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Mammary tissue microenvironment determines T cell-dependent breast cancer-associated inflammation

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Key words

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Although the importance of the host tissue microenvironment in cancer progression and metastasis has been established, the spatiotemporal process establishing a cancer metastasis-prone tissue microenvironment remains unknown. In this study, we aim to understand the immunological character of a metastasis-prone microenvironment in a murine 4T1 breast tumor model, by using the activation of nuclear factor-κb (NF-κB) in cancer cells as a sensor of inflammatory status and by monitoring its activity by bioluminescence imaging. By using a 4T1 breast cancer cell line stably expressing an NF-κB/Luc2 reporter gene (4T1 NF-κB cells), we observed significantly increased bioluminescence approximately 7 days after metastasis-prone orthotopic mammary fat-pad inoculation but not ectopic s.c. inoculation of 4T1 NF-κB cells. Such *in vivo* NF-κB activation within the fat-pad 4T1 tumor was diminished in immune-deficient SCID or nude mice, or T cell-depleted mice, suggesting the requirement of host T cell-mediated immune responses. Given the fat-pad 4T1 tumor expressed higher inflammatory mediators in a T cell-dependent mechanism compared to the s.c. tumor, our results imply the importance of the surrounding tissue microenvironment for inflaming tumors by collaborating with T cells to instigate metastatic spread of 4T1 breast cancer cells.

Mounting evidence supports not only cancer cells themselves, but also the surrounding microenvironment, as important determinants for the progression of cancer.[1–3] The mature tumor microenvironment consists of transformed cancer cells and numerous other stromal cells including infiltrating host immune cells.[1,2,4] Such tumor stromal cells have been known to contribute to disease progression through either direct cell–cell interactions or the production of a variety of inflammatory mediators such as cytokines, chemokines, matrix-degrading enzymes and growth factors.[4–7]

Given the link between inflammation and cancer has been established, the accumulating evidence regarding the contribution of the inflammatory microenvironment in tumor progression, including angiogenesis and metastasis, has been explored.[8–10] Clearly, the establishment of an inflammatory tumor microenvironment requires host immune responses and, furthermore, the activation of inflammatory signal cascades within the tumor;[8,11–13] however, the exact behavior of such inflammatory signals and their roles during *in vivo* tumor progression remain unclear. Furthermore, there is almost no definitive evidence of any spatiotemporal requirement for the establishment of an inflammatory microenvironment during tumor growth and progression. While many molecular cues are known to be involved in cellular inflammatory signals, nuclear factor-κB (NF-κB) is certainly known as one of the critical transcriptional factors to regulate the expression of pro-inflammatory genes in many diseases.[10,12,14] Importantly, there is strong evidence that many oncogenes and molecules cause activation of NF-κB. Furthermore, NF-κB is constitutively and /or highly activated in malignant cancer cells and associated with their proliferation, survival, and metastasis.[13,15–17] Thus, the inflammatory signal mediated by NF-κB has been considered as a critical link between inflammation, immunity and cancer progression.

In this study, we aim to understand the physiological process of establishment of the metastasis-prone tumor microenvironment in the light of cancer-associated inflammation, using *in vivo* bioluminescence imaging. Using murine 4T1 cells stably expressing an NF-κB-mediated luciferase reporter gene, we explored the spatiotemporal requirement for establishing cancer-associated inflammation of 4T1 breast cancer *in vivo*. After orthotopic mammary fat-pad inoculation, but not ectopic s.c. inoculation, of 4T1 NF-κB cells, cancer-associated inflammation was preferentially observed, as seen in the increased bioluminescence from 4T1 tumors. Such cancer-associated inflammatory response was diminished in immune-deficient

SCID or nude mice, and further in both $CD4^+$ and $CD8^+$ T cell-depleted mice. Thus, our results indicate the importance of the surrounding tissue microenvironment to determine the degree of cancer-associated inflammation in 4T1 breast tumors by collaborating with T cells.

Materials and Methods

Reagents. Tumor necrosis factor-α (TNF-α) was purchased from PeproTech (Rocky Hill, NJ, USA). pGL4.50 (luc2/CMV/Hygro) vector, pGL4.32 (luc2P/NF-κB-RE/Hygro) vector and D-luciferin were obtained from Promega (Madison, WI, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Hygromycin B was obtained from Nacalai Tesque (Kyoto, Japan).

Cells. Mouse mammary carcinoma 4T1 cells (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 medium containing 10% bovine serum (Nissui, Tokyo, Japan).

