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Supporting Information

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$CD122^{+}CD8^{+}$ Treg suppress vaccine-induced antitumor immune responses in lympho-depleted mice

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Supplementary Text

Dose-dependent suppression of vaccine-induced proliferation of pmel-1 T cells in lymphodepleted mice by naïve spleen cells

To better understand the competition between lymphopenia-driven proliferation and vaccine-induced T cells proliferation and expansion, we first performed a titration of pmel-1 T cells to determine the minimal number of pmel-1 T cells that needed to be transferred in order for them to be easily identified in the blood. When 10^7 wt naïve spleen cells co-transferred with different number of naïve pmel-1 spleen cells into irradiated mice that were then vaccinated with dendritic cells pulsed with gp100 peptide (hgp9), the number of pmel-1 T cells in blood of mice at day 14 post vaccination could be easily detected even when the number of pmel-1 spleen cells were transferred at as low as 10^3 cells (Figure S1A). Because vaccine-induced proliferation and lymphopenia-driven proliferation of T cells were IL-7 dependent, vaccine-induced proliferation of pmel-1 T cells could be competed by lymphopenia-driven proliferation of wt naïve spleen cells. To test this possibility, one million naïve pmel-1 spleen cells were transferred together with titrated numbers of naïve wt spleen cells into irradiated mice, the number of pmel-1 T cells in the blood of vaccinated mice two weeks after vaccination was increased when 1 to 10 million naïve spleen cells were co-transferred; but the number decreased when 50 or 100 million spleen cells were co-transferred (Figure S1B). These results demonstrated that upon exposure to their cognate peptide antigen-specific T cells could expand to a great degree even when only a small number (10^4 spleen cells with 20% $CD8^+$ pmel-1 T cells) were transferred; however, their expansion was inhibited when a large number of filler spleen cells (>10 million) were co-transferred. These results indicate that lymphopenia-driven proliferation of non antigen-specific T cells in naïve wt mice might hinder vaccine-induced proliferation of tumor-specific T cells in vaccinated lymphopenic hosts.

Spleen cells from IL-15 knock out mice were less capable to suppress vaccine-induced melanoma-specific pmel-1 T cells in lymphodepleted mice

During our study of the role of IL-15 in homeostatic proliferation, we demonstrated that expansion of pmel-1 T cells in non-irradiated IL-15 knockout mice was dramatically higher than in non-irradiated wt mice (Figure s2). In fact the peak expansion of pmel-1 T cells in IL-15 ko mice was similar to irradiated wt mice. Moreover, irradiation of IL-15 ko mice did not further improve pmel-1 T-cell expansion. These results indicated that the IL-15 ko recipient was lymphopenic and lymphocytes in IL-15 ko mice were less able to compete for IL-7 than lymphocytes in wt mice. To determine whether spleen cells from IL-15 ko mice were less able to compete with pmel-1 T cells for IL-7, one million pmel-1 naïve spleen cells were co-transferred into irradiated wt mice with 50 million spleen cells from wt or IL-15 ko mice followed by vaccination with peptide-pulsed DC (Figure S2A). A second vaccine was given 10 weeks later to determine if there was an effect on memory responses of pmel-1 T cells. Co-transfer of wt spleen cells suppressed both the primary and recall responses of pmel-1 T cells. However, co-transfer of the same number of spleen cells from IL-15ko mice resulted in a much smaller reduction of the primary response, and this reduction was detectable only during the first two weeks after immunization. Interestingly, the secondary pmel-1 response was actually augmented rather than suppressed by the co-transfer of IL-15 ko spleen cells. These results further support the hypothesis that the lymphocytes that compete with antigen-driven T-cell proliferation in lymphodepleted mice were absent in IL-15 ko mice.

