might exert antitumor effect directly and indirectly. However, at present, the mechanism of neutrophil infiltration after BCG treatment is not fully understood.

IL-17 (also known as IL-17A) is a T-cell-derived proinflammatory cytokine, which is involved in various pathogenesis where neutrophils are involved. IL-17 induces mobilization of neutrophils indirectly *via* production of several cytokines, growth factors, and CXC chemokines [8]. IL-17 is produced by a recently identified subset of helper $CD4^+$ T cells, Th17 cells, which contribute to various inflammatory disorder as well as host defense [9]. However, increasing evidence revealed that another subset of T cells, namely γδ T cells, could even play a dominant role as the source of IL-17 *in vivo*. We found that γδ T cells in the peritoneal cavity produced IL-17 immediately after *Escherichia coli* infection, which is critical to the infiltration of neutrophils [10]. Furthermore, it was reported that IL-17 production in pulmonary infection with BCG was mediated by γδ T cells [11]. In the present study, we found BCG treatment in murine bladder also induced IL-17 production by γδ T cells, which play essential role in local neutrophil infiltration and antitumor effect against bladder cancer.

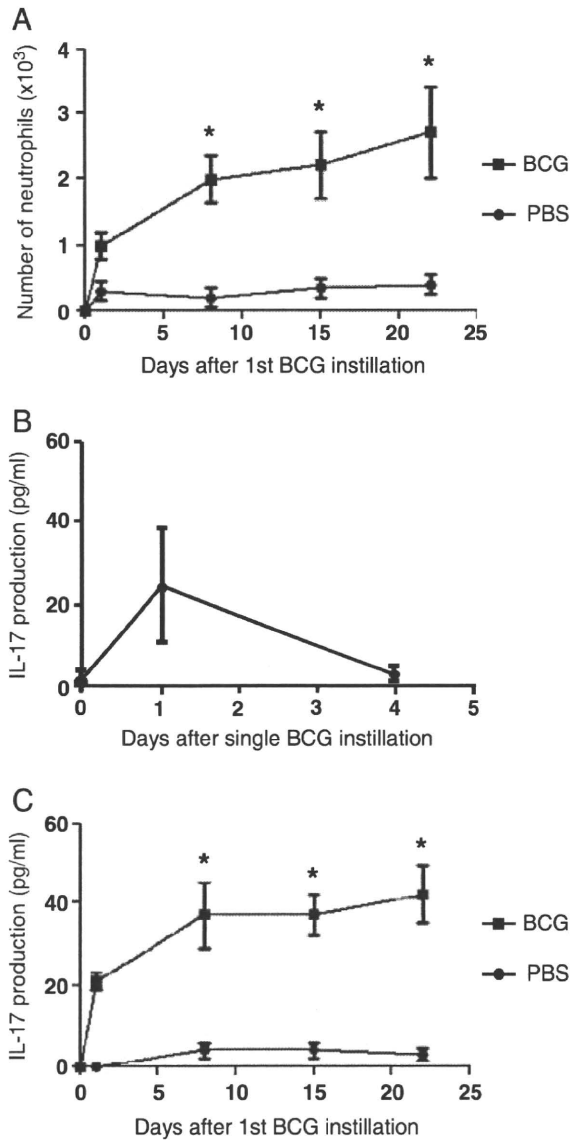

Figure 1. Intravesical neutrophil infiltration and IL-17 production after BCG treatment. B6 mice were intravesically injected with BCG or PBS on days 0, 7, 14, and 21. On days 0, 1, 8, 15, and 22, the number of neutrophils (A) and the level of IL-17 production (C) in the bladder were analyzed. Each group consisted of five mice. (B) Kinetics of the level of IL-17 production after single injection of BCG in the bladder ($n = 3$). Representative data of three separate experiments are shown. *$p<0.05$, Student's *t*-test.