Establishment of luciferase-expressing 4T1 breast cancer cell lines. The 4T1 cells stably expressing luciferase with a CMV-promoter (4T1 CMV) and 4T1 cells stably expressing an NF-κB-mediated luciferase gene (4T1 NF-κB) were established as previously described.[18] Briefly, 4T1 cells (5×10^5/well) were seeded in a 6-well plate and transfected with pGL4.50 vector or pGL4.32 vector using Lipofectamine 2000. The cells were selected with Hygromycin B (100 μg/mL) and cloned by limiting dilution. To evaluate the response of NF-κB *in vitro*, 4T1 NF-κB transfectants or 4T1 CMV control cells (1×10^5/well) were cultured in a 96-well plate and treated with TNF-α (100 ng/mL). After 6 h of incubation, luminescence was measured with a multiplate reader (2030 ARVO X; Perkin Elmer Life Science, Boston, MA, USA). *In vitro* characterization of 4T1 CMV or 4T1 NF-κB cells is shown in the Figure S1. There was a clear positive correlation between luciferase activity and cell number in both 4T1 CMV cells (Fig. S1a; $R^2 = 0.9922$) and 4T1 NF-κB cells (Fig. S1b; $R^2 = 0.9862$) under stable culture conditions, therefore, luminescence of both 4T1 CMV cells and 4T1 NF-κB cells represents a viable cell number in the absence of stimulation. *In vitro* TNF-α treatment specifically induced luciferase activity in 4T1 NF-κB cells but not in 4T1 CMV cells (Fig. S1c), confirming the activity of the NF-κB-specific luciferase reporter system.

Experimental mouse model. Inbred wild-type BALB/c mice, BALB/c-nu/nu (nude) mice, and C.B-17/lcrHsd-*Prkdc*^{scid} (SCID) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) or Japan SLC (Tokyo, Japan). All experiments were approved and carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences of the University of Tokyo (Tokyo, Japan) and the Care and Use of Laboratory Animals of University of Toyama (Toyama, Japan). The 4T1 CMV cells or 4T1 NF-κB cells were inoculated s.c. or into mammary fat-pad (i.f.p.). Mice were injected with D-luciferin (150 mg/kg i.p.; Promega) and luminescence was measured with an *in vivo* imaging system (IVIS Spectrum; Caliper Life Sciences, Hopkinton, MA, USA) 20 min after the D-luciferin injection. For $CD4^+$ or $CD8^+$ T cell depletion, mice were injected i.p. with 0.25 mg anti-CD4 antibody (clone GK 1.5) or anti-CD8 antibody (clone 53.6.2) on Day −2, −1, and 7 relative to the tumor implantation. The index of relative induction of luminescence was calculated as: (Day 7 luminescence of 4T1 NF-κB tumor/Day 0 luminescence of 4T1 NF-κB)/(Day 7 luminescence of 4T1 CMV tumor/Day 0 luminescence of 4T1 CMV tumor).

Tumor-infiltrating lymphocyte isolation and flow cytometry. The 4T1 NF-κB cells were inoculated s.c. or i.f.p., and 7 days after inoculation tumor tissues were dissected, minced, and digested with 2 mg/mL collagenase (Roche Diagnostics, Mannheim, Germany) and 0.1 mg/mL DNase I (Roche Diagnostics) in serum-free RPMI-1640 for 1 h at 37°C. Samples were further homogenized through wire mesh and mononuclear cells were isolated by Percoll gradient (30%). For flow cytometry analysis, mononuclear cells were first pre-incubated with CD16/32 (2.4G2) mAb to avoid non-specific binding of antibodies to FcγR. The cells were then incubated with a saturating amount of fluorophore-conjugated mAb. Antibodies to CD3e (2C11), NKp46 (29A1.4), CD4 (GK1.5), CD8 (2.43), CD62L (MEL-14), CD44 (IM7), CD25 (PC61), CD11b (M1/70), CD11c (N418), F4/80 (BM8.1), and CD206 (C068C2) were purchased from Biolegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or Tombo Bioscience (San Diego, CA, USA). Intracellular staining of Foxp3 was carried out using a Foxp3/Transcription Factor Staining Kit (eBio-

