Cancer Science
Japanese Cancer Association JCA
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IL-17A-producing CD30$^+$ Vδ1 T cells drive inflammation-induced cancer progression

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Key words
CD30, IL-17, IL-1β, neutrophil, γδ T cell

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Funding Information
Grant-in-Aid for Scientific Research (C) 26430158, the MEXT (Y.H.) and a Research Grant of Tokyo Biochemical Research Foundation (TBRF).

Received May 5, 2016; Revised June 29, 2016; Accepted July 4, 2016

Cancer Sci **107** (2016) 1206–1214

doi: 10.1111/cas.13005

Although it has been suspected that inflammation is associated with increased tumor metastasis, the exact type of immune response required to initiate cancer progression and metastasis remains unknown. In this study, by using an *in vivo* tumor progression model in which low tumorigenic cancer cells acquire malignant metastatic phenotype after exposure to inflammation, we found that IL-17A is a critical cue for escalating cancer cell malignancy. We further demonstrated that the length of exposure to an inflammatory microenvironment could be associated with acquiring greater tumorigenicity and that IL-17A was critical for amplifying such local inflammation, as observed in the production of IL-1β and neutrophil infiltration following the cross-talk between cancer and host stromal cells. We further determined that γδT cells expressing Vδ1 semi-invariant TCR initiate cancer-promoting inflammation by producing IL-17A in an MyD88/IL-23-dependent manner. Finally, we identified CD30 as a key molecule in the inflammatory function of Vδ1T cells and the blockade of this pathway targeted this cancer immune-escalation process. Collectively, these results reveal the importance of IL-17A-producing CD30$^+$ Vδ1T cells in triggering inflammation and orchestrating a microenvironment leading to cancer progression.

The tumor microenvironment (TME) has been increasingly recognized as a critical regulator of malignant progression of cancer cells driving their growth, survival and dissemination.[1] Recent evidence strongly suggests that the inflammatory microenvironment supports the generation of tumor and disease progression, including metastatic dissemination.[2] Thus, inflammatory TME can be a novel therapeutic target of cancer, and understanding the precise mechanism by which such inflammation can be triggered and maintained should be clinically relevant.

To date, the role of inflammation in cancer development has been studied using chemically induced and spontaneous carcinogenesis models.[3] Although those studies provide us with important information regarding the mechanism of inflammation during cancer initiation and development, it remains unclear whether inflammation can actively drive cancer cells to obtain malignant phenotypes. Immune promotion of cancer malignancy is particularly obvious in cancers with chronic inflammation, since the failure to resolve the initial inflammatory response results in the continuous recruitment of inflammatory cells to the TME.[3] Those inflammatory cells often release genotoxic factors, such as reactive oxygen species to accelerate the alteration of cancer cell genetics and promote malignancy. However, it is very important to understand the other cellular and molecular mechanisms by which such chronic inflammation in TME can drive malignant progression.

In contrast to anti-cancer immune mediators such as interferon-gamma (IFN-γ), the role of immune cytokines that promote malignant progression is less understood. It has been well established that IL-17A contributes to the various aspects of acute inflammation by inducing the subsequent release of inflammatory cytokines, such as IL-1, IL-6 or IL-8, and the accumulation of neutrophils into the site of inflammation.[4] Although IL-17A is also known to contribute to chronic inflammation,[5] there is limited information regarding its importance in the late stage of inflammation. Since the frequency of IL-17A-producing cells is generally very low in the pathology of chronic inflammation, it is believed that IL-17A is produced acutely and orchestrates molecular alteration of the surrounding cells to maintain chronic inflammation. In terms of the source of IL-17A, Th17 cells were discovered to promote chronic inflammation and tissue damage in

autoimmune diseases.[5] The earliest source of IL-17A is critical for tissue stress responses and host defense against infectious diseases.[6] Early IL-17A-mediated immune responses are induced within hours, so innate immune cells are the likely source of IL-17A.[6] Among those innate sources of IL-17A, γδT cells have been shown to be important IL-17A-producing cells during autoimmune inflammation and infectious diseases.[7,8] γδT cells also have an important role in tissue surveillance, particularly in epithelial barrier tissues, such as mucosa, skin and lung.[9–11] The functional character of γδT cells is mostly influenced by their unique development and selection process in the thymus.[12,13] In contrast to the definitive role of IL-17A and γδT cells in inflammation and protection against infectious diseases, respectively, it is still controversial whether IL-17A and γδT cells play a positive or negative role in cancer progression.[14]

