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3 Immune-mediated antitumor effect of a transplanted lymph node

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7

8 (8) Novelty & Impact Statement

9 In the treatment of melanoma, lymph node (LN) dissection is necessarily performed for
10 patients with LN metastasis. However, inadvertent damage to lymphoid organs by LN
11 dissection can cause intractable lymphedema and decrease antitumor immunity. Here,
12 the authors demonstrated that a transplanted LN served as a tumor-draining LN and
13 exerted an immune-mediated antitumor effect. Following local tumor control by
14 surgical resection, an additional lymph node transplant might be practical for preventing
15 lymphedema and reduced immunity.

16

1 **Abstract**

2 Lymph node (LN) transplantation is a recognized method for reconstruction of the
3 lymphatic system and is used in the clinical setting to treat lymphedema. However, it is
4 unclear whether transplanted LNs contribute to immune surveillance. In this study, we
5 investigated whether a single transplanted non-vascularized LN, defined as a
6 tumor-draining transplanted lymph node (TDTLN), could exert an immune-mediated
7 antitumor effect. LN and lung metastases and primary tumor enlargement were
8 evaluated in mice that were inoculated with B16-F10-luc2 melanoma cells in a hindlimb
9 footpad without (group 1) and with (group 2) popliteal lymph node (PLN) resection and
10 in mice that underwent LN transplantation after PLN resection (group 3). The function
11 of a TDTLN (group 3) and a tumor-draining popliteal lymph node (TDPLN; group 1)
12 was evaluated in the context of cancer. LN and lung metastases were significantly
13 aggravated by PLN resection but were significantly decreased by LN transplantation.
14 Immunohistochemistry showed that the TDTLNs retained T-cells and B-cells and
15 fluorescence-activated cell sorting analysis confirmed expansion of lymphocytes in
16 these nodes; however, the degree of expansion in TDTLNs was different from that in

1 TDPLNs. Expression of cytokines associated with immunostimulation was confirmed in
2 the TDTLNs as well as in the TDPLNs. One of the differences in the immune-mediated
3 antitumor effect of the TDPLNs and TDTLNs was ascribed to a difference in the site of
4 lymphocyte homing to peripheral LNs through high endothelial venules.
5 Non-vascularized LN transplantation had an immune-mediated antitumor effect.
6

1 **Introduction**

2 The lymphatic system consists of lymphatic vessels and lymphoid organs of the
3 immune system, including lymph nodes (LNs). The main function of the lymphatic
4 vessels in the lymphatic system is to collect excess protein-rich fluid that has
5 extravasated from blood vessels and transport it back into the blood circulation.
6 Therefore, impairment of lymphatic transport can cause accumulation of lymph in
7 tissues, tissue swelling, and fibrosis, known as lymphedema. However, the lymphatic
8 vessels also have an important role in immune surveillance, in that they import various
9 antigens and activated antigen-presenting cells into the LNs and export immune effector
10 cells and humoral response factors into the blood circulation (1, 2). Sentinel LNs are the
11 first to receive lymph from a primary tumor and are the preferential sites of initial tumor
12 metastases. They are also strongly influenced by tumor-derived factors, such as
13 cytokines (3). Therefore, impairment of these lymphoid organs can decrease antitumor
14 immunity (4, 5).

15 The standard strategy used to manage lymphedema is decongestion, which consists of
16 exercise, wearing a compression garment, skin care, and manual lymph drainage.

1 Surgery can be an option in resistant and/or severe cases (6). Lymphovenous
2 anastomosis using a super-microsurgical technique creates another route from the
3 lymphatics to the vein in the peripheral region of the affected limb (7). Recently,
4 vascularized LN transfer has emerged as a physiologic surgical treatment for
5 lymphedema. The goal of this procedure is to provide a bridge between the lymphatic
6 system distal and proximal to the LN dissection. Becker et al. have reported promising
7 outcomes in the clinical setting (8). There is also considerable evidence from animal
8 models showing the effectiveness of LN transplantation (9). However, this technique is
9 still in the exploratory stages and there is no direct evidence of how LN transplantation
10 acts on the lymphatic system, so its effect on immunity in particular is still unclear.

11 Kwon et al. showed that resection of a sentinel popliteal lymph node (PLN) was
12 associated with changes in the lymphatic drainage pathway, and thus the pathway for
13 dissemination of tumor cells (10). It is very important to consider changes in the
14 lymphatic pathway of tumor invasion and metastasis after damage to lymphatic vessels,
15 as occurs with LN dissection, because the lymphatic vessels provide a route for tumor
16 cells to metastasize (1). Thus, reversal of any changes in the lymphatic drainage

1 pathway could reduce the risk of dissemination of tumor cells. We recently reported the
2 possibility that LN transplantation could promote reconnection of lymphatic vessels and
3 restore lymphatic flow (11).

4 The aims of this study were to evaluate whether changes in lymphatic flow can affect
5 the dynamics of tumor cells and if LN transplantation can reduce the risk of
6 dissemination of these cells by reversing the changes in lymphatic flow that could likely
7 occur after LN dissection. We also compared the ability of a tumor-draining
8 transplanted lymph node (TDTLN) to exert an immune-mediated antitumor effect with
9 that of a tumor-draining popliteal lymph node (TDPLN) functioning as a sentinel LN in
10 a mouse model of melanoma.

11

12 **Materials and Methods**

13 *Experimental model*

14 Male C57BL/6N mice (age 6–8 weeks, weight 18–20 g) were sourced from Sankyo
15 Labo Service (Tokyo, Japan). All experiments were performed under general anesthesia.
16 The surgical procedure was performed as previously reported (11). The mice were

1 allocated to serve as controls (group 1), undergo resection of a single PLN (group 2), or
2 to receive a single autotransplanted, non-vascularized, resected PLN (group 3). The
3 protocols used in this study were approved by the Institutional Animal Care and Use
4 Committee, Hokkaido University School of Medicine and conducted in accordance with
5 the Guidelines for the Care and Use of Laboratory Animals, Hokkaido University.

6

7 *Cell lines*

8 The B16-F10-luc2 cell line was used to create the melanoma tumors (Caliper Life
9 Sciences Inc., Hopkinton, MA). This luciferase-expressing cell line is stably transfected
10 with the firefly luciferase gene (luc2). The parental line was B16-F10 (American Type
11 Culture Collection [ATCC, Rockville, MD]), and B16-F10-luc2 was established by
12 transduction of a lentivirus containing the luc2 gene under control of the human
13 ubiquitin C promoter. Red fluorescent protein (RFP)-expressing cells (B16-F10-RFP)
14 were prepared by transduction with a pFB-Neo retroviral vector (Stratagene, La Jolla,
15 CA) containing the gene for *Discosoma* sp. RFP (DsRed, Clontech, Mountain View,
16 CA).

1 A cell suspension of the B16-F10-luc2 and B16-F10-RFP cells was injected into a
2 hindlimb footpad at a dose of 4×10^5 in 0.05 mL of PBS to create the B16-F10
3 melanoma hindlimb footpad model. Saline was injected into the hindlimb footpad as the
4 control.

5 In group 1, the PLN receiving direct lymphatic drainage after tumor inoculation was
6 designated the TDPLN and in group 3 the LN transplanted to the area of the PLN was
7 designated the TDTLN. The PLN in the hindlimb opposite to the side on which the
8 footpad was inoculated with tumor cells was designated the non-TDPLN. PLNs from
9 non-tumor-bearing mice were designated as naïve popliteal lymph nodes (NPLNs).