Results and discussion

Vesical neutrophil infiltration after BCG treatment is mediated by IL-17

Recent studies demonstrated that neutrophils infiltrated in the bladder after BCG treatment played a key role in the antitumor effect [2]. In this study, we first examined the kinetics of neutrophil infiltration induced by weekly treatment with BCG. Significant infiltration of neutrophils was observed from one wk

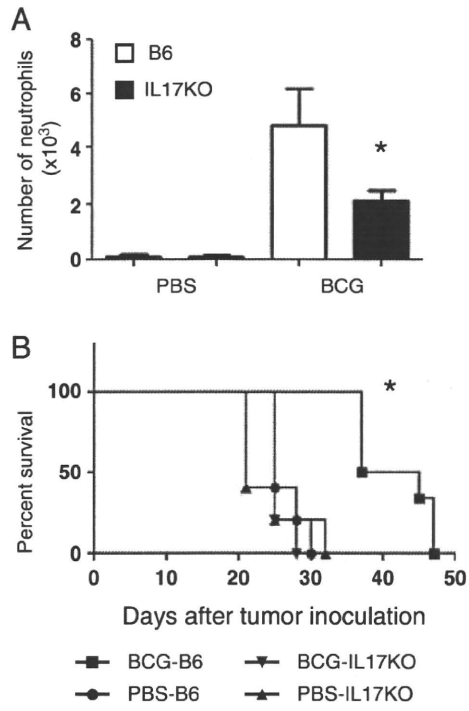

Figure 2. Involvement of IL-17 in the neutrophil infiltration and antitumor effect of BCG treatment. (A) B6 and IL-17KO mice were intravesically injected with BCG or PBS on days 0, 7, 14, and 21. On day 22, the number of neutrophils in the bladder was analyzed. Each group consisted of five mice. *$p<0.05$, Student's t-test. (B) B6 and IL-17KO mice were intravesically inoculated with 1×10^5 MB49 tumor cells on day 0 and received weekly intravesical injections of BCG or PBS from day 1 to 22. Each group consisted of five mice. Representative data of three separate experiments are shown. *$p<0.05$ compared with other three groups, log-rank test.

after starting BCG treatment, and it gradually increased during the observation period (Fig. 1A). We then examined intravesical IL-17 production after single BCG administration. As shown in Fig. 1B, IL-17 production was induced as early as 1 day after BCG injection, but lasted less than 5 days. During the course of repeated BCG administration, similar level of IL-17 production was induced after each injection (Fig. 1C). In order to determine the importance of IL-17 in the infiltration of neutrophils after BCG treatment, we examined the number of intravesical neutrophils in IL-17-deficient mice 22 day after starting BCG treatment. Infiltration of neutrophils was significantly reduced in IL-17-deficient mice (Fig. 2A). Therefore, IL-17 was involved in the infiltration of neutrophils into the bladder after BCG treatment.

Requirement for IL-17 in the antitumor effects of intravesical BCG treatment

To examine the significance of IL-17-induced neutrophil infiltration in the antitumor effect of BCG therapy, IL-17 KO mice were inoculated with MB49 bladder cancer cells before BCG treatment (Fig. 2B). The control B6 mice treated with BCG exhibited significantly longer survival compared to PBS-treated mice. On the other hand, there was no difference in the survival between BCG- and PBS-treated IL-17-deficient mice. There was also no difference in the survival of PBS-treated B6 and IL-17-deficient mice. We confirmed that depletion of neutrophils completely abrogated the antitumor effect of BCG therapy (data not shown), as was previously demonstrated by others [2]. Thus, it was revealed that IL-17-induced neutrophil infiltration was essential for the antitumor effect of intravesical treatment of BCG.