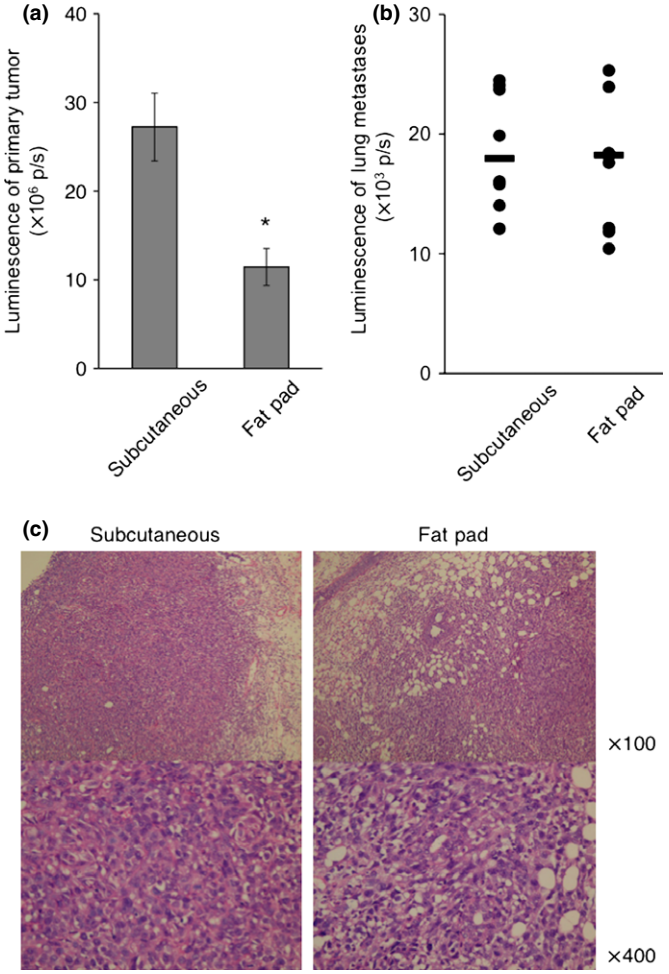

Fig. 1. Effect of distinct tissue microenvironments on the metastatic behavior of 4T1 breast cancer cells. 4T1 CMV cells were inoculated s.c. (right back) or into mammary fat pad with syngenic BALB/c mice (10^5 cells). On day 14, the luminescence of primary tumor (a) or lung metastasis (b) were quantified by the IVIS imaging system. Data are presented as the mean ± SEM. $*P < 0.05$ (c) 4T1 cells were inoculated s.c. or i.f.p. and tumor samples were harvested 7 days after the tumor inoculation. Paraffin-embedded tumor sections were subjected to H&E staining and representative images are shown.

science) according to the kit protocol. Briefly, cells were fixed with the fixation/permeabilization buffer, washed with permeabilization buffer, and incubated with APC-conjugated anti-Foxp3 Ab. Flow cytometry analysis was carried out with a FACS Canto (BD Biosciences, San Jose, CA, USA) and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Histological analysis. The 4T1 NF-κB cells were inoculated s.c. or i.f.p. and then tumor samples were harvested 7 days after tumor implantation. After the animals were killed, tumor samples were immediately fixed with 4% paraformaldehyde for 1–2 days. The tumor sample was then sliced sequentially into sections 3–5 mm thick. Representative sections of the tumor and adjacent tissue 2–3 mm thick were selected and embedded in paraffin for routine histopathological analysis with H&E staining.

Cytokine and chemokine protein array. The 4T1 NF-κB cells were inoculated s.c. or i.f.p., and 7 days after inoculation tumor tissues were dissected, minced, and digested with 2 mg/mL collagenase (Roche Diagnostics) and 0.1 mg/mL DNase I (Roche Diagnostics) in serum-free RPMI-1640 for 1 h at 37°C. Samples were further homogenized through wire mesh and cultured with complete RPMI-1640 medium for 24 h. The cell-free culture supernatants were collected and stored at −80°C. The protein expression of inflammatory cytokines and chemokines was measured by HQPLEX Analyte Kit (Mouse Inflammation 17-Plex; Cellector, San Diego, CA, USA) according to the manufacturer's instructions and data were analyzed using BeadLogic software (Inivai Technologies, Mentone, Australia).

Statistical analysis. All data were obtained from a group of four or five mice and are representative of at least two independent experiments. Data were analyzed for statistical significance using Student's *t*-test. *P*-values < 0.05 were considered significant.

Results

Tissue microenvironment affects metastatic behavior of 4T1 tumors. It has been recognized that the surrounding tissue microenvironment of the primary tumor collaborates with cancer cells for metastasizing to distant organs.[2,6] Murine 4T1 breast cancer cells are known to spontaneously metastasize to multiple distant organs after *in vivo* inoculation either s.c. (ectopic) or into mammary fat pad (orthotopic, i.f.p.). Although 4T1 cells grew more slowly in the orthotopic mammary fat pad tissue compared to s.c. tissue (Fig. 1a), the i.f.p. 4T1 tumor metastasized more efficiently to the lung than the s.c. tumor (Fig. 1b). Interestingly, the tissue architecture of i.f.p. 4T1 tumors and s.c. 4T1 tumors was significantly different (Fig. 1c). While the s.c. 4T1 tumor mostly consisted of cancer cells, the mammary fat-pad 4T1 tumor showed larger tissue heterogeneity, with the involvement of adipocytes and fibrotic lesions (Fig. 1c). These results imply that the mammary fat pad tissue may positively influence 4T1 cancer cells to metastasize to the lung to a greater degree than the s.c. tissue.

Formation of inflammatory microenvironment in mammary fat-pad but not s.c. 4T1 tumor. We next monitored the spatiotemporal changes of luminescence from 4T1 NF-κB cells to investigate the inflammatory status of 4T1 breast cancer cells in a different tumor microenvironment. Considering the tissue origin of 4T1 cells, we implanted 4T1 luciferase reporter cells in the s.c. tissue either orthotopically (mammary fat-pad, i.f.p.) or ectopically (right back, s.c.). Although both 4T1 CMV cells and 4T1 NF-κB cells after s.c. inoculation showed linear increase in the bioluminescence (Fig. 2a), 4T1 NF-κB cells inoculated into mammary fat-pad showed a transient increase in bioluminescence approximately 7 days after tumor inoculation (Fig. 2b). Considering the behavior of the bioluminescence of i.f.p. inoculated 4T1 CMV cells was similar to that of