10

11 *In vivo optical imaging*

12 Twelve mice in each group were used for in vivo bioluminescence imaging, and
13 representative images are shown in Fig. 1. Before imaging, the mice were anesthetized
14 with 2.5% isoflurane, after which 150 mg/mL of in vivo-grade D-luciferin (VivoGlo
15 Luciferin, Promega, Madison, WI) in PBS was injected intraperitoneally at a dose of
16 150 mg/kg body weight. Images were obtained using a Lumazone bioluminescence

1 imager (Nippon Roper, Tokyo, Japan) with a 1-min exposure time. The fluorescence
2 intensity values correlate with tumor volume and weight and allow for reliable and
3 robust quantification of the entire tumor compartment (12). The emission intensity of
4 bioluminescence was taken as the integrated value of all photographs detected in the
5 target area (of tumor growth in the hindlimb). Detection of bioluminescence was
6 performed 4 weeks after tumor implantation. The data were analyzed using SlideBook
7 software (Intelligent Innovations Inc., Denver, CO).

8

9 *Luciferase assay*

10 The PLNs (transplanted LNs in group 3), inguinal and axillary LNs, and the lungs of
11 mice that were inoculated with B16-F10-luc2 cells were harvested from each of the 12
12 mice in groups 1, 2, and 3 at 5 weeks after tumor induction for the ex vivo luciferase
13 reporter assay. Harvested organs were crushed at 3500 rpm for 15 s with a
14 5.5-mm-diameter stainless steel bead using a Micro Smash MS-100 centrifuge (Tomy
15 Company Ltd, Tokyo, Japan) and lysed in Glo lysis buffer. The cell lysate was
16 centrifuged at 13,000 rpm at 4 °C, and the supernatant was collected. Luciferase activity

1 was assayed using a GloMax 20/20 luminometer (Promega), following the previously
2 reported technique (4).

3

4 *Confocal microscopy*

5 In groups 1 and 3, 10 μ L of 10 mg/mL fluorescein isothiocyanate-dextran (molecular
6 weight 2000 kD; Molecular Probes Inc., Eugene, OR) were injected subcutaneously into
7 the footpad of each hindlimb at 2, 3, 4 weeks after the B16-F10-RFP tumor induction
8 surgery. Dextran of this size is too large to enter the bloodstream but does enter the
9 lymphatic vessels, so is used widely in studies of the lymphatic system (13). The
10 TDTLNs and TDPLNs were then removed, embedded in tissue-freezing medium, and
11 flash-frozen in partially frozen isopentane. Cryosections (30 μ m thick) were obtained
12 from each specimen for scanning and observation by confocal microscopy.

13

14 *Histology*

15 Four weeks after tumor induction surgery, the NPLNs, TDTLNs, and TDPLNs on the
16 left side were removed, fixed in 4% paraformaldehyde, and embedded in paraffin.

1 Sections (4 μ m thick) were then incubated with purified rat anti-mouse CD3 (CD3-12;
2 GeneTex, Irvine, CA), purified rat anti-mouse CD45R/B220 (RA3-6B2; WuXi
3 Biosciences (now 'WuXi AppTec, San Diego, CA), and rabbit anti-mouse lymphatic
4 vessel endothelial receptor 1 (LYVE-1) antibody (Abcam, Cambridge, MA),
5 respectively.

6 Two weeks after tumor induction surgery, the NPLNs, TDTLNs, and TDPLNs were
7 removed as above to assess the high endothelial venules (HEVs) of the PLNs in the
8 pre-metastatic state. Sections were incubated with purified rat anti-mouse MECA-79
9 (BD Biosciences, San Jose, CA).

10

11 *RNA isolation and quantitative reverse transcription polymerase chain reaction*
12 *analysis*

13 Total RNA was extracted from the frozen NPLN, TDTLN, and TDPLN tissues (n = 6–7
14 per group) at 1, 2, 3, and 4 weeks after tumor induction using a RNeasy Fibrous Tissue
15 Mini Kit (QIAGEN, Hilden, Germany) and then reverse transcribed to cDNA using an
16 iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the

1 manufacturers' protocols. Real-time reverse transcription polymerase chain reaction
2 (RT-PCR) was performed using Fast SYBR Green Master Mix (Applied Biosystems,
3 Foster City, CA) on a StepOnePlus Real-Time PCR system (Applied Biosystems). The
4 primer pair sequences are listed in Supplementary Table S1. Primers for interferon
5 gamma (IFN- γ), interleukin (IL)-2, IL-4, IL-10, IL-21, tyrosinase-related protein 1
6 (TYRP1), and hypoxanthine phosphoribosyl transferase (HPRT) were purchased from
7 Takara Bio (Mountain View, CA). The TYRP1 gene was selected as a representative
8 melanocyte/melanoma-specific marker to identify melanoma cells that had metastasized
9 to the draining LNs (14).

10 Relative expression of the PCR products was determined using the $\Delta\Delta C_t$ method. The
11 results are represented as the fold change as compared with the NPLNs. Each sample
12 was examined in triplicate.

13

14 *Flow cytometry and cell sorting*

15 Single-cell suspensions were prepared from the NPLNs, TDTLNs, and TDPLNs (n = 18
16 mice per group) at 4 weeks after tumor induction surgery by standard gentle mechanical

1 disruption and filtered through a sterile 75- μ m nylon mesh. NPLNs rather than
2 non-TDPLNs were used for the control because non-TDPLNs on the non-injected side
3 were affected by the systemic immune response, indicating a change in the cell
4 populations in the LNs (Supplementary Fig. S1). In addition, splenocytes were
5 harvested simultaneously in groups 3 and 1 (n = 12 mice per group). The LN cells and
6 splenocytes were washed and resuspended in PBS containing 0.2% bovine serum
7 albumin (BSA). The number of lymphocytes was calculated using the cell counter. The
8 cells were incubated with the following fluorescently conjugated antibodies: CD4, CD8,
9 CD25, CD44, CD62L, CD 127, B220, CXCR5, PD-1 (all antibodies from BD
10 Biosciences). Assessment of the combined expression of CXCR5 and PD-1 is the
11 technique most commonly used to identify follicular helper T-cells (15). CD 127 was
12 used as a surface marker of regulatory T cells (16).

13 The samples were run on a FACS Aria III sorter (BD Biosciences). Identically
14 conjugated isotype controls were utilized. The stained cells were analyzed using a BD
15 LSR flow cytometer (BD Biosciences) and WinList software (Verity Software House
16 Inc., Topsham, ME). Cell sorting was performed using the FACS Aria III sorter, the

1 enzyme-linked immunospot (ELISpot) assay, and the LIVE/ DEAD cytotoxicity assay.

2

3 *ELISpot assay*

4 The ELISpot assay was performed using a mouse IL-2 single color ELISpot kit

5 (Cellular Technology Ltd., Cleveland, OH) according to the manufacturer's instructions.

6 CD4⁺ T lymphocytes sorted from the NPLNs, TDTLNs, and TDPLNs at 4 weeks after

7 tumor induction (n = 7 mice per group) were added (1×10^5 cells/well) to plates coated

8 with the cytokine-specific capture antibody with or without the T-cell mitogen,

9 concanavalin A (Sigma-Aldrich, St Louis, MO) (17). B16-F10-luc2 melanoma cells

10 were added (1×10^4 cells/well). The plate was scanned to count the spot-forming cells

11 (Immunospot[®] Cellular Technology Limited, Cleveland, OH).