Deficiency of innate lymphocytes in spleens of IL-15 ko mice

We first determine the absolute number of $CD4^+$, $CD8^+$, NK, $CD8^+CD122^+$, and $CD4^+CD25^+$ T cells in the blood of wt and IL-15 ko mice and blood from irradiated wt mice was also included for comparison. Consistent with what was observed in the spleen, total $CD8^+$, particularly $CD8^+CD122^+$ T cells, and NK cells, but not total $CD4^+$ or $CD4^+CD25^+$ T cells, were greatly reduced in IL-15 deficient mice (Figure S2B and C). $CD4^+$ (including $CD25^+$) T cells, $CD8^+$ T cells (including $CD122^+$), and NK cells were almost totally ablated after irradiation. To investigate the difference in the composition of lymphocyte populations in wt and IL-15 ko mice, the percentage of $CD4^+$, $CD4^+CD25^+$, $CD8^+$, $CD8^+CD122^+$ T cells, NK, and NKT in the spleen was determined by 8-color polychromatic flow cytometry analysis (Figure S2D). In wt mice, 4.2% of the CD19 and CD11b negative cell population expressed high levels of CD122 ($CD122^{hi}$). The majority of $CD122^+$ cells in spleen of wt mice were either NK cells (50%) or $CD8^+$ T cells (30%), and only 4% are $CD4^+$ T cells. The percentage of total $CD122^{hi}$ cells was reduced by more than 90% in IL-15 ko mice (0.4%) and the majority of $CD122^+$ cells are $CD4^+$ (45%). Among the $CD122^+$ cells, $CD8^+$ T cells, especially the $CD8^+CD122^{hi}$ subset, NKT and NK cells, were all significantly reduced in IL-15 ko mice compared to wt mice. In contrast, the percentage of $CD4^+$ T cells that expressed CD25 did not differ between wt (8%) and IL-15 ko mice (9%) (Figure S1F). Because spleens from IL-15 ko mice contain normal number of both naïve and $CD4^+CD25^+$ regulatory T cells, but lack $CD122^+$ lymphocytes, it appeared that a deficiency of $CD122^+$ lymphocytes rather than $CD25^+$ lymphocytes was responsible for the heightened expansion of pmel-1 T cells observed in IL-15 ko mice. It is possible that $CD122^+CD8^+$ T cells, in addition to $CD4^+CD25^+$ T cells, could also inhibit pmel-1 T cell expansion in lymphopenic hosts after DC vaccination.

$CD122^+CD8^+$ T cells are the major population that underwent lymphopenia-driven proliferation

Numerous studies have demonstrated that lymphopenia-driven proliferation occurs among naïve T cells, memory T cells, regulatory cells, NK cells, and NKT cells (20, 26, 34-36). However, in each study the populations were all examined individually. Therefore their relative capacity to proliferate together

under lymphopenic conditions has not been compared. We reasoned that different lymphocyte subsets, particularly $CD122^+$ and $CD122^-$ $CD8^+$ T cells, would differ not only in their abundance, but also in their relative capacity to proliferate under lymphopenic conditions. To this end, spleen cells from wt mice were separated into $CD122^+$ and $CD122^-$ populations and labeled with CFSE before adoptive transfer into normal and irradiated mice. Seven days after transfer, the CFSE profile of $CD8^+$ and $NK1.1^+$ cells from the peripheral blood was analyzed by flow cytometry (Figure S3A). Minimal cell division of $CD122^+$ or $CD122^-$ (10%) was observed following transfer to non-irradiated mice. In irradiated mice, the majority of $CD122^+$ $CD8^+$ T cells had divided (96%) and divided significantly more $CD122^-CD8^+$ T cells (64%). About half of NK cells, which are all $CD122^+$, had divided in irradiated mice (49%).

Previously, we showed that both vaccine-induced proliferation of tumor-specific T cells and lymphopenia-driven proliferation of co-transferred T cells were IL-7 dependent, IL-15 appeared to play a minor role under these conditions. To examine the role of these two cytokines in lymphopenia-driven proliferation of $CD122^+$ and $CD122^-$ $CD8^+$ T cells, spleen cells were separated according to CD122 expression and labelled with CFSE before adoptive transfer into irradiated wt mice, irradiated IL-15 ko mice, and irradiated IL-15 ko mice injected with an IL-7 neutralizing antibody (Figure S3B). As expected, $CD122^+CD8^+$ T-cell proliferation in irradiated mice was IL-15 dependent; both the number of divisions and the absolute number of $CD8^+$ T cells were greatly reduced in IL-15 ko recipients (Figure S3C). IL-7 blockade diminished the division of $CD122^+$ T cells even further in IL-15 ko recipient mice but exhibited very little effect on the absolute number of $CD122^+$ T cells. This could be due to the low number of $CD122^+$ T cells that could be sustained in IL-15 ko mice; IL-15 is absolutely required for $CD122^+$ T cell renewal and survival. $CD122^+CD8^+$ T cells were rescued in IL-15 ko mice that over-express IL-7, both IL-7 and IL-15 jointly regulate lymphopenia-driven proliferation of $CD122^+CD8^+$ T cells. Somewhat surprisingly, $CD122^-$ $CD8^+$ T cells exhibited IL-7- and IL-15-independent proliferation (Figure 3B, left

panel); however, the number of these cells was much lower than that of $CD122^+$ $CD8^+$ T cells (Figure S3C). IL-7 and IL-15 independent proliferation of $CD4^+$ regulatory cells and a subset of $CD8^+$ memory T cells ($CD44^{hi}CD122^-$) was demonstrated previously.