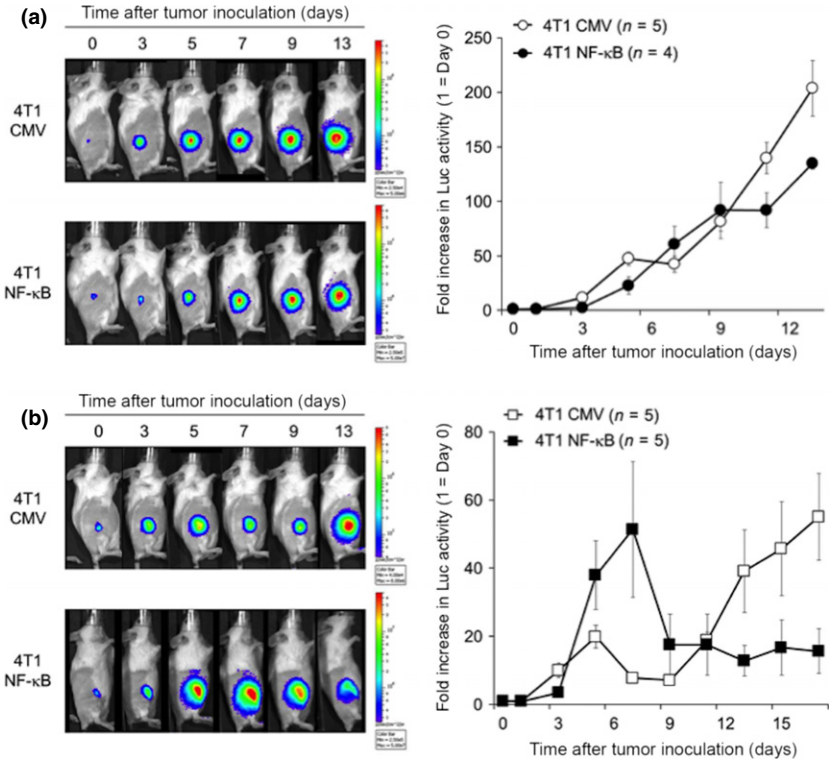

Fig. 2. Time-course bioluminescence imaging of s.c. 4T1 tumor in mice. 4T1 CMV cells or 4T1 nuclear factor-κB cells were inoculated s.c. (right back) (a) or into mammary fat pad (b) with syngenic BALB/c mice and the luminescence was monitored using the IVIS imaging system. The tumor volume of 4T1 CMV cells and 4T1 NF-κB cells were similar (data not shown). The images and time-course of luminescence are shown. The data are presented as the mean ± SEM.

s.c. inoculated 4T1 CMV cells, these results imply that NF-κB activity in 4T1 cells was specifically induced within the mammary fat-pad tissue microenvironment. As the status of NF-κB activity in 4T1 cells is proportional to the bioluminescence of 4T1 NF-κB cells and should reflect the surrounding inflammatory microenvironment, the microenvironment in mammary tissue, but not in ectopic s.c. tissue, most likely determined the inflammatory status of 4T1 breast cancer cells.

Requirement of host immune response for establishing inflammatory 4T1 tumor microenvironment in mammary fat-pad. To determine the requirement of the host immune response for establishing an inflammatory 4T1 tumor microenvironment in the mammary fat-pad, we carried out bioluminescence imaging of 4T1 NF-κB cells in the immune-deficient mice. To normalize the inflammatory status to tumor growth rate in the different immunological environments, the luciferase activity of 4T1 NF-κB cells relative to that of 4T1 CMV cells under different conditions was calculated and represented as an inflammatory induction index (NF-κB/CMV) (Fig. 3). Induction of inflammation was observed in i.f.p. 4T1 tumor, but not in s.c. 4T1 tumor (Fig. 3a); such induction was totally diminished in both SCID mice (Fig. 3b) and nude mice (Fig. 3c) that are deficient in both T and B cells, or T cells alone, respectively. These results clearly indicate the critical requirement of T cell-depen-