In contrast to our results, there have been reports implicating IL-17 with tumor progression. By acting on stromal cells and fibroblasts, IL-17 induces angiogenesis factors, which enhances tumor growth [12, 13]. It is possible that the antitumor effect of neutrophil infiltration overwhelmed the tumor-promoting effect of IL-17 in the case of bladder tumor. Alternative explanation for the discrepancy was the short duration of IL-17 production after each injection of BCG, which might not be enough for the tumor-promoting effect (Fig. 1B). In addition, there are reports showing

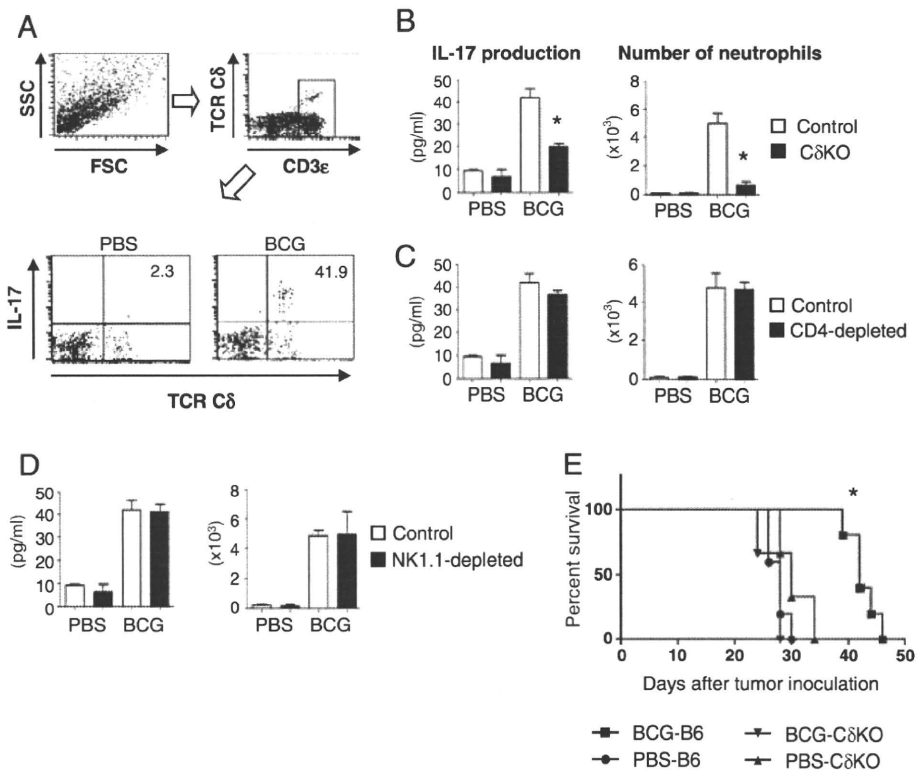

Figure 3. Importance of γδ T cells in IL-17 production, neutrophil infiltration, and the antitumor effect of BCG treatment. (A) Flow cytometric analysis of IL-17 production by the lymphocytes in the bladder of PBS (left)- or BCG (right)-treated mice was performed after brief *in vitro* culture without stimulation. Representative dot plots of IL-17 and TCR Cδ expression are shown after gating on $CD3^+$ lymphocytes. The number in upper right quadrant indicates the percent of $IL\text{-}17^+$ cells in TCR $C\delta^+$ cells. (B) B6 (control) and CδKO mice were intravesically injected with BCG or PBS on days 0, 7, 14, and 21. A group of PBS (control) or BCG-treated B6 mice received repeated i.p. injection of anti-CD4 mAb (C), anti-NK1.1 mAb (D), or the respective isotype control mAbs on days −1, 6, 13, and 20. Depletion of the target cell populations after mAb administration was confirmed by flow cytometry (Supporting Information Fig. 1). The level of IL-17 production (left) and the number of neutrophils (right) were measured on day 22. Each group consisted of five mice. Representative data of three separate experiments are shown. $^*p<0.05$, Student's t-test. (E) B6 and CδKO mice were intravesically inoculated with 1×10^5 MB49 tumor cells on day 0 and were received weekly intravesical injection with BCG or PBS from day 1 to 22. Each group consisted of five mice. Representative data of two separate experiments are shown. $^*p<0.05$ compared with other three groups, log-rank test.

tumor-inhibitory effects of IL-17 [14–17]. Further investigation is necessary to identify factors that dictate anti- *versus* pro-tumor effects of IL-17 [18].

