TUMORIGENESIS AND NEOPLASTIC PROGRESSION

B Cells Promote Tumor Immunity against B16F10 Melanoma

Tadahiro Kobayashi,* Yasuhito Hamaguchi,* Minoru Hasegawa,[†] Manabu Fujimoto,[‡] Kazuhiko Takehara,* and Takashi Matsushita*

From the Department of Dermatology,* Faculty of Medicine, Institute of Medical, Pharmaceutical, and Health Sciences, Kanazawa University, Kanazawa; the Department of Dermatology,[†] University of Fukui, Fukui; and the Department of Dermatology,[‡] Faculty of Medicine, University of Tsukuba, Tsukuba, Japan

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Address correspondence to
Takashi Matsushita, M.D.,
Ph.D., Department of Derma-
tology, Faculty of Medicine,
Institute of Medical, Pharma-
ceutical, and Health Sciences,
Kanazawa University, 13-1
Takaramachi, Kanazawa,
Ishikawa 920-8641, Japan.
E-mail: t-matsushita@med.kanazawa-u.ac.jp.

B cells are known to be critical mediators of tumor immunity; however, the mechanisms through which they exert this function remain unclear. B-cell linker protein (BLNK) is an essential component of the B-cell antigen receptor signaling machinery and is required for B-cell development, as evidenced by *BLNK*-deficient (*BLNK*^{-/-}) mice, in which the development and function of B cells are severely impaired. Herein, we evaluated the role of B cells in the development of tumor immunity against B16F10 melanoma using *BLNK*^{-/-} mice. B16F10 melanoma grew more aggressively in *BLNK*^{-/-} mice, resulting in a twofold increase in tumor volume compared with wild-type mice. As predicted, tumor-infiltrating B-cell numbers were decreased in *BLNK*^{-/-} mice. Paradoxically, tumor-infiltrating T-cell numbers were decreased in *BLNK*^{-/-} mice, although inguinal lymph node T-cell numbers were increased. Adoptive transfer of B cells from wild-type mice into *BLNK*^{-/-} mice attenuated B16F10 melanoma growth, with increasing numbers of B and T cells infiltrating into tumors. In addition, percentages of interferon- γ - and tumor necrosis factor- α -producing tumor-infiltrating T cells were restored. Taken together, our study supports the concept that B cells enhance tumor immunity against B16F10 melanoma by promoting T-cell infiltration into tumors and cytokine production within the tumor microenvironment. (*Am J Pathol* 2014; 184: 3120–3129; <http://dx.doi.org/10.1016/j.ajpath.2014.07.003>)

In addition to providing humoral immunity, B cells have multiple other functions, including antigen (Ag) presentation to and activation of T cells.¹ It is also widely recognized that certain B-cell subpopulations exhibit potent regulatory properties and are involved in immune pathological conditions, such as autoimmunity and malignancy.^{2,3} For example, in murine autoimmune models, adoptive transfer of B cells can ameliorate chronic inflammatory responses, even when disease is already established.^{2,4}

In tumor immunity, B cells can exert both negative and positive effects. For example, tumor immunity against B16F10 melanoma, EL4 thymoma, and MC38 colon carcinoma was found to be enhanced in B-cell-deficient μ MT mice compared with wild-type (WT) controls.⁵ Thus, both CD4⁺ T-cell priming and anti-tumor type 1 helper T-cell (Th1) cytokine production as well as CD8⁺ cytotoxic T-lymphocyte responses were enhanced in μ MT mice.⁶ Notably, reconstitution of μ MT mice with WT B cells abrogated tumor resistance.⁵ However, a caveat of studies

conducted in μ MT mice is that there are severe immune system abnormalities and skewing toward Th1 responses.^{7,8}

B cells are also capable of promoting anti-tumor immunity by functioning as antigen-presenting cells (APCs) and/or via cross presentation of Ag to other APCs to regulate cellular immune responses.^{9,10} In addition, B cells activated by CD40 ligand (CD40L) can induce an Ag-specific anti-tumor immune response by CD8⁺ T cells.⁹ Furthermore, depletion of mature B cells using an anti-CD20 monoclonal antibody (mAb) results in enhanced B16F10 melanoma tumor growth that was correlated with impaired activation of CD4⁺ and CD8⁺ effector-memory T cells and inflammatory and cytotoxic cytokine-secreting T cells. B-cell depletion was also shown to impair tumor Ag-specific CD8⁺ T-cell proliferation.¹¹ Thus,

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μ MT mice and anti-CD20 mAb-treated mice both lack mature B cells, although divergent tumor responses are elicited in these models.