12

13 *Cytotoxicity assay*

14 The TDTLNs and TDPLNs were harvested 4 weeks after tumor induction and

15 single-cell suspensions were prepared (n = 5 mice per each group). CD8⁺ T-cells were

16 sorted using the FACS Aria III cell sorter to evaluate the cytotoxic activity of the

1 antigen-specific T-cells.

2 Cytotoxicity was assayed using a cell-mediated cytotoxicity kit (Molecular Probes)

3 according to the manufacturer's instructions. Target B16-F10-luc2 cells were incubated

4 overnight at 37°C with a staining solution containing DiOC₁₈. The stained target cells (1

5 × 10⁶ cells/mL) were resuspended in complete culture medium and then mixed with

6 CD8⁺ T-cells to yield effector to target (E:T) ratios of 20:1 and 5:1. Cytolytic CD8⁺

7 T-cells were generated by activation of T-cells primed in vivo by concanavalin A as

8 described above. Fluorescence microscopy was used to evaluate cytolytic CD8⁺ T-cells.

9 The percentage of dead target cells in the presence of effector cells (+effectors)

10 corrected for spontaneous death of target cells in the absence of effector cells (–

11 effectors) is calculated according to the following equation:

12 Corrected % cytotoxicity = [(#G + R cells/#G cells)_{+effectors} – (#G + R cells/#G

13 cells)_{–effector}] × 100, where G indicates green and G + R indicate both green and red.

14

15 *Western blot analysis*

16 Protein was extracted from the TDTLN and TDPLN tissues (n = 7 mice per group) 2

1 weeks after tumor induction, frozen immediately in liquid nitrogen after isolation, and
2 then homogenized. Protein from the NPLN tissue was used as the control. Using a
3 previously reported method (18), total cellular protein extraction was performed and
4 proteins were separated by using sodium dodecyl sulfate polyacrylamide gel
5 electrophoresis. MECA-79 (Santa Cruz Biotechnology, Santa Cruz, CA) was used for
6 the primary antibody. Loading levels were equalized by β -actin (Cell Signaling
7 Technology, Beverly, MA). Image J software (National Institutes of Health, Bethesda,
8 MD) was used to determine the relative signal densities after normalization to β -actin.

9

10 *Enzyme-linked immunosorbent assay*

11 The LN tissue lysates were prepared as described earlier in the Western blot analysis
12 section. The CC-chemokine ligand 21 (CCL21) concentration in LN tissue was
13 estimated using a CCL21 enzyme-linked immunosorbent assay (ELISA) kit (R&D
14 Systems, Minneapolis, MN) in accordance with the manufacturer's instructions and
15 calculated according to the standard curve generated.

16

1 *Statistical analysis*

2 The Student's *t*-test was used to compare differences between two groups. Multi-group
3 comparisons were performed using two-way analysis of variance with the Tukey–
4 Kramer post-hoc test. The Wilcoxon rank-sum test was used unless the assumption of
5 normal distribution was required. A P-value < 0.05 was considered statistically
6 significant. In the bar graphs, the bars indicate the means and the error bars indicate the
7 standard error of the mean. In the box-and-whisker plots, the boxes represent the
8 medians with upper and lower quartiles and the whiskers represent maximum and
9 minimum values. The statistical analysis was performed using JMP 13.0 software (SAS
10 Institute Inc., Cary, NC).

12 **Results**

13 *LN and lung metastases were increased by LN resection and decreased by LN* 14 *transplantation*

15 In a previous study we found that resection of a PLN led to a change in lymphatic flow
16 and LN transplantation to the area of the resected PLN reversed this change (Fig. 1a).

1 All mice allocated to serve as controls (group 1), undergo resection of a single PLN
2 (group 2), or receive a single autotransplanted, non-vascularized, resected PLN (group
3 3) were inoculated with melanoma cells.

4 Fluorescence intensity values correlate with tumor volume and weight, so the tumors
5 produced by luciferase-expressing B16-F10-luc2 melanoma cells transplanted into the
6 left hindlimb footpad were imaged in all three groups of mice using bioluminescence
7 imaging. Four weeks after tumor inoculation, there were no intergroup differences in
8 tumor volume or weight (Fig. 1b and 1c).

9 To determine the total burden of tumor metastasis to the lung, inguinal LNs, and
10 axillary LNs, whole tissue was homogenized and total luciferase activity in the tissue
11 extracts was examined using luminometry. In group 2, macroscopic evaluation
12 confirmed an increase in size and a color change to black in the inguinal LN tissue,
13 which was thought to reflect the change in lymphatic flow (Fig. 1a). Also, the axillary
14 LN tissue in group 2 was slightly expanded when compared with that in groups 1 and 3.

15 The relative light units for the total lung, inguinal LN, and axillary LN tissues were
16 significantly higher in mice that had undergone LN resection compared with the other

1 groups ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively; Fig. 1d), indicating that these
2 mice had a greater burden of lung and LN metastases. Furthermore, mice that had
3 undergone LN transplantation had a significantly decreased burden of LN metastases (P
4 < 0.05). The burden of lung metastases also tended to be less, but the difference in
5 comparison with groups 2 and 3 was not statistically significant.

6

7 ***LN transplantation had antitumor activity in the lymphatic drainage basin***

8 Tumor-draining lymph nodes (TDLN) play a central role in orchestrating the immune
9 response against melanoma. The B16-F10 melanoma hindlimb footpad model enables
10 evaluation of tumor-specific changes in TDPLNs and TDTLNs when compared with
11 NPLNs injected with saline only.

12 On macroscopic evaluation, the TDPLNs were enlarged in response to the melanoma
13 antigen (Fig. 2a). This enlargement was also observed in TDTLNs, although these
14 nodes were smaller than the TDPLNs. This macroscopic difference was in accordance
15 with the total luciferase activity in TDPLN, TDTLN, and NPLN tissue (Fig. 2a).

16 Although TDTLNs showed a statistically significant increase in burden of LN

1 metastasis when compared with NPLN, a large difference in metastatic burden was also
2 confirmed between the TDTLNs and TDPLNs (indicated by the overwhelmingly greater
3 number of relative light units for TDPLNs than for TDTLNs).

4 As a tumor progresses, its microenvironment becomes increasingly
5 immunosuppressive, supporting outgrowth of metastases (3, 19). In our mouse model,
6 metastases to TDPLNs were seen about 3 weeks after tumor induction, and these
7 metastases gradually expanded thereafter. This expansion was detected by confocal
8 microscopy and relative TYRP1 mRNA expression (Fig. 2b and 2c). Tumor progression
9 was confirmed in TDTLNs about 4 weeks after tumor inoculation, reflecting a change
10 in the immunologic microenvironment, accounting for the difference between TDTLNs
11 and TDPLNs.

12

13 ***Lymphocyte expansion with a change in lymphocyte population following an adaptive***
14 ***immune reaction after LN transplantation***

15 On macroscopic evaluation 4 weeks after tumor inoculation, the diameters of the
16 TDPLNs, TDTLNs and NPLNs were approximately 3, 1.5, and 1 mm, respectively (Fig.

1 3a).

2 The total number of lymphocytes in the TDTLNs was 7.7-fold that in NPLNs;

3 however, the total number in the TDPLNs was 28.7-fold that in the NPLNs (Fig. 3b).