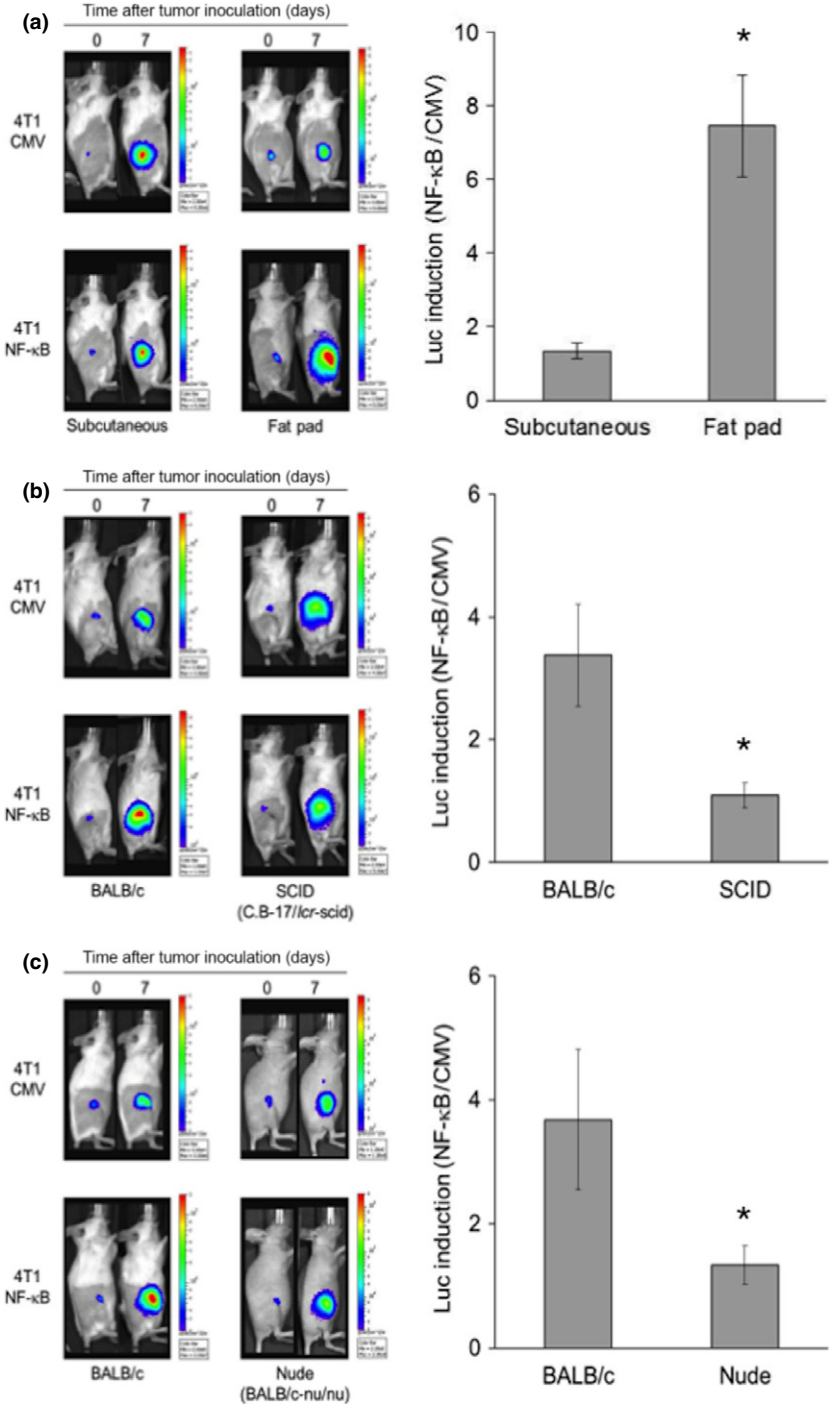

Fig. 3. Requirement of host immune responses for establishing inflammatory 4T1 tumor microenvironment in mammary fat pad. 4T1 CMV cells or 4T1 nuclear factor-κB (NF-κB) cells were inoculated s.c. or into mammary fat pad (i.f.p.) with wild-type BALB/c (a), i.f.p. with SCID (b), or nude mice (c). The images of luminescence on day 0 and 7 after tumor inoculation are shown. The index of relative induction of luminescence in either ectopic or orthotopic 4T1 NF-κB was calculated and is shown as a bar graph. All graphs are shown as a summary of two independent experiments and presented as the mean ± SEM. Total animal numbers: (a) s.c., $n = 8$; fat pad, $n = 10$; (b) BALB/c, $n = 8$; SCID, $n = 10$; (c) BALB/c, $n = 9$; nude, $n = 10$. $*P < 0.05$.