B-cell linker protein (BLNK) is a B-cell-specific adaptor molecule that contains multiple protein-binding motifs. Disturbed expression of BLNK results in severely altered development and function of B cells in mice and in human patients.^{12–15} In *BLNK*-deficient (*BLNK*^{−/−}) mice, B cells are impaired in their ability to produce the immunosuppressive cytokine IL-10 and develop more severe contact hypersensitivity and experimental autoimmune encephalomyelitis compared with WT mice. IL-10 message levels in B cells after contact hypersensitivity or experimental autoimmune encephalomyelitis induction are significantly decreased in *BLNK*^{−/−} mice compared with WT mice, whereas interferon (IFN)- γ , IL-2, IL-4, and IL-6 mRNA expression levels are not significantly different.¹⁶ Thus, *BLNK*^{−/−} mice are a good model for the investigation of B-cell function in tumor immunity. Herein, we show that B cells enhance tumor immunity by promoting CD4⁺ and CD8⁺ T-cell infiltration into tumors and cytokine production by these cells in the tumor microenvironment.

Materials and Methods

Mice and Cell Culture

Female C57BL/6 mice (9 to 12 weeks old) were purchased from SLC (Shizuoka, Japan); *BLNK*^{−/−} mice on the C57BL/6 background were from Jackson Laboratory (Bar Harbor, ME). All mice were housed and cared for in a pathogen-free barrier facility. All studies and procedures were approved by the Animal Committees of Kanazawa University, Institute of Medical, Pharmaceutical, and Health Sciences (Kanazawa, Japan). B16F10 melanoma cells were purchased from ATCC (Manassas, VA). Cells were cultured at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum and penicillin/streptomycin. The cells were passaged twice a week with trypsin. All cell culture reagents were obtained from Sigma-Aldrich (St. Louis, MO). Only early passages of the cell line were used in experiments.

In Vivo Tumor Growth

B16F10 cells (1.0×10^6) in 100 μ L sterile phosphate-buffered saline (PBS) were injected s.c. into the shaved right flank of anesthetized mice. Tumor growth was monitored on days 4, 7, 11, 14, 18, and 21 after tumor injection. The tumor volume was calculated using the following equation:

$$V = 4\pi(L_1 \times L_2^2)/3, \quad (1)$$

where V = volume (mm³), L_1 = longest diameter (mm), and L_2 = shortest diameter (mm).

Histological Examination and IHC

For immunohistochemical (IHC) staining of CD3 and B220, s.c. tumors harvested with surrounding circumference skin on day 11 after inoculation, freshly dissected spleens, and freshly dissected inguinal lymph nodes were fixed in 3.5% paraformaldehyde and then paraffin embedded. Sections (4 μ m thick) were then incubated with rat mAbs specific for mouse CD3 (CD3-12; AbD Serotec, Oxford, UK) and B220 (RA3-6B2; BD Biosciences, San Jose, CA).

For IHC staining of CD4, CD8, Foxp3, and NK1.1, s.c. tumors harvested with surrounding circumference skin on day 11 after inoculation, freshly dissected spleens, and freshly dissected inguinal lymph nodes were covered with Tissue-Tek OCT compound (SAKURA Finetek USA, Torrance, CA) and quickly frozen in liquid nitrogen. Frozen sections (4 μ m thick) were fixed in acetone and incubated with rat mAbs specific for mouse CD4 (RM4-5), CD8a (53-6.7; BD Biosciences), Foxp3 (FJK-16s; eBioscience, San Diego, CA), and NK1.1 (PK136; BioLegend, San Diego, CA).

Sections of spleens and inguinal lymph nodes were developed with 3,3'-diaminobenzidine tetrahydrochloride to stain targeted cells brown and then counterstained with hematoxylin. Sections of s.c. tumors were developed with New Fuchsin (Daco Japan, Tokyo, Japan) to stain targeted cells red, and thus, differentiate from brown melanin pigment, and then counterstained with hematoxylin. The numbers of stained cells in 10 high-power microscopic fields (0.07 mm²) were averaged, and each section was examined independently by two investigators in a blind manner (T.K. and T.M.).