The progression of cancer cells refers to the conversion of benign cells into malignant cells through acquiring more aggressive characteristics such as genetic instability, increased tumorigenicity and increased metastatic ability.[1,15] To understand the contribution of inflammation to cancer progression, we established a unique *in vivo* model for investigating malignant progression of a benign tumor cell line, QR-32, by exposing it to chronic inflammatory immune responses.[16] QR-32 is derived from 3-methyl-cholanthrene (MCA)-induced BMT-11 fibrosarcoma cells and is poorly tumorigenic and non-metastatic when injected in normal syngeneic C57BL/6 (B6) mice.[17] However, when pre-malignant QR-32 cells are co-implanted with an inflammation initiator, such as a gelatin sponge, the inflammation not only promotes the local growth of the implanted QR-32 cells, but also converts them into highly aggressive cells with enhanced tumorigenicity and metastatic ability *in vivo*.[16] In a series of studies, we have demonstrated the importance of neutrophils[18] and oxidative stress pathways[19,20] in achieving progression of QR-32 cells following chronic inflammation. Considering that extra-cellular matrices (ECM) are well known to initiate wound healing processes[21] as well as tumor-associated inflammation in many physiologically relevant animal models or humans,[22,23] our model potentially mimics such an ECM-rich microenvironment that might initiate inflammation around pre-malignant QR-32 cells.

Here, we have dissected the sequential events and participating cells in the inflammation and malignant progression of cancer cells by using the aforementioned *in vivo* model. We found that IL-17A was a critical cue for escalating cancer cell malignancy by amplifying the local inflammation through production of IL-1β and neutrophil infiltration and cross-talk between cancer and host stromal cells. The source of this IL-17A was a γδT cell subset expressing Vδ1 semi-invariant TCR and the production was IL-23-dependent and MyD88-dependent. Finally, we identified CD30 as a key molecule regulating the inflammatory function of Vδ1T cells and the blockade CD30–CD153 interactions prevented malignancy. Collectively, these results reveal the importance of IL-17A-producing $CD30^+$ Vδ1T cells in triggering inflammation and orchestrating a microenvironment leading to cancer progression.

Materials and Methods

Mice. Wild-type C57BL/6 (B6) mice were purchased from CLEA Japan (Tokyo, Japan). IFN-$\gamma^{-/-}$ (IFN-γ KO), IL-$17^{-/-}$ (IL-17 KO) and IFN- $\gamma^{-/-}$ IL-$17^{-/-}$ (IFN- γ/IL-17 DKO) mice on B6 background were kindly provided by Dr Y. Iwakura (Tokyo University of Science, Chiba, Japan) and maintained at the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo. MyD$88^{-/-}$ (MyD88 KO) mice on B6 background were kindly provided by Dr S. Akira (Osaka University, Osaka, Japan) and maintained at the animal facility Graduate School of Pharmaceutical Sciences, University of Tokyo. p$19^{-/-}$ mice (IL-23 KO mice) on B6 background were generated as described previously[24] and maintained at the Department of Immunoregulation, Institute of Medical Science, Tokyo Medical University. In some experiments, groups of mice were treated with either anti-γδTCR mAb (UC7-13D5, 250 μg/mouse)[25] or anti-CD153 (RM153, 250 μg/mouse)[26] on day −1, day 0 and subsequently every 3–4 days. All experiments were approved and performed according to the guidelines of the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences of the University of Tokyo, the Care and Use Committee of the Laboratory Animals of the University of Toyama and the Animal Care and Use Committee of the Institute of Medical Science of the University of Tokyo.

