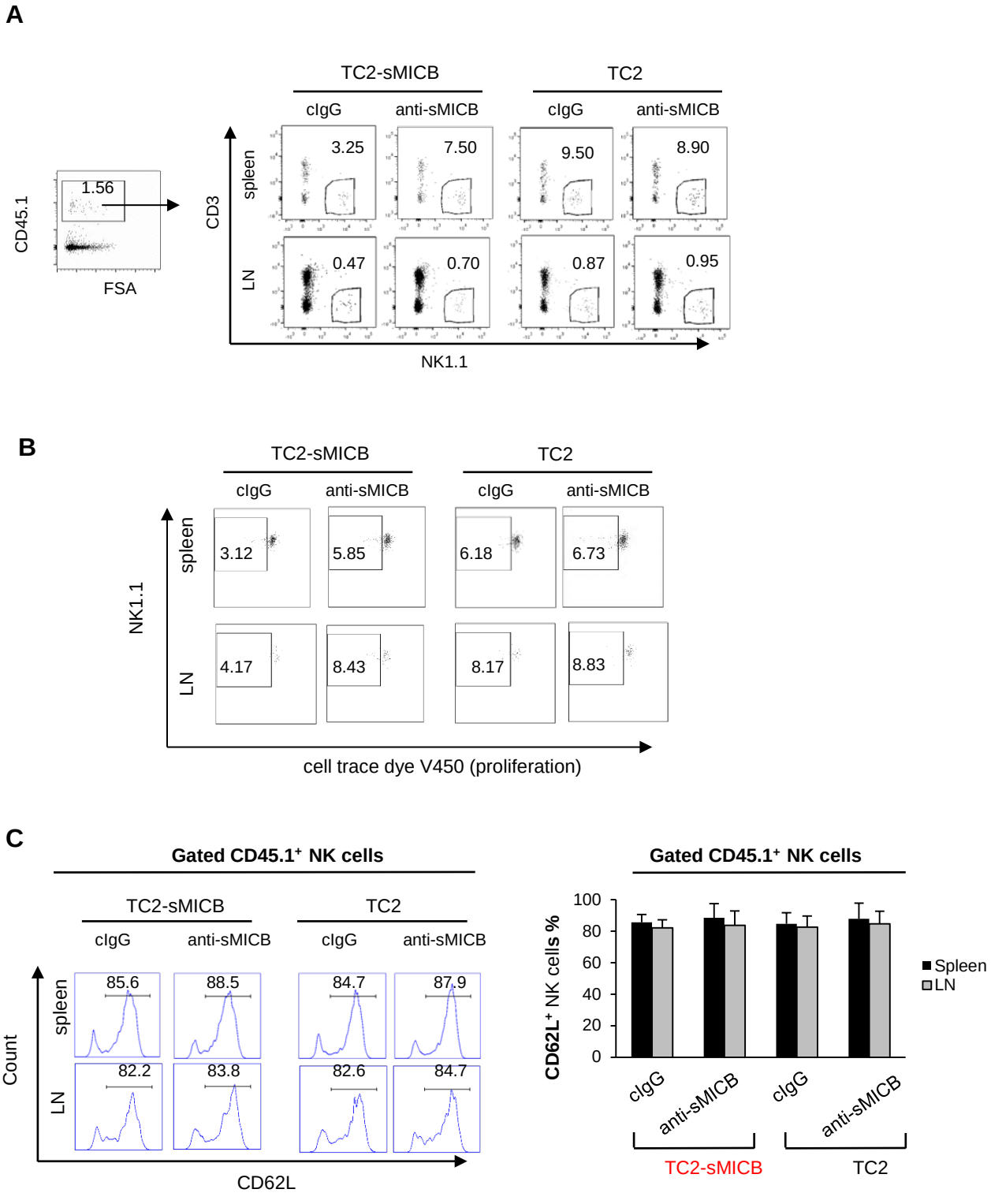

B
LN (TRAMP)
(no metastasis)
LN (TRAMP/MICB)
(with metastasis, arrows)
anti-NK1.1
anti-NK1.1
anti-SV40T

Figure S6 Legend

A and B, Representative dot plots of flow cytometry analyses of adoptively transferred V450-labeled congenic CD45.1^{+} NK1.1 population (**A**) and proliferation (**B**) in the spleen and lymph nodes of TRAMP-C2 (TC2) and TC2-sMICB metastasis model. Data shown that sustained congenic CD45.1^{+} NK1.1 cells were significant lower in mice implanted with TC2-sMICB cells than those implanted with TC2 cells. Data also show that sMICB neutralizing antibody treatment increases the sustainability of congenic CD45.1^{+} NK1.1 cells in mice implanted with TC2-sMICB cells by increasing NK cell proliferation. **C**, representative dot plots and pooled statistical data showing that sMICB does not influence the expression of NK cell homing receptor CD62L.

Figure S6


A
TC2-sMICB
TC2
cIgG
anti-sMICB
cIgG
anti-sMICB
CD45.1
1.56
FSA
CD3
spleen
LN
3.25
7.50
9.50
8.90
0.47
0.70
0.87
0.95
NK1.1
B
TC2-sMICB
TC2
cIgG
anti-sMICB
cIgG
anti-sMICB
NK1.1
spleen
LN
3.12
5.85
6.18
6.73
4.17
8.43
8.17
8.83
cell trace dye V450 (proliferation)
C
Gated CD45.1+ NK cells
TC2-sMICB
TC2
cIgG
anti-sMICB
cIgG
anti-sMICB
Count
spleen
LN
85.6
88.5
84.7
87.9
82.2
83.8
82.6
84.7
CD62L
Gated CD45.1+ NK cells
CD62L+ NK cells %
100
80
60
40
20
0
Spleen
LN
cIgG
anti-sMICB
cIgG
anti-sMICB
TC2-sMICB
TC2