Flow Cytometry

The following mAbs were used: fluorescein isothiocyanate-, phosphatidylethanolamine-, PerCP-Cy5.5-, APC-, APC-Cy7-, or Pacific Blue-conjugated mAbs to mouse B220 (RA3-6B2; BD Biosciences), CD3 ϵ (145-2C11), CD4 (RM4-5), CD25 (PC61.5; BioLegend), CD8a (53-6.7), NK1.1 (PK136), and Foxp3 (FJK-16s; eBioscience). LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Carlsbad, CA) was used to detect dead cells.

Single-cell suspensions of splenocytes, inguinal lymph nodes, and tumor-infiltrating lymphocytes were stained for 20 minutes using mAbs at predetermined optimal concentrations for multicolor immunofluorescence analysis using a FACSCanto II flow cytometer (BD Biosciences).¹⁷ Data were analyzed using FlowJo software version 8.8.7 (Tree Star, Ashland, OR).

Intracellular Cytokine Staining

For detection of cytokine production, T cells were stimulated for 4 hours with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich), 1 μ g/mL ionomycin (Sigma-Aldrich), and 3 μ mol/L brefeldin A (BioLegend). Natural killer (NK) cells were not stimulated for detection of granzyme B (GrB).

After cell-surface staining, the cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences), followed by staining with anti-IFN- γ (XMG1.2), anti-tumor necrosis factor α (TNF- α ; MP6-XT22), and anti-granzyme B (GB11) mAbs (BioLegend).

Single-Cell Suspensions of Tumor-Infiltrating Lymphocytes for Flow Cytometry

The s.c. tumors were minced on day 11 after inoculation and then digested in 10 mL of RPMI 1640 medium–10% fetal bovine serum containing 2 mg/mL crude collagenase (Sigma-Aldrich), 1.5 mg/mL hyaluronidase (Sigma-Aldrich), and 0.03 mg/mL DNase I (Sigma-Aldrich) at 37°C for 90 minutes to prepare tumor cell suspensions for flow cytometry. Digested cells were passed through a 70- μ m cell strainer (BD Biosciences) to generate single-cell suspensions. The cell suspension was centrifuged at $460 \times g$ for 5 minutes. The pellet was resuspended in 70% Percoll solution (GE Healthcare, Fairfield, CT) and then overlaid with a 37% Percoll solution (GE Healthcare), followed by centrifugation at $500 \times g$ for 20 minutes at room temperature. The cells were aspirated from the Percoll interface and passed through a 70- μ m cell strainer (BD Biosciences). Cells were harvested by means of centrifugation and washed with PBS.

Isolation and Adoptive Transfer of B Cells

B cells were isolated from WT splenocytes using the Pan B Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) with purities of approximately 95%. After isolation, purified B cells (1.0×10^7) in 500 μ L sterile PBS were transferred into recipient *BLNK*^{-/-} mice via i.v. injection through the lateral tail vein 1 day before tumor injection. The control group received PBS.

Statistical Analysis

All data are shown as means $\pm$ SEM. Statistical significance was determined by using the Student's *t*-test. Survival analysis was performed using a Kaplan-Meier estimate.

Results

B16F10 Melanoma Growth Is Significantly Increased in *BLNK*^{-/-} Mice

To determine the effect of B cells on tumor immunity against B16F10 melanoma, *BLNK*^{-/-} and WT mice were injected s.c. with 1.0×10^6 B16F10 melanoma cells in the flank and tumor growth was monitored. B16F10 melanoma growth in *BLNK*^{-/-} mice was significantly enhanced compared with WT mice. Statistically significant differences in tumor volume between *BLNK*^{-/-} and WT mice were observed beginning on day 11 after inoculation (Figure 1A), and enhanced tumor growth resulted in decreased survival of

Figure 1 B16F10 melanoma growth increases in *BLNK*^{-/-} mice. WT (white circles) and *BLNK*^{-/-} (black circles) mice were inoculated s.c. in the right flank with 1.0×10^6 B16F10 cells. **A:** Means $\pm$ SEM tumor volumes on the indicated days after B16F10 melanoma inoculation ($n = 7$ for each group). Significant differences between means for the same days. **B:** Survival of mice after B16F10 melanoma inoculation ($n = 7$ for each group). Similar results were obtained from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

BLNK^{-/-} mice (Figure 1B). Thus, tumor immunity against B16F10 melanoma is attenuated in *BLNK*^{-/-} mice.