Tumor malignant progression model. Tumor malignant progression model was performed as previously described.[16] Briefly, a subcutaneous pocket reaching up from a 10-mm incision to the thorax on the flank of the pelvic region was made in mice. Sterile gelatin sponge (Spongel [Astellas Pharma, Tokyo, Japan]) cut into 10 × 5 × 3 mm pieces was inserted into the pocket and the wound was closed with a sterile clip. QR-32 cell line was originally derived from MCA-induced BMT-11 fibrosarcoma cells, and was maintained and authenticated as previously described.[22–26] QR-32 cells (4–5 × 10^5 cells) in 100 μL PBS were injected into the pre-inserted gelatin sponge. Tumor growth was measured by a caliper square measuring along the longer axes (a) and the shorter axes (b) of the tumors. Tumor volumes (mm^3) were calculated using the following formula: tumor volume (mm^3) = $ab^2/2$.

To monitor *in vivo* proliferation of QR-32 cells, we established QR-32 cells stably expressing luciferase (QR-32-Luc2) as previously described.[27] Briefly, QR-32 cells were transfected with pGL4.50 vector or pGL4.32 vector using Lipofectamine 2000 and cells were selected with Hygromycin B (100 μg/mL), followed by cloning with the limiting dilution method. For measuring *in vivo* luminescence, mice were injected with D-luciferin (Promega, Madison, WI, USA, 150 mg/kg i.p.) and analyzed with an *in vivo* imaging system (IVIS Spectrum; Caliper Life Sciences, Waltham, MA, USA) 20 min after the D-luciferin injection.

Collection and analysis of gelatin sponge-infiltrating cells. Gelatin sponge implanted with 100 μL PBS or QR-32 cells (4 × 10^5 cells) in mice was excised and digested for 1 h at 37°C with 2 mg/mL collagenase (Roche, Basel, Switzerland) in serum-free RPMI. Obtained gelatin sponge-infiltrating cells were used in other experiments. Gelatin sponge-infiltrating cells were stained in the presence of anti-CD16/32 (2.4G2), with some of the following antibodies: anti-NK1.1 (PK136), CD3ε (145-2C11), CD4 (RM4-5), γδTCR (GL3), CD27 (LG.3A10), CCR6 (29-2L17), CD103 (2E7), CD11b (M1/70), Ly-6G (1A8), F4/80 (BM8), CD25 (7D4) and/or Siglec-F (E50-2440) antibodies purchased from Biolegend (San Diego, CA, USA) or BD Pharmingen (San Jose, CA, USA). Flow cytometry analysis was performed by FACSAria cell sorter, or FACS Canto (BD Bioscience, San Jose, CA, USA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). To analyze the intracellular level of IL-17, gelatin sponge-infiltrating cells were incubated in the presence of GolgiStop (BD Bioscience) for 4 h without *in vitro*

stimulation. After surface marker staining, cells were fixed and permeabilized with the BD Cytofix/Cytoperm Kit (BD Bioscience) according to the manufacturer's instructions. Then, cells were stained with anti-IL-17 (TC11-18H10.1) antibody purchased from Biolagend and analyzed by flow cytometry.

Isolation and analysis of gelatin sponge-infiltrating γδT cells. Gelatin sponge-infiltrating cells were first incubated with biotin-conjugated anti-CD11b antibody and then with Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD11b^+$ cells were removed from gelatin sponge-infiltrating cells by autoMACS (Miltenyi Biotec) and $NK1.1^-$ $CD3^+$ $\gamma\delta TCR^+$ cells were sorted by FACS Aria cell sorter so that the purity would be >90%. Total RNA was extracted from isolated gelatin sponge-infiltrating γδT cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and reverse-transcribed into cDNA by SuperScript III (Invitrogen, Carlsbad, CA, USA). cDNA was amplified by TaKaRa LA Taq (Takara, Shiga, Japan) under the following conditions: denaturation at 95°C, annealing at 55°C and extension at 72°C for 40 cycles. Real-time PCR was performed on the StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using Fast SYBR Green Master Mix (Applied Biosystems). mRNA expression level was normalized by the amount of β-actin mRNA. Products were visualized on 2% ethidium bromide-stained agarose gels with Image Quant LAS 4010 (GE Healthcare, Little Chalfont, UK).