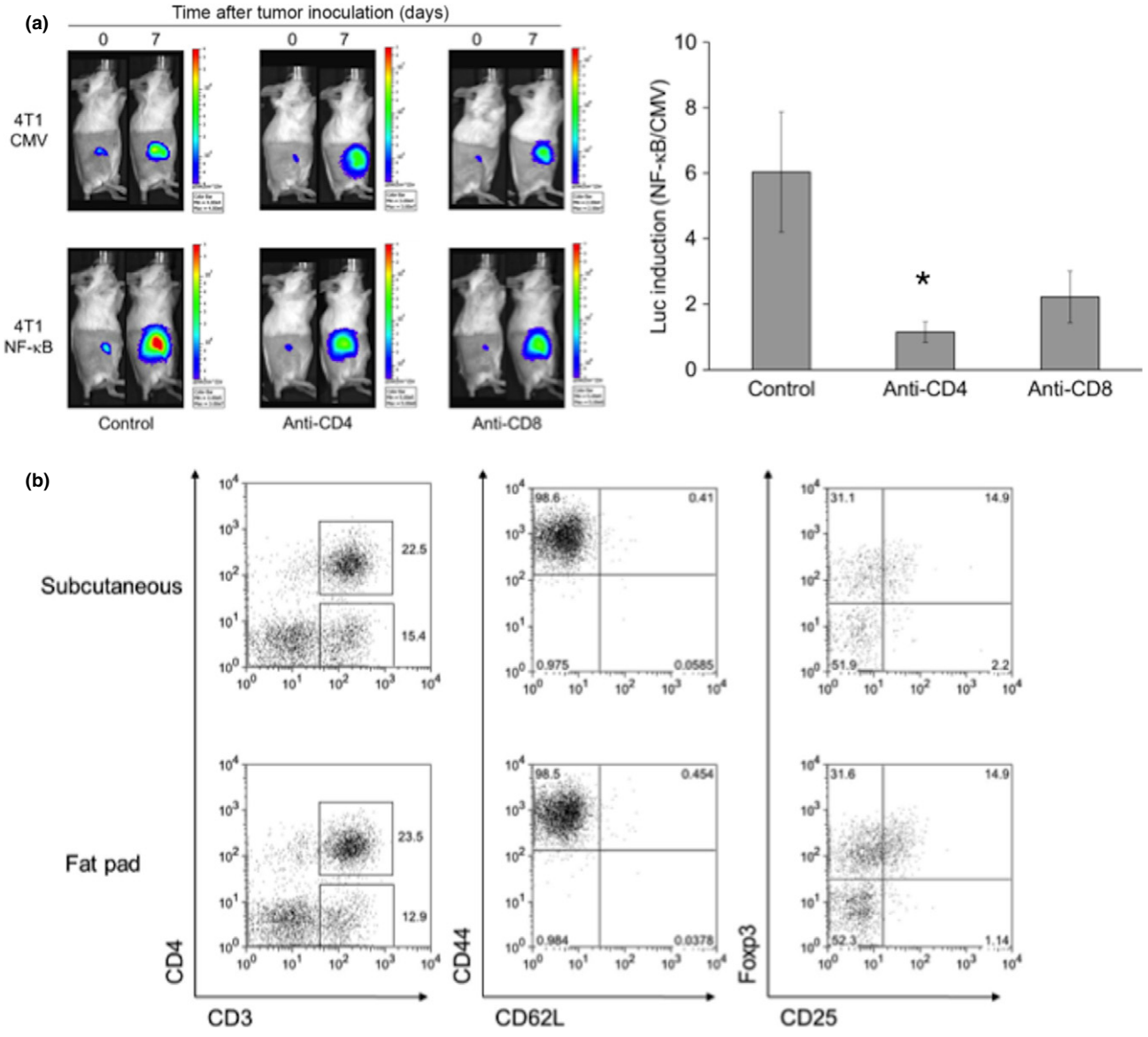

Fig. 4. Tissue microenvironment as a determinant for T cell-dependent inflammation in 4T1 tumor. (a) 4T1 CMV cells or 4T1 nuclear factor-κB (NF-κB) cells were inoculated into mammary fat pad (i.f.p.) in wild-type BALB/c mice. To deplete $CD4^+$ or $CD8^+$ T cells, mice were treated with anti-CD4 antibody or anti-CD8 antibody (250 μg/mouse, i.p.) on day −2, −1, and 7. The images of luminescence on day 0 and 7 are shown. The index of relative induction of luminescence in either control or antibody-treated mice was calculated. The graph is shown as a summary of two independent experiments (total animal numbers: control, $n = 10$; anti-CD4, $n = 10$; anti-CD8, $n = 9$) and presented as the mean ± SEM. (b) 4T1 NF-κB cells were inoculated s.c. or i.f.p. and tumor samples were harvested 7 days after tumor inoculation. Tumor-infiltrating lymphocytes were isolated and subjected to flow cytometry analysis. The dot plots electronically gated on lymphocytes (left), $CD4^+$ T cells (middle and right, $CD4^+$ $CD3^+$) are shown. $*P < 0.05$.

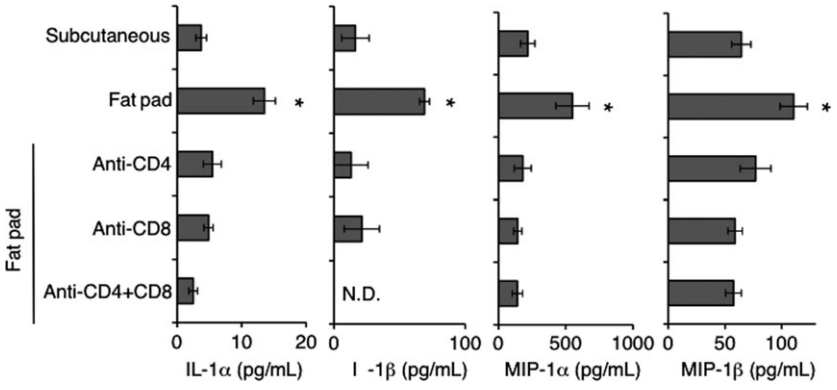

Fig. 5. T cell-dependent induction of inflammatory mediators within mammary fat pad 4T1 tumor microenvironment. 4T1 nuclear factor-κB cells were inoculated s.c. or into mammary fat pad and tumor samples were harvested 7 days after tumor inoculation. To deplete $CD4^+$ or $CD8^+$ T cells, mice were treated with anti-CD4 antibody or anti-CD8 antibody (250 μg/mouse, i.p.) on day −2 and −1. Tumor samples were harvested on day 7 and cells were cultured for 24 h to collect the supernatant. All graphs are presented as the mean ± SEM. $*P < 0.05$. IL, interleukin; MIP, macrophage inflammatory protein; N.D., no data.

dent host immune responses for establishing an inflammatory 4T1 tumor microenvironment.