B-Cell Numbers Are Significantly Decreased in *BLNK*^{-/-} Mice

Because *BLNK* is essential for B-cell development,¹³ we hypothesized that the alteration of B-cell populations in *BLNK*^{-/-} mice is a factor in the attenuation of tumor immunity. To evaluate lymphocyte populations in *BLNK*^{-/-} mice, spleen and inguinal lymph node (LN) cells were analyzed by IHC and flow cytometry.

A significant reduction in the number of splenocytes was seen in *BLNK*^{-/-} mice compared with WT mice [$(40.1 \pm 4.8) \times 10^6$ cells versus $(124 \pm 12.8) \times 10^6$ cells ($P < 0.001$)], whereas numbers of inguinal LN cells did not differ significantly between *BLNK*^{-/-} and WT mice [$(20.9 \pm 1.9) \times 10^5$ cells versus $(21.4 \pm 1.4) \times 10^5$ cells ($P = 0.848$)]. IHC analysis revealed that the number and size of lymphoid follicles and the number of B220⁺ cells are

clearly decreased in both the spleen and inguinal LNs of *BLNK*^{-/-} mice (Figure 2A). On the other hand, the distribution and number of CD3⁺ cells in spleen and inguinal LNs of *BLNK*^{-/-} mice were similar to WT mice (Figure 2B), as were CD4⁺, CD8⁺, Foxp3⁺, and NK1.1⁺ cells (data not shown). As shown previously,¹² flow cytometric analysis of *BLNK*^{-/-} mice revealed that B220⁺ B-cell numbers in the spleen were significantly decreased [by $(69.4 \pm 1.2) \times 10^6$ cells, $P < 0.001$] compared with WT mice, and B220⁺ B-cell numbers in inguinal LNs were also significantly decreased [by $(4.5 \pm 0.4) \times 10^5$ cells, $P < 0.001$] (Figure 2C). We also assessed T-cell and NK cell numbers in inguinal LNs and spleens. Reflective of the decrease in B-cell numbers, T and NK cells make up most of the cells in the spleen of *BLNK*^{-/-} mice, as shown by the increased percentage of CD4⁺, CD8⁺,

CD4⁺CD25⁺Foxp3⁺, and NK1.1⁺CD3⁻ cells compared with WT mice [$39.6\% \pm 1.1\%$ versus $20.2\% \pm 0.9\%$ ($P < 0.001$), $23.4\% \pm 0.9\%$ versus $10.9\% \pm 0.3\%$ ($P < 0.001$), $11.7\% \pm 0.5\%$ versus $10.2\% \pm 0.2\%$ ($P < 0.05$), and $5.0\% \pm 0.3\%$ versus $2.1\% \pm 0.1\%$ ($P < 0.001$), respectively] (Figure 2, D–F). However, numbers of these cells are lower in *BLNK*^{-/-} mice than in WT mice, reflecting the decreased cellularity of the *BLNK*^{-/-} spleen. Numbers of splenic CD4⁺, CD8⁺, CD4⁺CD25⁺Foxp3⁺, and NK1.1⁺CD3⁻ cells in *BLNK*^{-/-} mice are decreased by $(9.1 \pm 1.2) \times 10^6$ cells ($P < 0.001$), $(4.2 \pm 0.5) \times 10^6$ cells ($P < 0.001$), $(0.7 \pm 0.1) \times 10^6$ cells ($P < 0.001$), and $(0.7 \pm 0.2) \times 10^6$ cells ($P < 0.01$), respectively (Figure 2, D–F).

In contrast, the percentages and numbers of inguinal LN CD4⁺, CD8⁺, CD4⁺CD25⁺Foxp3⁺, and NK1.1⁺CD3⁻ cells

Figure 2 B-cell numbers decrease in *BLNK*^{-/-} mice. Representative IHC images in spleen and inguinal LN sections. B220⁺ (A) and CD3⁺ (B) cells stain brown. The percentages (left, flow cytometry plots) and numbers (right, bar graphs) of B220⁺ (C), CD4⁺ and CD8⁺ (D), CD25⁺Foxp3⁺ regulatory T (E), and NK1.1⁺CD3⁻ NK (F) cells within spleens and inguinal LNs in tumor-free WT and *BLNK*^{-/-} mice were determined by immunofluorescent staining followed by flow cytometric analysis ($n = 6$ for each group). Histograms represent means $\pm$ SEM. Significant differences between sample means. Similar results were obtained from two independent experiments. ** $P < 0.01$.