Further support for the notion that $CD122^+CD8^+$ lymphocytes were the predominant population that underwent lymphopenia-driven proliferation was the finding that depletion $CD122^+$ cells dramatically reduced the number of $CD8^+$ T cells recovered from irradiated mice (Figure S3D). Based on the CFSE dilution assay both NK cells and $CD122^-$ T cells exhibited a relatively moderate degree of cells division; however their contribution to the total number of cells was minor compared to $CD122^+CD8^+$ T cells (Figure S3E). The number of $CD8^+$ T cells in irradiated mice greatly exceeded that of NK cells and the difference increased continually from week 1 to week 4.

Co-transfer of CD122 depleted spleen cells did not suppress pmel-1 mediated antitumor response

Previously, pmel-1 spleen cells were always transferred together with naïve congenic spleen cells. To determine whether co-transfer of CD122depleted spleen cells could affect pmel-1 mediated tumor regression, we treated melanoma-bearing mice with irradiation, adoptive transfer of either 10^4 naïve pmel-1 spleen cells or pmel-1 spleen cells plus 10^7 CD122 depleted spleen cells (sFig. 4). Melanom-bearing mice received irradiation alone or adoptive transfer of CD122 depleted spleen cells were included as control mice. Consistent with the notion that $CD122^+$ spleen cells are the main subset of suppressor cells in our adoptive immunotherapy model, co-transfer of CD122 depleted spleen had no effect on pmel-1 T-cell mediated tumor regression.

Supplementary Methods

Development of Murine T-Cell Staining Panel for Flow Cytometry

Fluorescent antibodies were purchased or fluorescenated in-house with the use of commercially available QDot antibody conjugation kits (Invitrogen, Molecular Probes, Eugene, OR). The staining panel consisted us the following: TCRβ chain-FITC, (Becton Dickinson, Pharmingen, San Diego, CA), CD25-PE, (eBioscience, San Diego, CA), CD8α-PE-TR, (Invitrogen, Caltag, Burlingame, CA), CD19-PE-Cy5 (eBioscience, San Diego, CA) F4/80-PE-Cy5 (eBioscience, San Diego, CA), NK1.1-PE-Cy7, (Becton, Dickinson, Pharmingen, San Diego, CA), CD45.2-APC (eBioscience, San Diego, CA), CD4-APC-Cy7, (eBioscience, San Diego, CA), CD122-biotin (eBioscience, San Diego, CA), Sav-Qdot 605 (Invitrongen, Molecular Probes, Eugene, OR), The pairing of flurochromes to antibodies was determined by establishing staining profiles of each antibody to allow for detection of bright, dim, and negative populations. Spectral overlaps between fluorescent dyes were also considered. Each fluorescent antibody was tittered carefully at optimal PMT voltage settings to determine the antibody concentration that produced fluorescence with minimal non-specific background staining. The use of a "dump cocktail" of CD19&F4/80-PE-Cy5 in combination with 5ug/ml of 7-amionactinomycin D (7-AAD) (Invitrogen, Molecular Probes, Eugene, Oregon) in 1X PBS was employed to stain cells with high cell surface Fc receptor mediated non-specific binding of antibodies, and to discriminate between live and dead cells. Bright, high-density surface markers were selected to counter broadening distributions of fluorescence in the far-red channels of PE-Cy7 and APC-Cy7. All samples were aquired using Summit 4.2 software on a Dako Cyan ADP Flow Cytometer. (Dako, Ft. Collins, CO). Data was analyzed using Winlist 5.0 software (Verity House Software, Topsham, ME) Computer-assisted digital compensation was performed using single-color staining controls via the Hyperlog transform. FMO ("Fluorescence minus one") controls were used to set hinged-gating and define histograms regions that distinguish positive from negative

events for experimental samples and fidelity controls. Fidelity controls were used to ensure that there was no loss of staining frequency and intensity between lower order panels and corresponding fluorescence for each mAb in the 8-color panel.