Mammary tissue microenvironment as a determinant for T cell-dependent inflammation in 4T1 tumor. Given that a T cell requirement was indicated (Fig. 3), we then further determined which subset of T cells is involved in establishing an inflammatory 4T1 tumor microenvironment within the mammary fat pad. Using anti-CD4 mAb or anti-CD8 mAb depletion, we observed a significant reduction of NF-κB activation in mammary fat-pad 4T1 tumor in CD4-depleted mice (Fig. 4a). Although CD8 depletion was not statistically significant ($P = 0.084$), there was a substantial reduction of NF-κB activation in CD8-depleted mice (Fig. 4a). These results support that predominantly $CD4^+$ subsets of T cells were required for the establishment of an inflammatory 4T1 tumor microenvironment *in vivo*. To explain the tissue specificity of mammary fat-pad for establishing an inflammatory 4T1 tumor microenvironment in the context of T cell immune responses, we then compared i.f.p. 4T1 tumors and s.c. 4T1 tumors for the differences in the infiltration of T cells into the tumors. Contrary to the apparent differences in the inflammatory status of 4T1 cells, as seen by imaging analysis, there were no differences in either total infiltration of T cells, the population of effector memory $CD4^+$ T cells ($CD44^+$ $CD62L^-$), or the population of regulatory $CD4^+$ T cells ($Foxp3^+$ $CD25^{+/-}$) between i.f.p. and s.c. 4T1 tumor-infiltrating lymphocytes (Fig. 4b). In addition to the above cellular analysis of 4T1 tumor infiltrating T cells, the spatial localization of T cells within the tumor tissue was examined by immunohistochemistry, however, this did not show any apparent difference (data not shown). These results suggest that the presence of T cells within the 4T1 tumor microenvironment was critical but not solely responsible for establishing an inflammatory 4T1 tumor microenvironment in mammary tissue.

To further explore the molecular mechanism that is responsible for establishing an inflammatory 4T1 tumor microenvironment in mammary tissue collaborating with T cells, we used the inflammatory cytokine/chemokine protein array to determine the factors involved in such T cell-dependent inflammation in mammary 4T1 tumor. Of 17 cytokines /chemokines measured, the expression level of interleukin-1 (IL-1) and macrophage inflammatory protein (MIP) were significantly higher in the fat-pad 4T1 tumor compared to the s.c. tumor (Fig. 5). Given T cell depletion diminished such induction of both IL-1 and MIP expression within fat-pad 4T1 tumor, the upregulation of those inflammatory cytokines and chemokine expression requires both mammary tissue microenvironment and T cell-dependent immune responses. In accordance with such higher expression of inflammatory mediators, we also observed higher frequency of the tumor-associated macrophage (TAM) population ($CD11b^+$ $F4/80^+$ cells) in fat-pad 4T1 tumor compared to s.c. tumor (Fig. 6) Considering there were no differences in the cell surface phenotype of both fat-pad and s.c. TAMs (Fig. 6), the higher expression of inflammatory mediators, IL-1 and MIP, and/or infiltration of TAMs likely correspond to more inflamed 4T1 breast tumor microenvironment within orthotopic mammary tissue compared to ectopic s.c. tissue.