γδ T cells are the major source of IL-17 production after BCG treatment

In order to identify the cell subset(s) responsible for the IL-17 production after BCG treatment, we harvested mononuclear cells in the bladder of BCG- or PBS-treated mice at day 22 and performed flow cytometric analysis of *ex vivo* intracellular staining for IL-17. We detected $CD3^+$ cells producing IL-17 in BCG-treated bladder, and the $IL\text{-}17^+$ cells were mostly TCR $\gamma\delta^+$ (Fig. 3A). To directly address which cell population is important as the source of IL-17, we measured IL-17 production and neutrophil infiltration in the bladder of γδ T-cell-deficient mice (CδKO), and CD4 or NK1.1-depleted mice (Fig. 3B and D). We found that BCG-treated CδKO mice showed significant reduction of IL-17 production and neutrophil infiltration compared with BCG-treated control mice. On the other hand, there was no difference in either IL-17 production or neutrophil count between CD4 or NK cell-depleted mice and the control mice. These results revealed that γδ T cells significantly contributed to IL-17 production that induced recruitment of neutrophlis to the bladder after BCG treatment. Similar to our results, IL-17 production by tumor infiltrating γδ T cells was recently reported in a model of mouse sarcoma, although IL-17 supported tumor progression *via* angiogenesis in this case [19]. In order to define the cellular source of the remaining IL-17 production in BCG-treated CδKO mice, we performed flow cytometric analysis but failed to detect cells positive for IL-17 (data not shown).

We lastly examined the importance of γδ T cells in the antitumor effect of BCG treatment. As shown in Fig. 3E, BCG treatment prolonged the survival of the control B6 mice inoculated with MB49 tumor cells. However, survival of CδKO mice was not improved by BCG treatment. There was also no difference in the survival of PBS-treated WT and CδKO mice, indicating that antitumor effect of γδ T cells depends on BCG treatment. Taken together, these results indicated that IL-17 produced by γδ T cells plays a key role in the recruitment of neutrophlis to the bladder after BCG treatment, which is important for the antitumor effect against bladder tumor. Although the mechanism of IL-17 production by γδ T cells is not fully elucidated yet, an involvement of IL-23-signaling has been suggested [10, 11, 20]. In agreement with this, we detected a significant level of IL-23 production in the bladder after BCG treatment (data not shown). However, because the antigens recognized by γδ T cells remain unclear, possible involvement of antigenic stimulation on BCG-induced IL-17 production by γδ T cells is not excluded.

Concluding remarks

We found in this study that γδ T cells were involved in the antitumor effect of intravesical BCG treatment *via* IL-17 production. Interestingly, Yuasa *et al.* reported that intravesical administration of γδ T cells exerted antitumor activity against bladder tumor, which is thought to be mediated by the direct cytotoxic activity to the tumor cells [21]. Importantly, human γδ T cells are also known for their antitumor effect [22]. Because γδ T cells exert effector function in an MHC-unrestricted manner, these findings suggest that γδ T cells could be a good target of universally applicable immunotherapy against bladder cancer.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from Japan SLC (Hamamatsu, Japan). CδKO and IL-17KO mice (B6 background) were kindly provided by Dr. S. Itohara and Dr. Y. Iwakura, respectively. The mice were bred in specific pathogen-free conditions in our institute. 6- to 8-wk-old female mice were used for the experiments. This study was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine, Kyushu University. Experiments were conducted under the control of the Guideline for Animal Experiment.

Tumor and tumor inplantation

The murine bladder cancer cell line, MB49, was kindly provided by Dr. T. L. Ratliff. The cells were cultured in RPMI-1640 containing 10% FCS at 37°C in a humidified 5% CO_2 atmosphere and passaged 2–3 times weekly.