Supplementary Figure legend

Figure S1. Dose titration of spleens used to suppress vaccine-induced pmel-1 T cell expansion in lymphodepleted mice.

(A) C57BL/6 ($CD45.2^+$) mice were irradiated (500 rads) and adoptively transferred with indicated number of pmel-1/GFP double Tg spleen cells together with 10 million wt congenic spleen cells ($CD45.1^+$). Transferred mice were immediately vaccinated with $1x10^6$ DCs pulsed hgp-9 peptide.

(B) Experiments were repeated as above except that one million pmel-1 spleen cells and indicated number of congenic spleen cells were transferred. Blood was collected at day 14 after vaccination. The absolute number of pmel-1 T cells ($CD8^+GFP^+CD45.2^+$) in pooled blood samples (n=3-5) was determined by flow cytometry analysis and qualified with fluorescent beads and presented as the mean in the bar graphs. The number of pmel-1 T cells was significant different among different groups ($p < 0.05$, student's paired *t*-test). Data represent typical result from one of three independent experiments.

Figure S2. Reduced competition by cotranferred spleen cells of IL-15ko mice and lack of $CD8^+CD122^+$ T cells compared to wt mice.

(A) Reconstitution with wt but not IL-15ko spleen consistently inhibited the primary and secondary responses of pmel-1 T cells in irradiated mice. Irradiated mice (n=3~5) were adoptively reconstituted with 10^4 pmel-1 T cells alone or together with 5×10^7 spleen cells from wt mice or IL-15ko mice, followed immediately by peptide-pulsed DCs vaccination. Booster vaccinations were given 10 weeks after

adoptive transfer. Absolute number of pmel-1 T cells in blood samples at the indicated weeks was determined and presented in the graphs as the mean with the error bars (SEM). The number of pmel-1 T cells found in the blood at two wks post the primary DC vaccination or 3, 12 days post the secondary vaccination was significantly lower in blood from mice co-transferred with wt spleen than IL-15 ko spleen cells or pmel-1 only transfer ($p < 0.05$, student paired *t*-test).

(B) The IL-15ko mice had reduced number of $CD8^+$ T cells and NK cells, but normal number of $CD4^+$ T cells. Blood from wt, IL-15ko, and irradiated mice (n=5) were stained for CD4, CD8, and NK1.1 respectively.

(C) $CD8^+CD122^+$ T cells but not $CD4^+CD25^+$ regulatory T cells were significantly reduced in blood from IL-15 ko mice. Blood from wt, IL-15ko, irradiated wt mice (n=5) were stained for CD4, CD8, CD122, and NK1.1 respectively and the absolute numbers of designated cells was determined.

(D) Multiple color flow cytometry analysis of spleens of nonirradiaed wt, irradiated wt, and IL-15ko mice (n = 5). Spleens collected from wt, IL-15 KO and 3-day post irradiation mice (500 rads) were stained for CD4, CD8, CD11b, CD19, CD25, CD122 and NK1.1, TCRβ and analysed.

Figure S3. $CD8^+CD122^+$ memory-like T cells are the major population that undergo lymphopenia-driven proliferation.

(A) $CD8^+CD122^+$ memory-like T cells have more extensive division upon adoptive transfer into irradiated mice than $CD122^-CD8^+$ naïve T cells or NK cells. Total or CD122 depleted congenic spleen cells (5×10^6) were labelled with CFSE and adoptively transferred into irradiated mice. Splenocytes from indicated mice were stained with CD45.1 and CD8 or NK1.1 and the CFSE profile of corresponding cells were determined one-week post transfer.

(B and C) Lymphopenia-driven proliferation of $CD8^+CD122^+$ memory-like T cells were IL-15 and IL-7 dependent. Congenic spleen cells were separated into $CD122^+$ and $CD122^-$ populations with anti-CD122

antibody and MACS beads as above. $CD122^+$ or $CD122^-$ cells containing equal number of CD8 T cells were CFSE-labeled and adoptively transferred into irradiated B6 mice, irradiated IL-15ko mice with or without treatment of anti-IL-7 Ab, or nonirradiated control mice, respectively. Two weeks later, splenocytes from treated mice were gated on $CD45.1^+$ $CD8^+$ T cells, and then analysed for CFSE division (B) and absolute number per 10^3 plenocytes (C).