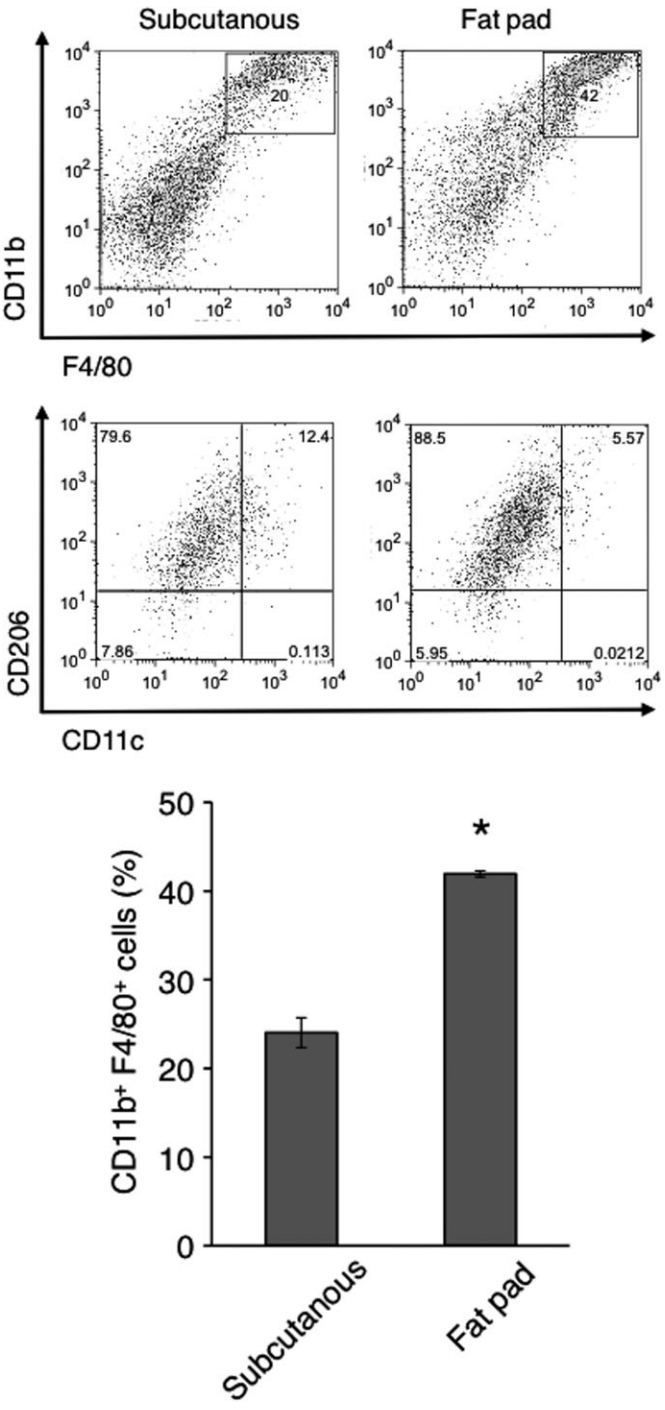

Fig. 6. Distinct tissue infiltration of tumor-associated macrophages into 4T1 tumor. 4T1 nuclear factor-κB cells were inoculated s.c. or into mammary fat pad and tumor samples were harvested 7 days after tumor inoculation. Tumor-infiltrating lymphocytes were isolated and subjected to flow cytometry analysis. The dot plots electronically gated on myeloid cells (top) or macrophages (middle, $CD11b^+$ $F4/80^+$) are shown. The bar graphs are presented as the mean ± SEM. $*P < 0.05$.

Discussion

Although the importance of the tissue microenvironment and its inflammatory status have been recognized as an essential component of tumor progression and metastasis,[8,9,11,14] the spatiotemporal process for establishing tissue-dependent cancer-associated inflammation is not well understood. In the present study, by establishing *in vivo* bioluminescence imaging of the NF-κB status in 4T1 breast cancer cells as a sensor of inflammation, we showed the requirement of the tissue micro-

environment for establishing an inflammatory 4T1 tumor in collaboration with T cell-dependent immune responses.

Our results strongly indicate that the mammary tissue microenvironment can contribute to determining the inflammatory status of 4T1 breast cancer cells collaborating with the host immune system, particularly with $CD4^+$ T cells (Figs 3,4). Notably, it is also suggested from our results that the immune response *per se* is not sufficient for establishing mature cancer-associated inflammation, but rather a heterogeneous interaction between immune cells, cancer cells, and tissue stromal cells seems to be required. Even though the precise underlying mechanism for establishing an inflammatory microenvironment in mammary fat-pad 4T1 tumors is not clear, there have been studies that suggest various players might be involved in cancer-associated inflammation, such as TAMs or cancer-associated fibroblasts, together with tumor-infiltrating lymphocytes.[4,6]

In the specific context of the breast cancer tissue microenvironment, much attention has been given to mammary adipocytes for playing a key role in tumor progression.[19] Accumulating experimental evidence has shown that obesity increases the risk of cancer and adipocytes secrete high levels of pro-inflammatory mediators called adipokines, including monocyte chemo-attractant protein-1, TNF-α, IL-6, IL-8, and leptin.[19–22] Such adipokines are known to induce inflammatory signals in cancer cells, including NF-κB-mediated signals, and to play an important role in cancer-associated inflammation.[19,23] Moreover, a recent study showed that adipocytes were able to differentiate into fibroblast-like cells within the tumor microenvironment and promote tumor progression and fibrosis in a manner similar to cancer-associated fibroblasts.[24] Other studies showed that adipocytes promoted tumor cell growth, invasion, and metastasis through adipokines.[25–30] In addition, tumor-promoting macrophage recruitment and angiogenesis were also induced by tumor-associated adipocytes.[31] Considering we observed upregulation of inflammatory mediators, such as IL-1 or MIP, and higher frequency of TAM populations in fat-pad 4T1 tumor compared to s.c. tumor (Fig. 6), and greater tissue heterogeneity in the mammary fat pad, as seen in the involvement of adipocytes and fibrotic lesions (Fig. 1c), we speculate that a 4T1 tumor can be inflamed by collaborating with the mammary tissue microenvironment and T cell-dependent immune response to facilitate the malignant behavior of 4T1 cells.

Regarding the physiological importance of the inflammatory tumor microenvironment, many previous studies have implied that pathological inflammation contributes to tumor progression and metastatic spread to distant organs.[11,32] For instance, systemic inflammation induced by lipopolysaccharide injection has been shown to promote cancer cell invasiveness and therefore increase lung metastasis *in vivo*.[33,34] In our present findings, the luminescent signal from 4T1 CMV tumors seems to transiently decrease approximately 5–7 days after i.f.p. but not s.c. inoculation of 4T1 cells (Fig. 2). Considering the signal from the 4T1 CMV tumor directly reflects the number of viable cancer cells, it is possible that the inflammatory 4T1 tumor microenvironment could be a result of endogenous protective antitumor immune responses against 4T1 cancer cells. Although further study is clearly required to elucidate the details of the molecular mechanisms, our present findings postulate an important clue, that distinct tissue environments and /or tissue architecture might have different impacts on immune responses to cancer cells, facilitating the establishment of tumor-promoting inflammation. Alternatively, it is possible that the combination of tissue microenvironment and immune response is required for inducing the inflammatory tumor microenvironment. Nevertheless, further investigation is clearly required to understand the exact determinant for establishing the metastasis-prone inflammatory microenvironment.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Establishment of nuclear factor-κB-dependent luciferase gene expressing 4T1 breast cancer cells.