4 The total number of B lymphocytes in the TDTLNs was 9.4-fold that in the NPLNs,

5 whereas that in the TDPLNs was 45.6-fold that in the NPLNs. The total number of T

6 lymphocytes in the TDTLNs was 8.6-fold that in the NPLNs, and that in the TDPLNs

7 was 27.3-fold that in the NPLNs.

8 The change in the populations of B and T lymphocytes, including CD4⁺ (helper) and

9 CD8⁺ (killer) T lymphocytes in the LNs, was confirmed by fluorescence-activated cell

10 sorting (FACS) analysis (Fig. 3c). The greater expansion of B lymphocytes following an

11 adaptive immune response when compared with T lymphocytes was confirmed in the

12 TDTLNs as well as in the TDPLNs. In contrast, the T lymphocyte population decreased

13 in the TDTLNs as well as in the TDPLNs. Although the total T lymphocyte population

14 was decreased, the population of killer T-cells and helper T-cells was also changed in

15 both the TDTLNs and TDPLNs. The populations of naïve helper and killer T

16 lymphocytes were also examined. Interestingly, a statistically significant decrease in the

1 number of naïve helper T lymphocytes was confirmed in both TDTLNs and TDPLNs in
2 comparison with NPLNs ($P < 0.05$). This finding could indicate that there were more
3 diverse memory/effector populations in both TDTLNs and TDPLNs.

4 The TDTLNs were stained with antibodies against T-cells (CD3), B-cells (B220), and
5 lymphatic endothelial cells (LYVE-1) to determine if the components of the LNs
6 changed after the adaptive immune reaction when compared with the TDPLNs (Fig. 3d).

7 The T-cell populations were distributed in the T-cell zones of the paracortex and the
8 B-cell populations in the B-cell follicles of the cortex. However, the number of
9 lymphatic endothelial cells appeared to be increased and had an irregular appearance,
10 reflecting lymphatic regeneration.

11

12 *Transplanted LNs served as a secondary lymphoid organ in the immune response via*

13 ***CD4 T-cell activation***

14 We examined the IFN- γ , IL-2, IL-4, IL-10, and IL-21 levels produced by the immune
15 reaction in both TDTLNs and TDPLNs by quantitative RT-PCR after tumor antigen
16 presentation. It has been suggested that increased secretion of cytokines is induced in

1 sentinel LNs that are melanoma-free (19, 20), so we harvested these LNs, which were
2 tumor-free based on the above data (Fig. 2b and 2c), 2 weeks after tumor inoculation.
3 Higher IFN- γ mRNA level was observed in TDTLNs than in NPLNs (Fig. 4a); IL-4
4 mRNA level was similarly higher. In contrast, mRNA level of IL-2 was lower in both
5 TDTLNs and TDPLNs.

6 The ELISpot assay was used to ascertain the secretion of IL-2 at the cellular level by
7 monitoring cell-mediated immunity in both TDTLNs and TDPLNs (Fig. 4b). An
8 individual cytokine-producing cell was represented by a blue-colored end product
9 (Supplementary Fig. S2). None of the lymphocytes in the NPLNs, TDTLNs, or
10 TDPLNs produced IL-2 in the absence of the T-cell mitogen; on addition of
11 concanavalin A, lymphocytes in the TDTLNs produced more IL-2 (23.8 ± 3.2
12 spot-forming cells per 10^5 cells) than the lymphocytes in the NPLNs (5.6 ± 1.7
13 spot-forming cells per 10^5 cells) and less IL-2 than the lymphocytes in the TDPLNs
14 (128.7 ± 70.2 spot-forming cells per 10^5 cells; $n = 5$). On addition of melanoma cells,
15 lymphocytes in the TPLNs, TDTLNs, and TDPLNs produced more IL-2 (43.4 ± 9.2
16 spot-forming cells per 10^5 cells in TDTLNs, 12.4 ± 1.5 spot-forming cells per 10^5 cells

1 in NPLNs, and 174.3 ± 84.0 spot-forming cells per 10^5 cells in TDPLNs).

2 We observed that expression of IL-21 was higher in TDTLNs and TDPLNs than in
3 NPLNs although these findings were not statistically significant (Fig. 4a). Interestingly,
4 FACS analysis did not detect any follicular helper $CD4^+$ T (T_{fh}) cells in either the
5 TDTLNs or the TDPLNs (Fig. 4c). In contrast, regulatory helper $CD4^+$ T (T_{reg}) cells
6 were identified in both TDTLNs and TDPLNs with a significant difference between
7 them (Fig. 4d). Higher IL-10 mRNA level was observed in TDPLNs than in TDTLNs
8 (Fig. 4a).

9

10 ***Transplanted LN functioned as a secondary lymphoid organ in the immune response***
11 ***via CD8 T-cell activation***

12 The most direct way to analyze the activity of tumor-reactive T-cells is to extract these
13 cells and transfer them to the tumor itself. This is possible because the melanoma
14 antigens most commonly recognized are not tumor-specific, but rather are
15 tissue-specific melanocyte antigens, such as tyrosinase, MART-1/MelanA, and
16 gp100(21). $CD8^+$ killer T-cells can destroy target cells without requiring any additional

1 accessory signals, which are usually necessary for these cells to become primed (22). To
2 examine cytotoxic T-cell responses, we harvested TDTLNs and TDPLNs 28 days after
3 subcutaneous injection of melanoma cells, and cell suspensions from these organs were
4 tested for lytic activity against the tumor cells. Dead target cells are represented as green
5 cells with red-colored nuclei, whereas live target cells are represented as entirely green
6 cells (Supplementary Fig. S3). CD8⁺ T-cells purified from TDTLNs were cytotoxic to
7 melanoma cells as were those from TDPLNs, although the cytotoxic activity of CD8⁺
8 T-cells in TDTLNs was significantly decreased at an E:T ratio of 20:1 ($P < 0.05$; Fig.
9 4e).

10

11 ***The spleen could also be affected as a secondary lymphoid organ by the changed***
12 ***local immune response***

13 The T-cell and B-cell populations in the spleens from tumor-bearing mice were
14 investigated to determine if lymphoid organs other than TDLNs show lymphocytes
15 alterations in the hindlimb footpad melanoma model.

16 The population of B-cells in the spleens of mice with TDTLN was $56.9\% \pm 4.3\%$ and

1 that in mice with a TDPLN was $61.9\% \pm 4.4\%$ (Fig. 5); the difference was statistically
2 significant ($P < 0.01$, Student's *t*-test). There was also a statistically significant
3 difference between the T-cell populations in the spleens of mice with TDTLN and those
4 with TDPLN ($33.3\% \pm 4.1\%$ vs. $28.5\% \pm 5.8\%$, $P < 0.05$). In mice with TDTLN, the
5 expansion of B-cells was less activated than in mice with TLPLN. There was no
6 significant difference in the populations of $CD4^+$ helper T-cells, $CD8^+$ killer T-cells,
7 effector memory, central memory, or naïve $CD4^+$ helper T-cells between the two groups,
8 nor in the populations of Tfh cells.

9 These findings demonstrate that the change in local immune response brought about
10 by a transplanted LN could change the immune response in the spleen as secondary
11 lymphoid organ.

12

13 ***HEVs in transplanted LNs could have a role in the adaptive immune response***

14 T-cell recruitment in most peripheral lymphoid organs occurs through the walls of
15 HEVs in a stepwise sequence of interactions between lymphocytes and HEV cells (23).
16 HEVs are blood vessels and could be damaged directly by non-vascularity. CCL21 is

1 present on the luminal surface of HEVs and is important for the recruitment and
2 migration of T-cells in the LNs. Immunohistochemical staining and Western blotting of
3 MECA79 were performed to evaluate HEVs in NPLNs, TDTLNs, and TDPLNs. ELISA
4 of CCL21 was also performed.