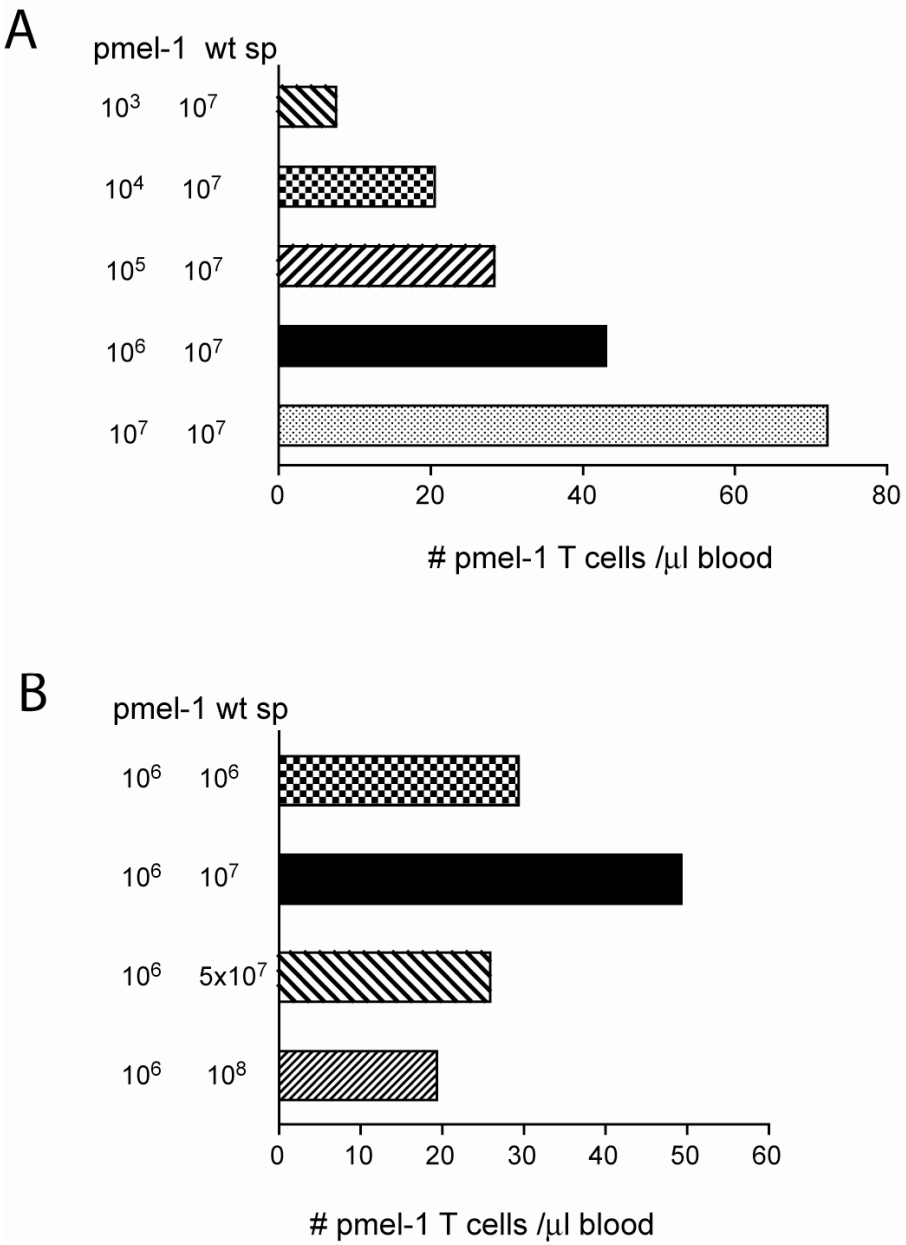
(D and E) $CD8^+$ T cells account for the majority of lymphopenia-driven proliferation and exhibited greater lymphopenia-driven proliferation than NK cells. Congenic spleen cells were transferred into irradiated mice and the number of congenic $CD8^+$ T cells and NK cells recovered in blood were determined at indicated times after adoptive transfer. Irradiated mice (n=5) were adoptively reconstituted with $10^7$of either congenic splenocytes or with depletion of $CD122^+$ cells, respectively. Blood samples were collected from each group at the indicated weeks. Absolute number of congenic CD8 T cells from mice in the indicated groups (D), congenic NK cell and CD8 T cells from mice transferred with congenic splenocytes (E) were determined and presented in the graphs as the mean with the error bars (SEM). The numbers in (D and E) were significantly different at all time points examined ($p < 0.05$, student paired *t*-test).