BCG treatment protocol

We used a well-defined murine syngeneic bladder tumor model [23]. Briefly, mice were catheterized to receive an intravesical inoculate of 1×10^5 MB49 tumor cells on day 0. On days 1, 8, 15, and 22, mice were treated intravesically with either 3×10^6 CFU of BCG Connaught strain (Immucyst, kindly provided by Nippon kayaku, Tokyo, Japan) or PBS. Just after BCG or PBS injection, the urethra of the mice was ligated by 3-0 silk and released 3 h later.

Isolation of neutrophils and lymphocytes for flow cytometric analysis

To harvest neutrophils and lymphocytes, the bladder was minced to yield 1–2 mm pieces and were incubated in a mixture of 1 mg/mL collagenase (Invitrogen, Carlsbad, CA, USA) and 20 μg/mL DNase (Sigma-Aldrich, St. Louis, MO, USA) in RPMI 1640 containing 10% FCS for 90 min at 37°C. The following antibodies were used for flow cytometric analysis: FITC-conjugated anti-Gr-1 (RB6-8C5), anti-TCR Cδ (GL3), and anti-CD4 (RM4-5)

mAbs, PE-conjugated anti-I-A/E (M5/114.15.2), anti-NK1.1 (PK136), anti-CD8 (53-6.7) mAbs, allophycocyanin-conjugated anti-CD3e (145-2C11) mAb (BD Biosciences, San Diego, CA, USA), and PE-conjugated donkey anti-mouse IgG polyclonal antibody (eBioscience, San Diego, CA, USA). Stained cells were run on a FACS Calibur flow cytometer (BD Biosciences) after adding propidium iodide (1 μg/mL) in order to exclude the dead cells. The data were analyzed using Cell Quest software (BD Biosciences).

Intracellular cytokine staining

Freshly isolated lymphocytes from the bladder were immediately incubated with 10 μg/mL befeldin A (Sigma-Aldrich) in RPMI containing 10% FCS at 37°C for 6 h. Cells were first stained with mAbs for surface molecules and were then fixed and permeabilized using BD Perm/Wash solution (BD Biosciences) and stained with PE-conjugated anti-mIL-17 (TC11-18H10.1) mAb (BD Biosciences).

In vivo depletion of neutrophils, $CD4^+$ cells, and $NK1.1^+$ cells

Two hundred micrograms of anti-Gr-1 mAb (RB6-8C5), anti-CD4 mAb (GK1.5), or anti-NK mAb (PK136) or the isotype control mAb was given i.p. every 7 days. Depletion of each cell subset was confirmed by flow cytometric analysis.

ELISA for measurement of IL-17 production

Bladders were dissected from BCG-treated or PBS-treated mice and minced in 200 μL of PBS. After a centrifugation, IL-17 in the supernatant was measured by mouse IL-17 DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Statistical analyses

Survival of mice was evaluated using Kaplan–Meier plots and the log-rank test. Difference in the amounts of IL-17 production or neutrophil counts were analyzed by Student's *t*-test using GraphPad Prism 5.0 software (Prism Graphpad, San Diego, CA, USA). Differences with p values of <0.05 were considered statistically significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviation: **CδKO:** γδ T-cell-deficient

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Audio-Visual Article

Transurethral resection of the bladder tumour (TURBT) for non-muscle invasive bladder cancer: Basic skills

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Abstract: Transurethral resection of the bladder tumour (TURBT) is the standard surgical procedure for non-muscle invasive bladder cancer. We believe that all urologists should be trained in this procedure. This DVD provides an overview of TURBT with particular focus on basic skills, including basic surgical techniques such as the obturator nerve block.