5 On immunohistochemical staining, HEVs were observed in both TDTLNs and
6 TDPLNs at the paracortex. Interestingly, the lumens of the HEVs in TDTLNs were not
7 dilated when compared with those in TDPLNs, although the HEVs were remodeled in
8 the anticancer context in the premetastatic state (Fig. 6a).

9 On Western blotting, the quantity of HEVs tended to be greater in TDTLNs than in
10 NPLNs (Fig. 6b). However, the quantity of CCL21 tended to be smaller in TDTLNs
11 than that in NPLNs (Fig. 6c). The quantity of HEVs in TDPLNs was significantly
12 greater than that in NPLNs, reflecting the premetastatic state.

13

14 **Discussion**

15 The sentinel LN is thought to be an important lymphoid organ that protects against
16 metastasis and plays a crucial role in antitumor immunity. The sentinel LN is the first

1 site of tumor antigen presentation associated with lymphocyte activation. Induction of a
2 T-cell response depends on antigen-specific priming of naïve T-cells by dendritic cells
3 (24). Activation of naïve T-cells is generally thought to take place within the highly
4 specialized microenvironment of secondary lymphoid organs such as LNs and the
5 spleen (25), so the sentinel LN could be regarded as the primary site for the immune
6 system to encounter tumor antigens (19). Subcutaneous implantation of B16-F10
7 melanoma cells in the rear footpad of mice induces hypertrophy of the draining
8 popliteal LN, which is regarded as the sentinel LN (26). Therefore, when we evaluated
9 the function of the transplanted LN, we focused on the PLN as the TDPLN in the
10 context of cancer in mice.

11 We found that there was a statistically significant difference in lung and LN metastasis
12 between mice with sentinel LN resection and mice with LN transplantation (TDTLNs)
13 to the area of the resected sentinel LN. One of the reasons for this difference in
14 metastasis was thought to be a change in lymphatic flow, as we have reported
15 previously (11). Mice with autotransplanted, non-vascularized, resected PLN showed
16 the same normal lymphatic flow as the controls without PLN removal. This restoration

1 of lymphatic flow played an important role in decreasing the degree of metastasis.
2 However, there was a statistically significant difference in the extent of metastasis
3 between TDPLN and TDTLN. We hypothesized that another reason for this difference
4 was the difference in the function of the LN itself.

5 It is apparent that a TDLN has an increased content of T lymphocytes and B
6 lymphocytes (26, 27) because the immune response to the tumor is likely mediated by
7 soluble material from the tumor or antigen-presenting cells entering the LN via the
8 lymphatic drainage pathway from the tumor to the PLN (28). On immunochemical
9 staining, the components of the LN changed after the adaptive immune reaction, and
10 T-cells (CD3), B-cells (B220), and lymphatic endothelial (LYVE-1) cells were
11 maintained in TDTLNs as well as TDPLNs. However, the size of the LN itself was
12 different, indicating that TDTLNs are not as large as TDPLNs. This was reflected in the
13 difference in the number of LN lymphocytes, eventually resulting in a difference in
14 function.

15 CD4⁺ helper T-cells, upon activation by antigen-presenting cells, differentiate into
16 cytokine-expressing effector helper T cells, which are classified as Th1, Th2, Th9, Th17,

1 and Tfh cell subsets (29-31). Th1 cells secrete type I cytokines, such as IFN- γ and
2 TNF- α . Th2 cells secrete type II cytokines, such as IL-4, IL-5, IL-13, which limit the
3 activation of antigen-presenting cells and enhance humoral immunity (29, 32). The
4 inflammation caused by these cells is dominated by IL-10-producing cells in tumor
5 tissue (33). FACS analysis showed the change in the cell populations of LN
6 lymphocytes, including these CD4⁺ helper T-cells and CD8⁺ killer T-cells, even in
7 TDTLNs.

8 Quantitative RT-PCR was performed to determine whether expansion of CD4⁺ T-cells
9 or CD8⁺ T-cells in TDTLNs could show an effective antitumor immune response
10 directly via cytokine secretion. The results showed that levels of IFN- γ and IL-4 were
11 increased in the context of cancer. However, level of IL-2 was decreased. Endogenously
12 activated T-cells produce either IFN- γ or IL-2, but not both simultaneously, which is
13 known as the threshold phenomenon(34). In this setting, a threshold phenomenon could
14 cause an increase in the IFN- γ level and a decrease in the IL-2 level. To ascertain the
15 existence of IL-2-producing lymphocytes in TDTLNs, we performed an ELISpot assay
16 by stimulating lymphocytes and changing the condition of the threshold phenomenon

1 and demonstrated that $CD4^{+}$ T-cells in TDTLNs produced IL-2. The increases in IFN- γ ,
2 IL-2, IL-4 levels by $CD4^{+}$ T-cells were confirmed in TDTLNs. Furthermore, the
3 responses of cytotoxic T-cells in the TDTLNs via $CD8^{+}$ T-cells could also support the
4 immunologic function of these transplanted LNs. Taken together, both TDTLNs and
5 TDPLNs could function as the sentinel LN in the presence of cancer.

6 As subsets of $CD4^{+}$ helper T cells, Tfh, and Treg were also examined. Tfh are
7 specialized cells that assist B-cells. IL-21 is highly expressed by Tfh and germinal
8 center Tfh, and is the most potent cytokine known in terms of driving plasma cell
9 differentiation in mice and humans (35, 36). Because B cell expansion was confirmed in
10 TDTLNs and TDPLNs (Fig. 3c), we speculated that IL-21 was secreted by Tfh. In this
11 setting, IL-21 mRNA expression was observed in TDTLNs and in TDPLNs than in
12 NPLNs (Fig. 4a), but the Tfh population was not detectable in either TDTLNs or
13 TDPLNs (Fig.4c). This finding was not associated with the timing of the harvest of the
14 LNs (data not shown). T reg cells play critical roles in the induction of peripheral
15 tolerance to self and foreign antigens, and IL-10 is a regulatory cytokine playing an
16 important role in controlling inflammatory processes (37). T reg cells were identified in

1 both TDTLNs and TDPLNs, demonstrating a significant difference in population (Fig.
2 4d). The IL-10 mRNA level was consistent with that of the findings from FACS (Fig.
3 4a). It was likely that TDTLNs had immune-mediated antitumor effect with
4 immune-suppressive functions.

5 We examined the function of the spleen, another secondary lymphoid organ, in terms
6 of its association with the local antitumor immunity of sentinel LNs. The spleen allows
7 generation of antigen-specific immune responses protecting the body against antigens
8 from the bloodstream (38). In this study, we demonstrated a statistically significant
9 difference in the T-cell and B-cell populations of the spleen between groups 3 and 1.
10 This finding indicates that the change in function of the sentinel LN contributing to
11 anti-tumor immunity could bring about a change in the antitumor function of the spleen,
12 especially the transition to expansion of B-cells.