Figure S4. Adoptive immunotherapy with co-tranasfer of pmel-1 and CD122 depleted spleen cells.

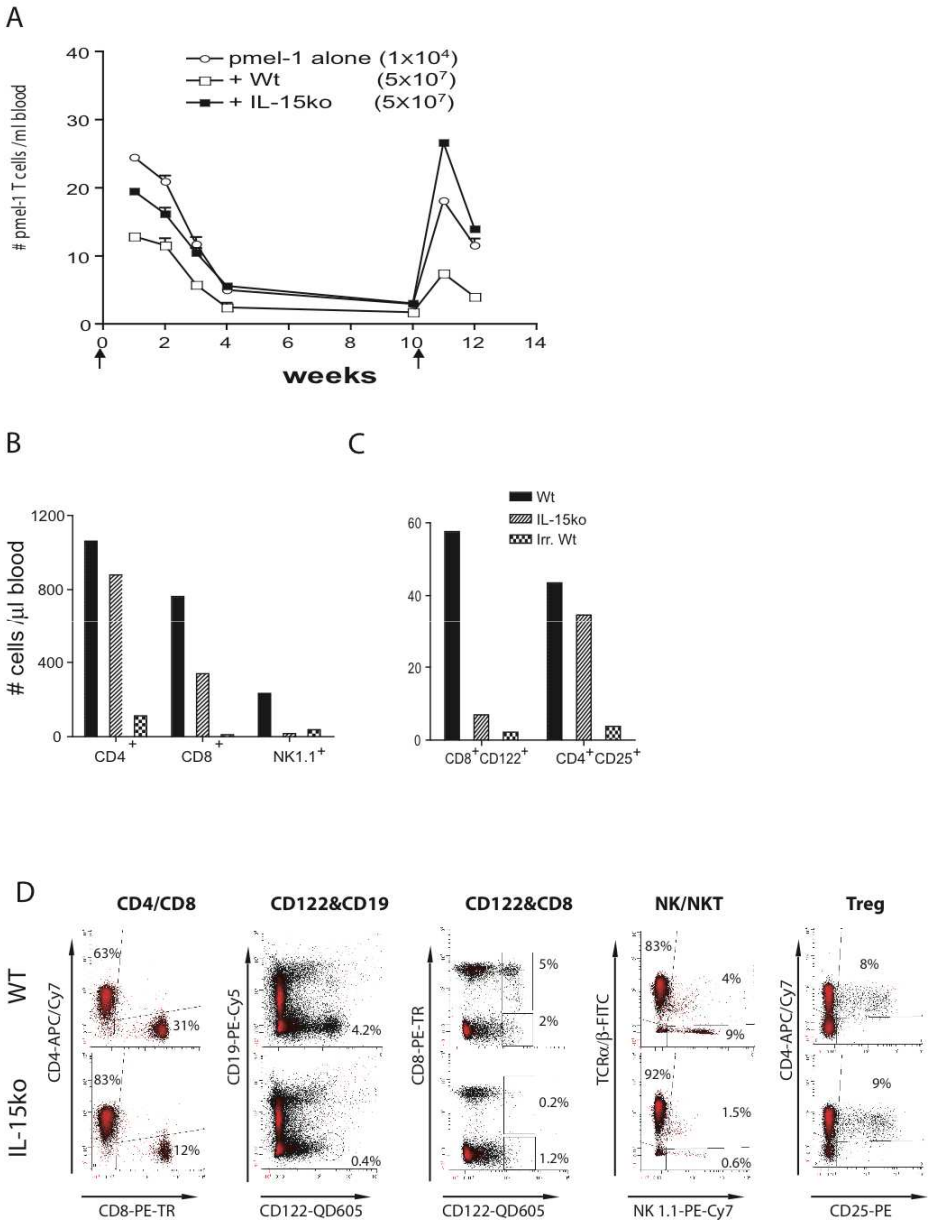
Naive C57BL/6 mice (n = 6 per group) were injected with 2×10^5 B16-F10 tumor cells s.c. at day 0. Five days later, the tumor-bearing mice were sublethally irradiated. At day 6 after F10 inoculation, irradiated mice were adoptively transferred with 10^4 pmel-1 T cells alone, or together with 10^7 of CD122 depleted spleen cells, in conjunction with peptide-pulsed DC vaccination at day 6 after F10 inoculation. Tumor growth and survival of mice were measured and monitored. There is a significant difference between control mice and mice treated with either pmel-1 T cells alone or pmel-1 T cells together with CD122 depleted spleen cells by log-rank (Mantel-Cox) test (P = 0.0012). There is no significant difference

between control mice and mice treated with CD122 depleted spleen cells, or mice treated with pmel-1 T cells and pmel-1 T cells together with CD122 depleted spleen cells.