in *BLNK*^{-/-} mice were well correlated because numbers of inguinal LN cells did not differ significantly between *BLNK*^{-/-} mice and WT mice. Inguinal LN CD4⁺, CD8⁺, and CD4⁺CD25⁺Foxp3⁺ cell numbers in *BLNK*^{-/-} mice were increased by $(2.1 \pm 0.4) \times 10^5$ cells ($P < 0.01$), $(1.7 \pm 0.3) \times 10^5$ cells ($P < 0.001$), and $(2.5 \pm 0.4) \times 10^5$ cells ($P < 0.001$) over WT mice, respectively (Figure 2, D and E). No significant difference was observed in the numbers of inguinal LN NK1.1⁺CD3⁻ NK cells (Figure 2F).

These results suggest the possibility that the alterations in lymphocyte populations may contribute to the attenuation of tumor immunity in *BLNK*^{-/-} mice.

Numbers of Infiltrating T and B Cells in B16F10 Melanoma Tumors Decrease in *BLNK*^{-/-} Mice

CD4⁺CD25⁺Foxp3⁺ regulatory T cells inhibit immune responses mediated by CD4⁺CD25⁻ and CD8⁺ T cells

Figure 3 The numbers of B and T cells infiltrating into B16F10 melanoma tumors decrease in *BLNK*^{-/-} mice. WT and *BLNK*^{-/-} mice were inoculated s.c. in the right flank with 1.0×10^6 B16F10 cells. The s.c. B16F10 melanomas were harvested on day 11 after inoculation. Representative IHC images and numbers of B220⁺ (A), CD4⁺ (B), CD8⁺ (C), and Foxp3⁺ (D) cells. The cells were stained red to differentiate from brown of the melanin pigment. Data were obtained from four mice in each group. Significant differences between sample means. Similar results were obtained from three independent experiments. $^{***}P < 0.01$. Original magnification, $\times 200$ (A–D). HPF, high-power field.

in vitro via contact-dependent and cytokine-independent mechanisms,^{18–20} and affect the development and progression of tumor growth.²¹ We used IHC to quantitate B220⁺ B, CD4⁺ T, CD8⁺ T, and Foxp3⁺ regulatory T cells infiltrating into s.c. primary B16F10 melanoma tumors on day 11 after inoculation. The numbers of B220⁺ B, CD4⁺ T, and CD8⁺ T cells in tumors were significantly decreased in *BLNK*^{−/−} mice compared with WT mice (Figure 3, A–C), although no significant difference was seen in the number of Foxp3⁺ regulatory T cells in tumors (Figure 3D). This is despite increased numbers of lymph node CD4⁺ and CD8⁺ T cells in *BLNK*^{−/−} mice (Figure 2D). Taken together, these results suggest that the differences in tumor immunity observed between WT and *BLNK*^{−/−} mice are not due to regulatory T cells, and raise the possibility that B cells promote T-cell infiltration into B16F10 melanoma tumors.

Adoptive Transfer of B Cells into *BLNK*^{−/−} Mice Improves Animal Survival and Attenuates B16F10 Melanoma Growth

To investigate whether B cells promote tumor immunity against B16F10 melanoma, we evaluated the effect of adoptive transfer of B cells into *BLNK*^{−/−} mice on tumor immunity. We found that B16F10 melanoma growth was significantly diminished in reconstituted *BLNK*^{−/−} mice compared with *BLNK*^{−/−} mice, and was comparable to tumor growth observed in WT mice (Figure 4A). Moreover, survival of reconstituted *BLNK*^{−/−} mice was improved compared with unreconstituted *BLNK*^{−/−} mice (Figure 4B). In addition, IHC analyses revealed increased numbers of B220⁺ B, CD4⁺ T, and CD8⁺ T cells infiltrating into tumors in reconstituted *BLNK*^{−/−} mice compared with *BLNK*^{−/−} mice (Figure 5, A–C). Notably, no significant differences were observed in the number of Foxp3⁺ regulatory T cells infiltrating into tumors between reconstituted *BLNK*^{−/−} mice and *BLNK*^{−/−} mice (Figure 5D). These results indicate that B cells attenuate the growth of B16F10 melanoma by promoting CD4⁺ and CD8⁺ T-cell infiltration into tumors.