13 HEVs are specialized vessels that are present in LNs and tertiary lymphoid organs.
14 CCL21, one of the molecular gatekeepers for entry of naïve T-cells from the
15 bloodstream into LNs, is present on the luminal surface of HEVs and regulates
16 migration of lymphocytes through HEVs (39). It is suggested that premetastasis

1 remodeling of HEVs in sentinel LNs is a common phenomenon in animal models (40).
2 The rate of proliferation of HEV endothelial cells is increased before metastasis, which
3 is consistent with the process of HEV remodeling. In our study, this change was
4 confirmed in TDPLNs 14 weeks after tumor inoculation and was supported by Western
5 blotting and ELISA of CCL21. The lumens of the HEVs were dilated in TDPLNs but
6 not in TDTLNs. This finding was attributed to the transient decrease in vascularity as a
7 result of non-vascularized LN transplantation. There was a suggestion of a relationship
8 between the less expanded lumens of the HEVs and the differences in the number of
9 lymphocytes infiltrating the LN and amounts of HEVs. Our study indicated that there
10 were more HEVs in TDTLNs than in NPLNs, although the quantity of CCL21 in
11 TDTLNs was less than that in NPLNs. It was speculated that because CCL21 was a
12 much smaller molecule, it was more directly affected by the decreased vascularity than
13 the HEVs. Taken together, the HEVs in TDTLNs could function following the adaptive
14 immune reaction under the limited condition of decreased vascularity.

15 In summary, TDPLN resection was associated with development of LN and lung
16 metastases, and LN transplantation to the area of the resected PLN suppressed these

1 metastases. Non-vascularized transplanted LN demonstrated immune-mediated
2 antitumor effect as a secondary lymphoid organ through activation of CD4 and CD8
3 T-cells, demonstrating expansion of lymphocytes and a change in the lymphocyte
4 population following the adaptive immune reaction. The function of the transplanted
5 LN was affected by the change in HEVs brought about by decreased vascularity.

6

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10

11 **Author Disclosure Statement**

12 The authors have no conflicts of interest or financial ties to disclose.

13

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- 14
- 15

1 **Figure legends**

2

3 Figure 1. LN resection associated with development of further LN and lung metastasis;

4 LN transplantation suppressed this metastasis. (a) Lymph drainage pattern following

5 popliteal LN resection and LN transplantation, and tumor metastasis along each

6 lymphatic pathway. In the mouse hindlimb, lymph drained into the popliteal LN in

7 group 1. After the popliteal LN resection, lymphatic drainage was rerouted to the

8 inguinal LN in group 2. LN transplantation to the site of the popliteal LN resulted in

9 lymph flow to the PLN, indicating normalization of lymph flow. Upper, scheme of right

10 lateral position; Lower, fluorescent images of right lateral position. White arrow

11 indicates area of the inguinal LN, white arrowhead indicates area of the popliteal LN.

12 (b) In vivo optical imaging of melanoma cells in the mouse hindlimb footpad 4 weeks

13 after inoculation. Representative images of each group are shown. Upper, inoculated

14 melanoma cells: Lower, fluorescent images in the supine position. Left, group 1; Center,

15 group 2; Right, group 3. (c) Twelve mice in each group were used for in vivo

16 bioluminescence imaging. Fluorescence intensities correlate with tumor volume and

1 weight after injection of D-luciferin in each group. (d) The lungs, inguinal LNs, and
2 axillary LNs were excised 5 weeks after tumor inoculation and homogenized; total
3 luciferase activity was determined with standard ex vivo luciferase assay.
4 Representative macroscopic views are shown in the bottom row. The graphs show the
5 data as the mean and the standard error of the mean. * $P < 0.05$ and ** $P < 0.01$
6 (Wilcoxon rank-sum test). LNs, lymph nodes; RLUs, relative light units.

7

8 Figure 2. Fewer inoculated melanoma cells metastasized to TDTLN than to the TDPLN.

9 (a) Total luciferase activity was determined using a standard ex vivo luciferase assay.

10 Representative macroscopic views are shown in the bottom row. The graphs show the

11 data as the mean and the standard error of the mean ($n = 9$ per group). * $P < 0.05$ and

12 ** $P < 0.01$ (Wilcoxon rank-sum test). (b) Identification of melanoma cells in a PLN and

13 transplanted LN using B16-F10-RFP cells. Fluorescein isothiocyanate-dextran was used

14 to depict the LNs. In the TDTLN, B16-F10-RFP melanoma cells were identified at 4

15 weeks after tumor induction but were identified in the TDPLN at 3 weeks. Scale bar 20

16 μm . (c) TYRP-1 mRNA transcript was determined from 1 to 4 weeks. Measurements

1 were obtained from the LNs in triplicate. The graphs show the data as the mean and the
 2 standard error of the mean ($n = 6-7$ per group). LNs, lymph nodes; RLUs, relative light
 3 units; NPLN, naïve popliteal lymph node; TDPLN, tumor-draining popliteal lymph
 4 node; TDTLN, tumor-draining transplanted lymph node.

5

6 Figure 3. Transplanted LN showing lymphocyte expansion with change in lymphocyte
 7 population following adaptive immune reaction. (a) Macroscopic images of NPLN,
 8 TDTLN, and TDPLN. Major axis of the NPLN measures about 1 mm, major axis of
 9 TDTLN about 1.5 mm, and major axis of TDPLN about 3 mm. (b) Total number of
 10 lymphocytes, B220⁺ B-cells and CD3⁺ T-cells ($n = 12-14$ per group. * $P < 0.05$, ** $P <$
 11 0.01 (two-way analysis of variance with the Tukey–Kramer post-hoc test). (c)
 12 Fluorescence-activated cell sorting analysis showing proportion of B220^{hi}CD3^{lo} B-cells
 13 and B220^{lo}CD3^{hi} T-cells (first line), CD8^{hi}CD4^{lo} killer T-cells and CD8^{lo}CD4^{hi} helper
 14 T-cells (second line), CD44^{lo}CD62L^{hi} naïve T-cells and CD44^{hi}CD62^{lo} effector/memory
 15 CD4⁺ helper T-cells (third line), and CD44^{lo}CD62L^{hi} naïve T-cells and CD44^{hi}CD62^{lo}
 16 effector/memory CD8⁺ killer T-cells (fourth line) in TDTLDs and TDPLNs. Numbers in

1 dot-plot quadrants represent percentages. Data are presented as box-and-whisker plots
2 (n = 18 per group). *P < 0.05 and **P < 0.01 (two-tailed two-sample unequal variance
3 Student's *t*-test). (d) Immunohistochemistry staining for CD3, B220, and LYVE-1 in
4 NPLN, TDTLN, and TDPLN. Scale bar 200 μ m. NPLN, naïve popliteal lymph node;
5 TDPLN, tumor-draining popliteal lymph node; TDTLN, tumor-draining transplanted
6 lymph node.