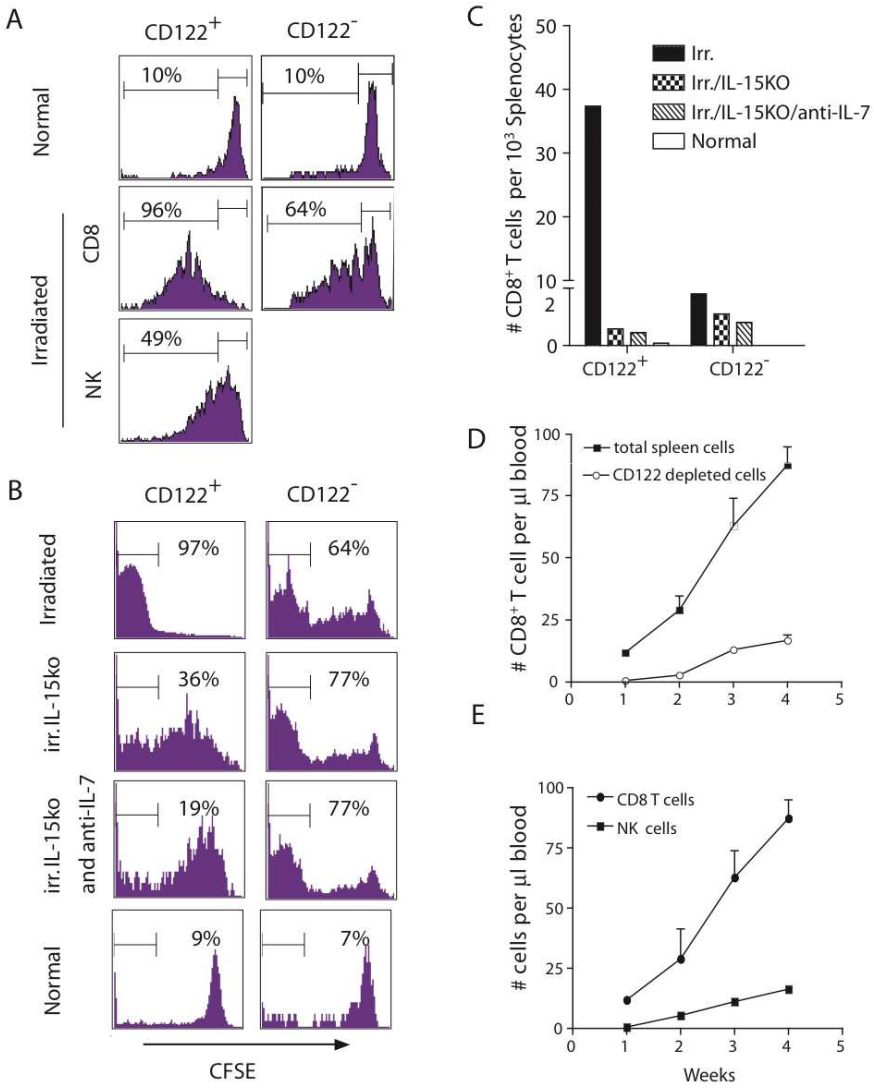
sFig1.


A
pmel-1 wt sp
10^3 10^7
10^4 10^7
10^5 10^7
10^6 10^7
10^7 10^7
0
20
40
60
80
pmel-1 T cells /µl blood
B
pmel-1 wt sp
10^6 10^6
10^6 10^7
10^6 5x10^7
10^6 10^8
0
10
20
30
40
50
60
pmel-1 T cells /µl blood