B Cells Are Required for Cytokine Production by Tumor-Infiltrating T cells

A previous study showed that the presence of B cells enhances T-cell cytokine production *in vitro*.¹¹ Thus, we assessed the cytokine production of tumor-infiltrating lymphocytes (TILs) in WT, *BLNK*^{−/−}, and reconstituted *BLNK*^{−/−} mice on day 11 after inoculation. TILs were stained for cell surface CD3, CD4, CD8, and NK1.1 as well as intracellular IFN- γ , TNF- α , and GrB expression, and analyzed by flow cytometry.

The phenotypes of the TILs included CD3⁺CD4⁺ T, CD3⁺CD8⁺ T, and CD3[−]NK1.1⁺ NK cells. Percentages of IFN- γ -secreting CD4⁺ T cells and IFN- γ - and TNF- α -secreting CD8⁺ T cells were significantly decreased in *BLNK*^{−/−} mice compared with WT mice (Figure 6, A and B), whereas percentages of TNF- α -secreting CD4⁺ T cells and

Figure 4 B16F10 melanoma growth in *BLNK*^{−/−} mice is attenuated by adoptive transfer of WT B cells. For reconstituted *BLNK*^{−/−} mice, purified B cells (1.0×10^7) from WT mice were transferred into *BLNK*^{−/−} mice by means of i.v. injection 1 day before tumor inoculation. WT (white circles), *BLNK*^{−/−} (black circles), and reconstituted *BLNK*^{−/−} mice (black squares) were inoculated s.c. on the right flank with 1.0×10^6 B16F10 cells. **A:** Means $\pm$ SEM tumor volumes on the indicated days after B16F10 melanoma inoculation ($n = 6$ for each group). Significant differences between means for the same days. **B:** Survival of mice after B16F10 melanoma inoculation ($n = 6$ for each group). Similar results were obtained from three independent experiments. ** $P < 0.01$ for WT mice versus *BLNK*^{−/−} mice; † $P < 0.05$, †† $P < 0.01$ for WT mice versus reconstituted *BLNK*^{−/−} mice.

GrB-secreting NK1.1⁺CD3[−] NK cells were similar in both *BLNK*^{−/−} and WT mice (Figure 6, A and C). Interestingly, percentages of IFN- γ -secreting CD4⁺ T cells and IFN- γ - and TNF- α -secreting CD8⁺ T cells in reconstituted *BLNK*^{−/−} mice were improved compared with unreconstituted *BLNK*^{−/−} mice (Figure 6, A and B). Furthermore, cytokine production by TILs correlated with the B16F10 melanoma growth and animal survival in WT, *BLNK*^{−/−}, and reconstituted *BLNK*^{−/−} mice. Therefore, cytokine production is an important factor contributing to the attenuation of tumor immunity in *BLNK*^{−/−} mice. In addition, these results suggest that B cells are required for T-cell cytokine production in the tumor microenvironment.

Discussion

Initial studies of mouse tumor models using B-cell-deficient μ MT mice suggested B cells negatively regulate

Figure 5 The numbers of B and T cells infiltrating into B16F10 melanoma tumors significantly increase in reconstituted *BLNK*^{-/-} mice compared with *BLNK*^{-/-} mice. For reconstituted *BLNK*^{-/-} mice, purified B cells (1.0×10^7) from WT mice were transferred into *BLNK*^{-/-} mice by means of i.v. injection 1 day before tumor inoculation. WT, *BLNK*^{-/-}, and reconstituted *BLNK*^{-/-} mice were inoculated s.c. on the right flank with 1.0×10^6 B16F10 cells. The s.c. B16F10 melanoma tumors were harvested on day 11 after inoculation. Representative IHC images and numbers of B220⁺ (A), CD4⁺ (B), CD8⁺ (C), and Foxp3⁺ (D) cells. The cells were stained red to differentiate from brown of the melanin pigment. Data were obtained from four mice in each group. Significant differences between sample means. Similar results were obtained from three independent experiments. * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 200$ (A–D). HPF, high-power field.

tumor immunity by inhibiting anti-tumor T-cell responses.^{5,6} In contrast, other murine tumor studies in which B cells were depleted by anti-CD20 mAb showed that B cells help to promote anti-tumor responses.¹¹ The current study investigates the role of B cells in tumor immunity using *BLNK*^{-/-} mice, which exhibit severely impaired development and function of B cells.^{12–16} The results show that tumor immunity against B16F10 melanoma tumors in *BLNK*^{-/-} mice is attenuated.