7

8 Figure 4. Transplanted LN functioning as secondary lymphoid organ in the immune
9 response via helper T-cell activation (a) IFN- γ , IL-2, IL-4, IL-10 and IL-21 mRNA
10 transcripts were determined 2 weeks after tumor inoculation. Results are expressed as
11 mean and standard error of the mean. Triplicate measurements were obtained from the
12 LNs (n = 9–10 per group). (b) ELISpot showing antigen-specific IL-2-secreting CD4⁺
13 helper T-cell responses from the NPLNs, TDTLNs, and TDPLNs (n = 7 per group). *P
14 < 0.05, **P < 0.01 (two-way analysis of variance with Tukey–Kramer post-hoc test). (c)
15 Fluorescence-activated cell sorting analysis showing proportion of PD-1^{hi}CXCR5^{hi}
16 CD4⁺ follicular helper T-cells in TDTLDs and TDPLNs. (d) Fluorescence-activated cell

1 sorting analysis showing proportion of CD127^{low}CD25^{hi} CD4⁺ regulatory helper T-cells
 2 in TDTLDs and TDPLNs. (e) Cytotoxic activity of CD8⁺ T-cells in TDTLNs and
 3 TDPLNs. Cell suspension was prepared from TDTLNs and TDPLNs 28 days following
 4 melanoma cell inoculation. Purified CD8⁺ T-cells were cultured with melanoma cells at
 5 effector to target ratios of 5:1 and 20:1. Cytotoxic activity was assessed with confocal
 6 microscopy. All values are presented as mean and standard error of the mean (= 5 per
 7 group). *P < 0.05 (two-tailed two-sample unequal variance Student's *t*-test). ELISpot,
 8 enzyme-linked immunospot; IFN- γ , interferon gamma; IL, interleukin; LN, lymph
 9 node; NPLN, naïve popliteal lymph node; TDPLN, tumor-draining popliteal lymph
 10 node; TDTLN, tumor-draining transplanted lymph node.

11

12 Figure 5. Immunologic state of the spleen affected by immunologic change in the LN
 13 associated with local immunity. Fluorescence-activated cell sorting analysis showing
 14 the proportion of B220^{hi}CD3^{lo} B-cells and B220^{lo}CD3^{hi} T-cells (first line), CD8^{hi}CD4^{lo}
 15 killer T-cells and CD8^{lo}CD4^{hi} helper T-cells (second line), CD44^{lo}CD62L^{hi} naïve T-cells
 16 and CD44^{hi}CD62^{lo} effector/memory CD4⁺ helper T-cells (third line), and

1 PD-1^{hi}CXCR5^{hi} CD4⁺ follicular helper T-cells (fourth line) in TDTLDs, and TDPLNs.

2 Numbers in dot-plot quadrants represent percentages. Data are presented as

3 box-and-whisker plots (n = 12 per group). *P < 0.05 and **P < 0.01(two-tailed

4 two-sample unequal variance Student's *t*-test). LN, lymph node; TDPLN,

5 tumor-draining popliteal lymph node; TDTLN, tumor-draining transplanted lymph

6 node.

7

8 Figure 6. TDTLNs retain high endothelial venules, but the quantity is limited. (a)

9 Immunohistochemical staining with MECA79 in NPLNs, TDTLNs, and TDPLNs. Scale

10 bar 100 μ m. (b) Western blot analysis for MECA79 levels in NPLNs, TDTLNs, and

11 TDPLNs. Images are representative of two independent experiments. Graph shows data

12 as mean and standard error of the mean. *P < 0.05 (two-way analysis of variance with

13 Tukey–Kramer post-hoc test). (c) Enzyme-linked immunosorbent assay of CCL21 (n =

14 10 per group). Graph shows data as mean and standard error of the mean. *P < 0.05

15 (two-way analysis of variance with Tukey–Kramer post-hoc test). NPLN, naïve

16 popliteal lymph node; TDPLN, tumor-draining popliteal lymph node; TDTLN,

1 tumor-draining transplanted lymph node.

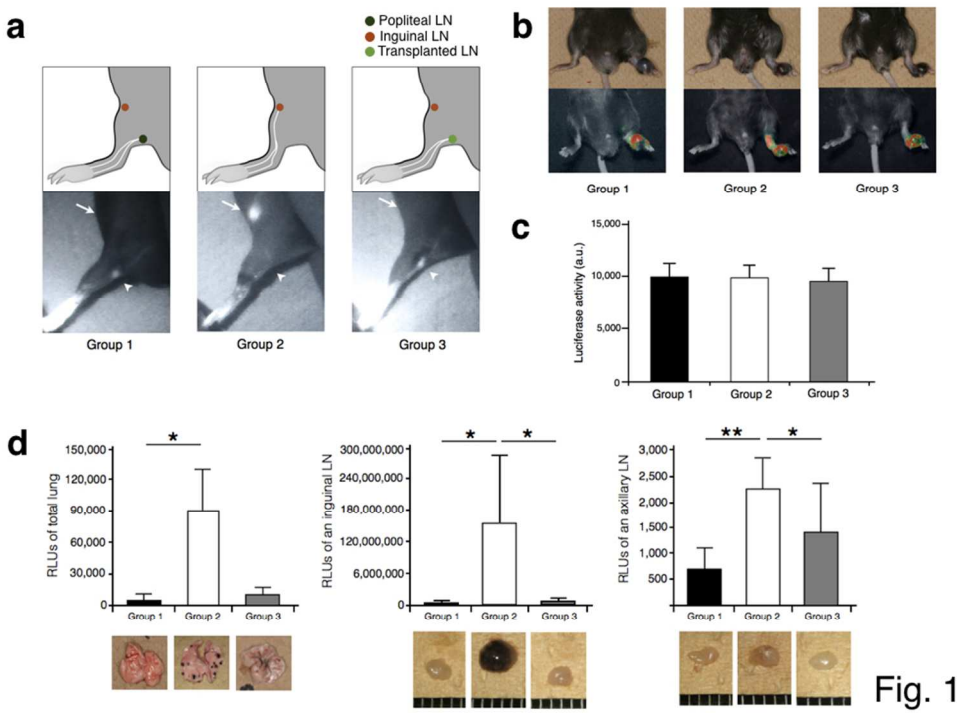


Fig.1

361x270mm (72 x 72 DPI)

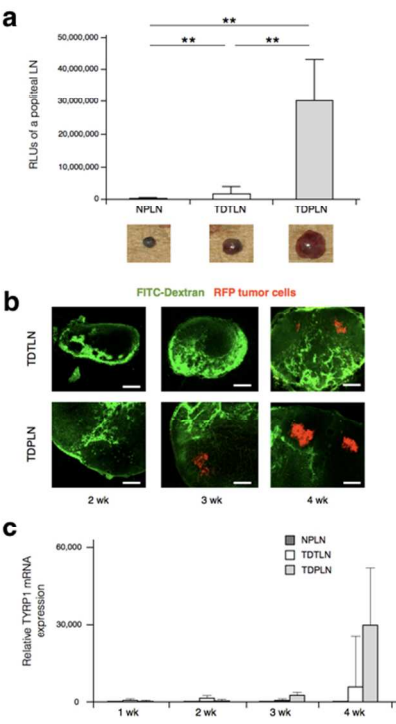


Fig. 2

Fig.2

361x270mm (72 x 72 DPI)