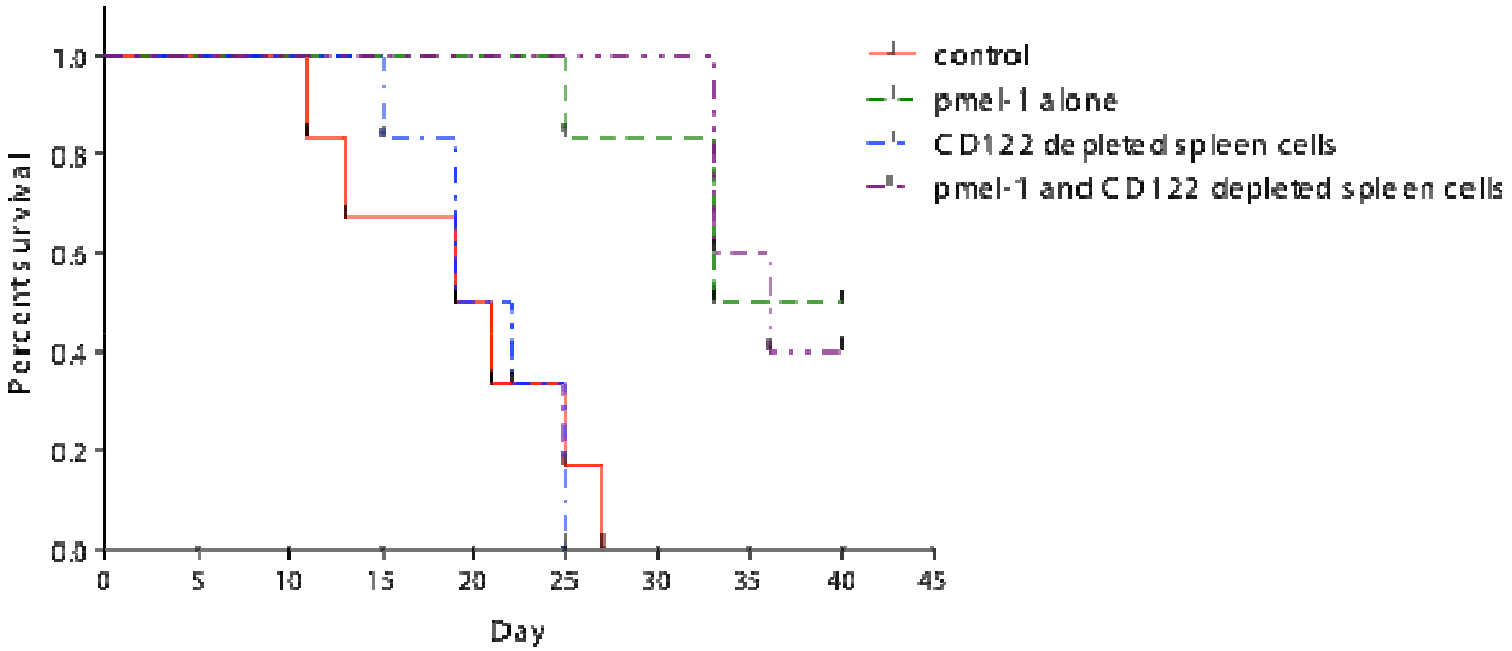
sFig2.


A
pmel-1 alone (1x10⁴)
+ Wt (5x10⁷)
+ IL-15ko (5x10⁷)
# pmel-1 T cells /ml blood
weeks
B
cells /µl blood
CD4+
CD8+
NK1.1+
C
Wt
IL-15ko
Irr. Wt
CD8+CD122+
CD4+CD25+
D
CD4/CD8
CD122&CD19
CD122&CD8
NK/NKT
Treg
WT
IL-15ko
CD4-APC/Cy7
CD8-PE-TR
CD19-PE-Cy5
CD122-QD605
TCRα/β-FITC
NK 1.1-PE-Cy7
CD25-PE

sFig3.


A
CD122+
CD122-
Normal
10%
10%
Irradiated
CD8
96%
64%
NK
49%
B
CD122+
CD122-
Irradiated
97%
64%
irr.IL-15ko
36%
77%
irr.IL-15ko and anti-IL-7
19%
77%
Normal
9%
7%
CFSE
C
CD8+ T cells per 10^3 Splenocytes
Irr.
Irr./IL-15KO
Irr./IL-15KO/anti-IL-7
Normal
CD122+
CD122-
D
CD8+ T cell per µl blood
total spleen cells
CD122 depleted cells
E
cells per µl blood
CD8 T cells
NK cells
Weeks

sFig4.


Percent survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
45
Day
control
pmel-1 alone
CD122 depleted spleen cells
pmel-1 and CD122 depleted spleen cells