The conflicting results regarding the function of B cells in tumor immunity may be attributable to the activation status of B cells in different model systems. For example, resting B cells impair dendritic cell-induced tumor immunity, whereas CD40L-activated B cells do not.²² B-cell-deficient

μ MT mice lack both resting and activated B cells, which could potentially yield conflicting results depending on the extent of B-cell activation in a particular model system. Most tumor-infiltrating B cells (TIL-Bs) are activated by antigen exposure in cancer-bearing patients, as evidenced by Ig class switching, somatic hypermutation, and oligoclonality.²³ In our model, B16F10 melanoma growth was suppressed in *BLNK*^{-/-} mice reconstituted with WT B cells (Figure 4), suggesting that the state of TIL-B activation induced by B16F10 melanoma is favorable to tumor immunity.

Furthermore, in *BLNK*^{-/-} mice, a few mature IgM^{lo}IgD^{hi} B cells are found in the periphery, and T-cell development and function are both normal. Adult *BLNK*^{-/-} mice (8 to 13 weeks old), which we used in the current study, have

Figure 6 Cytokine production by TILs in WT, *BLNK*^{-/-}, and reconstituted *BLNK*^{-/-} mice on day 11 after inoculation. **A** and **B**: TILs were stimulated *in vitro* with phorbol myristate acetate (PMA) and ionomycin for 4 hours in the presence of brefeldin A followed by staining for CD4 and CD8 and intracellular IFN- γ and TNF- α . **C**: TILs were stained *in vitro* for CD3, NK1.1, and intracellular granzyme B. Data were obtained from four mice in each group. Significant differences between sample means. Similar results were obtained from two independent experiments. **P* < 0.05, ***P* < 0.01.

increased numbers of B220⁺IgM⁺ B cells compared with younger *BLNK*^{-/-} mice (3 to 6 weeks old).¹⁴ Regarding lymphoid architecture, the numbers of splenocytes in *BLNK*^{-/-} mice are, on average, only three times less than in WT mice and the spleens contain lymphoid follicles that consist of B220⁺ B cells (Figure 2A). Thus, *BLNK*^{-/-} mice have a few mature B cells in the periphery, normal T cells, and partially similar lymphoid architecture to WT mice. By contrast, in μ MT mice, T-cell receptor diversity is significantly reduced and Th1 immune responses are enhanced.^{7,8} The size of spleens in μ MT mice is, on average, six times smaller than in WT mice, and there is an absence of mature B cells in the periphery.²⁴ Thus, it seems that in *BLNK*^{-/-} mice, T-cell repertoire, T-cell function, and lymphoid tissue architecture are established in an environment more similar to WT mice than occurs in μ MT mice. These critical

differences between *BLNK*^{-/-} and μ MT mice are the most likely explanations for the opposing findings between our study and previous studies using μ MT mice.

On the other hand, it was previously reported that an ability of B cells to negatively regulate antitumor immune responses was attributed to B-cell provision of IL-10.²⁵ The B-cell subset that produces IL-10 was recently identified as regulatory B cells (Bregs), and we have confirmed that the ability of Bregs to produce IL-10 is impaired in *BLNK*^{-/-} mice.¹⁶ Our current work does not eliminate the possibility of an inhibitory role for Bregs during tumor immune responses, but it does support the notion that the positive regulatory functions of B cells are dominant in this context.

TIL-Bs are an important component of the B-cell response to cancer^{1,26}; however, their significance in solid tumors remains unclear. In ovarian cancers, Dong et al²⁷

state that TIL-Bs are associated with a worse outcome. Another study indicated that tumor-infiltrating IL-21–induced GrB⁺ B cells play an immunosuppressive role in certain tumors by suppressing T-cell proliferation via GrB-dependent degradation of the T-cell receptor (TCR) ζ -chain, a known GrB substrate.^{28,29} On the other hand, Milne et al³⁰ showed that the presence of TIL-Bs is positively correlated with survival. In this regard, tumors containing both CD8⁺ and CD20⁺ TILs are associated with higher survival than tumors containing either CD8⁺ or CD20⁺ TILs alone,³⁰ suggesting that positive cooperative interactions between CD8⁺ and CD20⁺ TILs are necessary for tumor immunity.