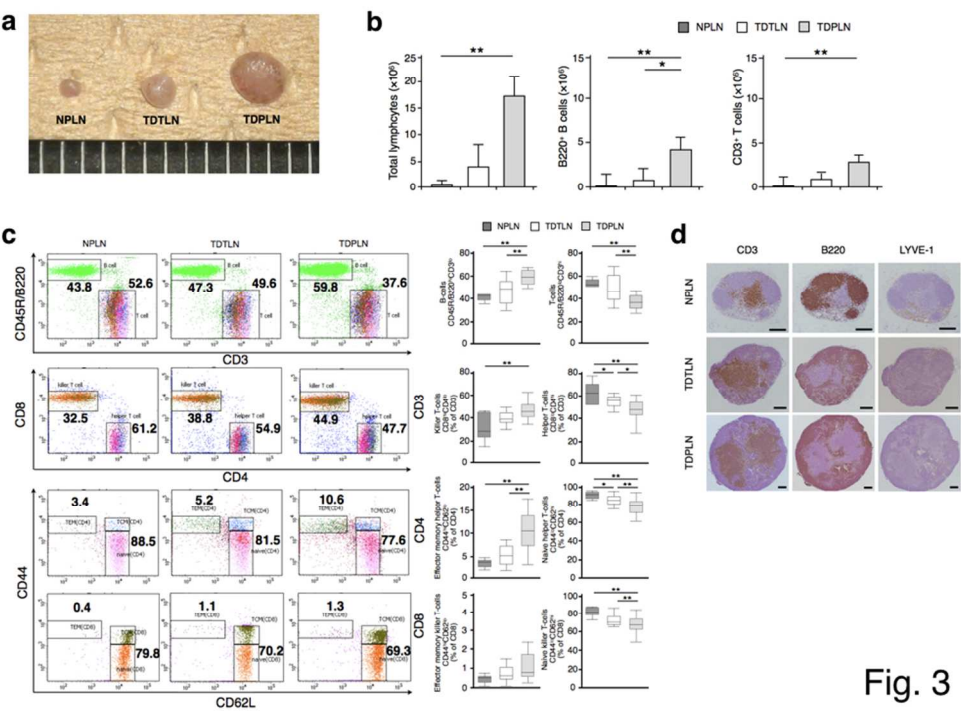


Fig. 3

Fig.3

361x270mm (72 x 72 DPI)

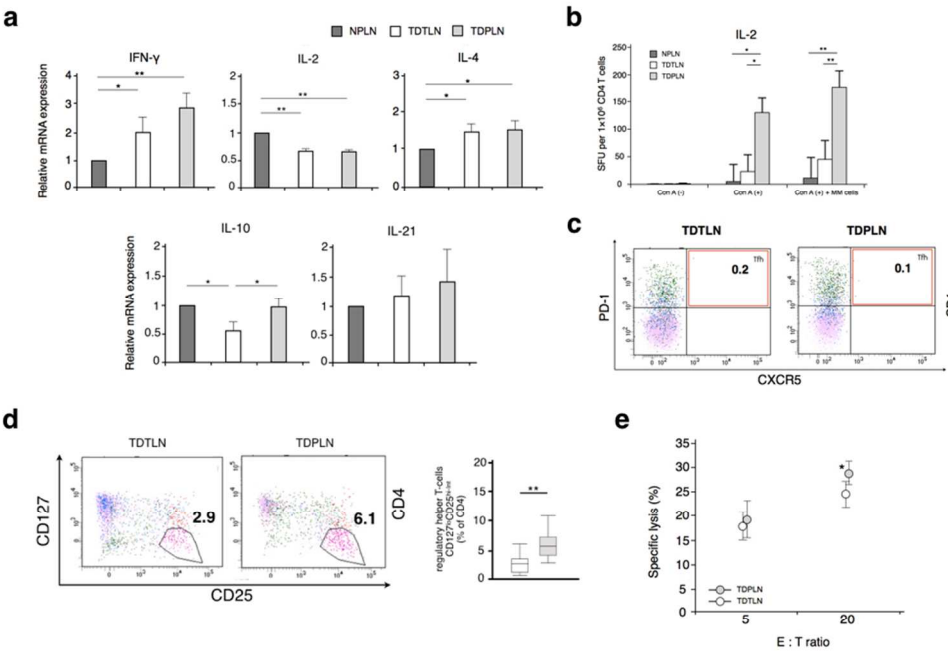


Fig. 4

Fig.4

361x270mm (72 x 72 DPI)

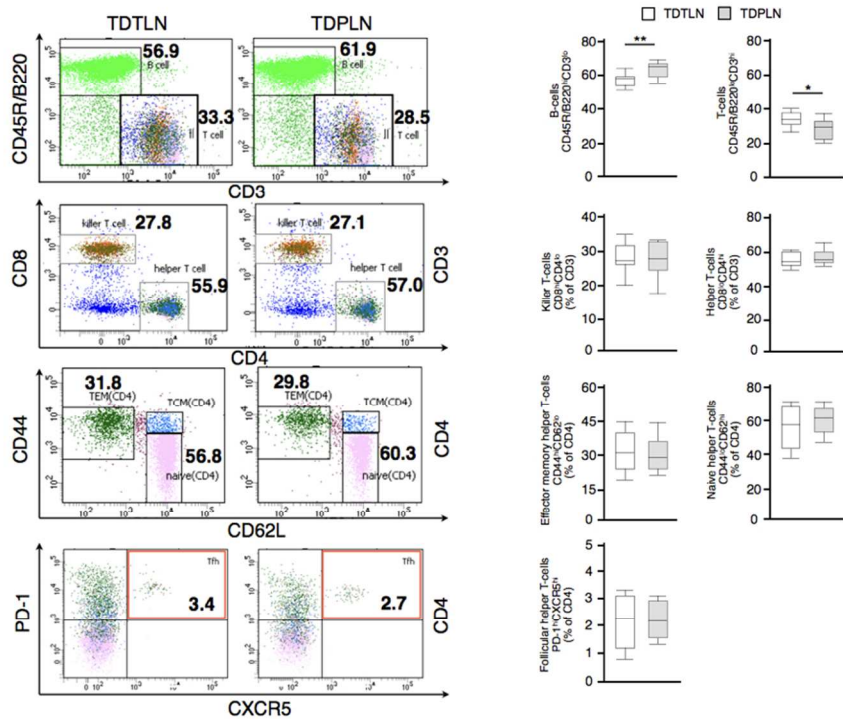


Fig. 5

Fig.5

361x270mm (72 x 72 DPI)

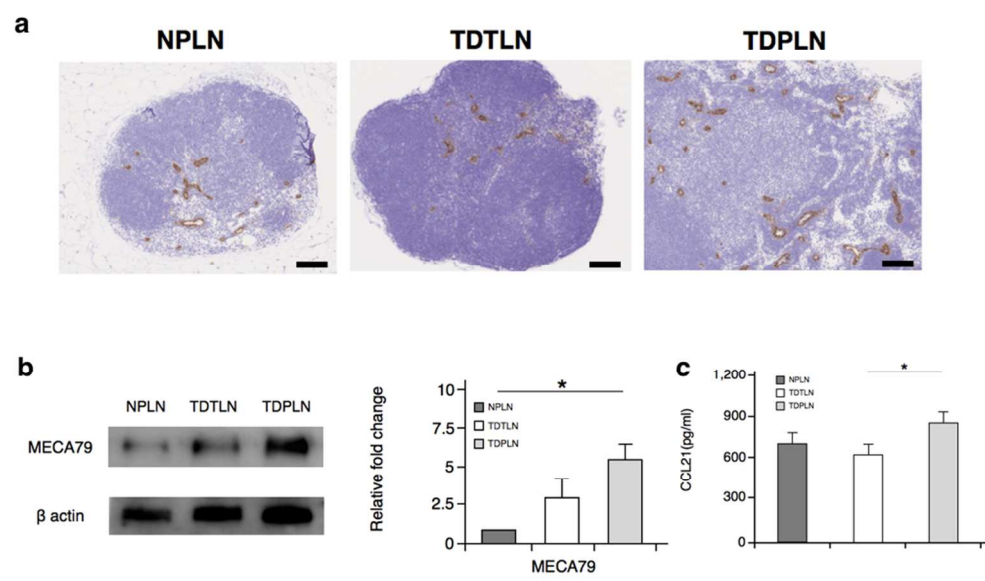


Fig. 6

Fig.6

361x270mm (72 x 72 DPI)