Important basic surgical skills required for complete TURBT in non-muscle invasive bladder cancer are: (i) resection of all visible tumors; (ii) resection of apparently normal mucosa on the border of the tumor; (iii) resection of the muscle layer at the base of the tumor until normal muscle fibers are visible; (iv) in applicable cases, random biopsy of apparently normal urothelium of the bladder wall and transurethral resection (TUR) biopsy of both sides of the prostatic urethra; and (v) when possible, after these procedures are completed, a different operating surgeon should inspect the bladder lumen to confirm that there are no remaining tumors. In particular, sampling resection should be implemented in apparently normal mucosa for approximately 1 cm around the tumor, and at the base of the tumor down to the superficial muscle layer. Resected specimens should be examined histopathologically in order to confirm the absence of malignant findings.

Fundamental procedures for TURBT include both one-stage and two-stage resection. One-stage resection is used for relatively small tumors and involves a single procedure with simultaneous resection of both the tumor and the tissue at the tumor base down to the superficial muscle layer. In the two-stage resection, the first resection exposes the lower level of the mucosa and the second resection removes that lower mucosal layer in order to sample the superficial muscle layer for cancer staging. At the start of the resection, the loop is electrified before it makes contact with the mucosa. Delicate movements of the sheath should be used, along with delicate movement of the loop itself to adjust the depth of resection.

The illustration of surgical techniques shows not only the basic techniques but also some points for caution during the resection. For actual resection, it is important to fully understand the properties of the tumor and to combine these techniques appropriately for each individual resection procedure. When resecting multiple tumors, the same basic resection techniques used for single tumors should be applied, and repeated as necessary. (This is a translated section of a video article originally published in Japanese as a DVD in the Audio-Visual Journal Vol.14 No.1. 2008 by The Japanese Urological Association.)

Video images

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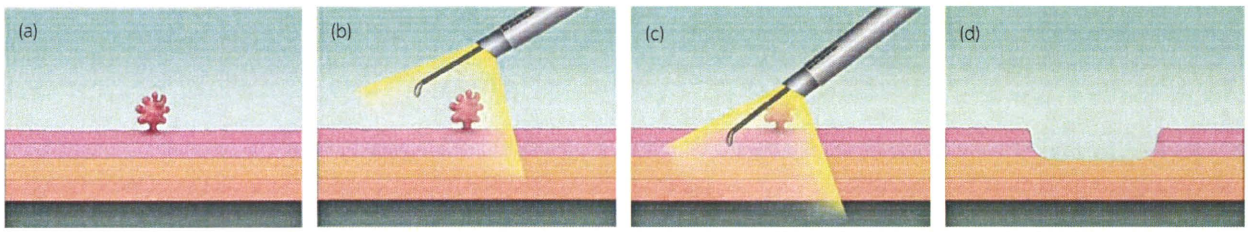

Fig. 1 One-stage resection is used for relatively small tumors. The tumor and the superficial muscle layer, including the tissue around the base of the tumor, are resected together in a single procedure.

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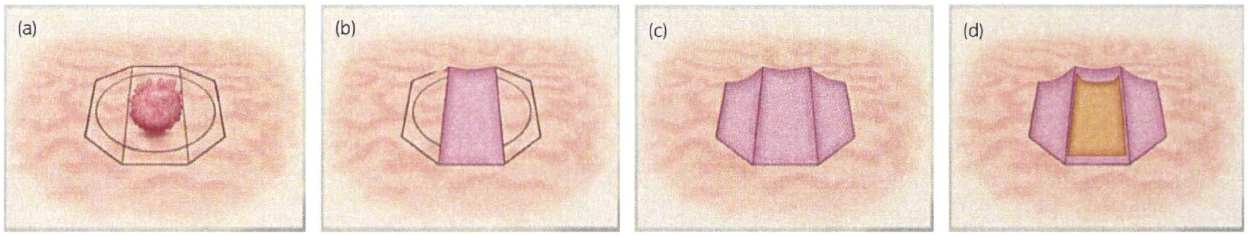

Fig. 2 The tumor and apparently normal mucosa on both sides around the tumor are resected, and then the base of the tumor is also resected.