In the current study, the percentages of IFN- γ –secreting CD4⁺ T cells and IFN- γ – and TNF- α –secreting CD8⁺ T cells infiltrating into tumors were found to be significantly decreased in *BLNK*^{−/−} mice, and were restored in reconstituted *BLNK*^{−/−} mice (Figure 6, A and B). A previous study demonstrated that B cells are required for T-cell cytokine production *in vitro*.¹¹ In addition, it is also known that activated B cells can contribute to CD4⁺ T-cell priming through engagement of their antigen receptors.³¹ Taken together, our results indicate that TIL-Bs contribute to tumor immunity by promoting CD4⁺ and CD8⁺ T-cell cytokine production in the tumor microenvironment. Although little is known about the mechanisms by which TIL-Bs promote favorable outcomes in cancer, several possibilities have been suggested, such as the production of cytokines and function of these cells as APCs.¹

B cells can present antigens more efficiently than other APCs by selectively presenting cognate antigens (Ags) collected through surface Ig molecules, which allows presentation of even low concentrations of Ags to T cells.^{32–35} In the case of weakly immunogenic tumors, such as B16F10 melanoma, B cells and other APCs are involved in optimal T-cell expansion.¹¹ Nielsen et al²³ showed that TIL-Bs are antigen-experienced B cells and express cell surface markers characteristic of APCs, such as major histocompatibility complex class I and class II molecules, as well as costimulatory molecules, such as CD80 and CD86. Accordingly, most tumor-infiltrating T cells show an activated effector phenotype. Intriguingly, CD8⁺ and CD20⁺ TILs are frequently seen in close proximity, often in loosely structured aggregates.²³

Another study showed that advanced ovarian cancers lack intratumoral dendritic cells and instead contain TIL-Bs in close association with T cells.³⁰ These studies suggest a positive role for TIL-Bs as APCs, thereby sustaining the survival and proliferation of tumor-infiltrating T cells over the long-term. In addition, the transfer of CD40L-activated tumor peptide-loaded B cells induces Ag-specific anti-tumor immune responses in mice via cross presentation of Ag to APCs and via direct T-cell activation,⁹ whereas *BLNK*^{−/−} B cells show diminished responses to anti-CD40 stimulation.^{12–15} Taken together, the function of *BLNK*^{−/−} B cells as APCs in our model is likely impaired, resulting in decreased infiltration of activated CD4⁺ and CD8⁺ T cells into tumors.

To prove that B cells serve as APCs in tumor immunity, it will be necessary to clarify their antigen specificity and assess recognition by tumor-infiltrating T cells. Prior studies in medullary breast cancer identified two antigens recognized by recombinant antibodies derived from TIL-Bs: ganglioside D3 and β -actin. The latter was identified by virtue of exposure of β -actin on the surface of apoptotic tumor cells.^{36,37} In lung cancer, target Ags of TIL-Bs include TP53, as well as many self-Ags that are overexpressed in tumor tissue.³⁸ These studies suggest that TIL-B responses reflect a breakdown of tolerance to self-proteins, possibly due to apoptosis of tumor cells. Melanoma antigens recognizable by TIL-Bs in our model have not been identified, although it is known that glycoprotein 100 and melanoma antigen recognized by T cells-1 are melanoma-associated antigens recognized by T cells.^{39,40} On the other hand, Moutai et al⁴¹ showed the transfer of B cells specific for a surrogate tumor Ag [hen egg lysozyme (HEL)] suppressed metastasis and growth of B16 melanoma cells transfected with a membrane-anchored form of the HEL expression vector (B16-mHEL). Therefore, we have already started to investigate the antigen specificity of TIL-Bs. Identification of additional antigens recognized by TIL-Bs may yield further insights into the mechanisms by which B-cell responses are triggered and contribute to tumor immunity.

In conclusion, our study supports the argument that B cells positively regulate tumor immune responses.^{1,11} Our findings strongly suggest that B cells contribute to tumor immunity by increasing the numbers of tumor-infiltrating T cells and promoting IFN- γ and TNF- α production by T cells in the tumor microenvironment. Therefore, optimization of these B-cell functions could be important for cancer immunotherapies. It remains unclear which underlying characteristics of B cells, such as antigen specificity, are critical during tumor immunity. Further investigation into the function of TIL-Bs will be required to facilitate the design of more effective immunotherapies for the treatment of cancer.

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