Analysis of cytokine production. Gelatin sponge-infiltrating cells were cultured for 24 h in complete RPMI 1640 medium without *in vitro* stimulation. Supernatants were collected and cytokine concentration was measured by Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad, Hercules, CA, USA) and Bio-Plex 200 Systems (Bio-Rad). IL-1β or IL-17 concentration was also measured using the Mouse IL-1β or IL-17 ELISA MAX Standard Kit (Biolegend), respectively. All obtained values were normalized as the concentrations of cytokines produced from gelatin sponge-infiltrating cells in 0.1 g of gelatin sponge.

Statistical analysis. Data were analyzed by two-tailed, unpaired Student's *t*-test or a Mann–Whitney test and a P-value < 0.05 was considered significant.

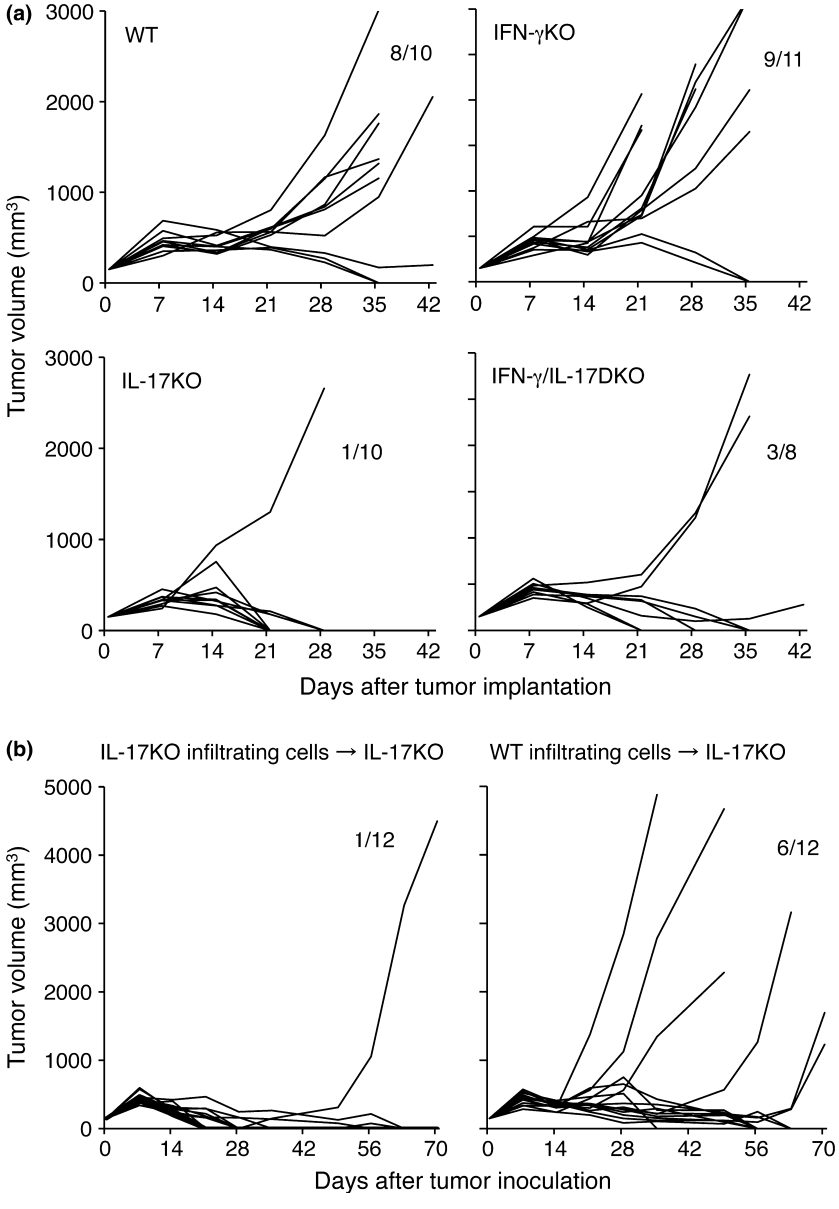

Fig. 1. IL-17-producing inflammatory cells are the critical component to promote gelatin sponge-induced malignant progression of QR-32. (a) Wild-type (WT), IFN-γ KO, IL-17 KO or IFN-γ/IL-17 DKO B6 mice were inoculated with QR-32 and gelatin sponge. Tumor volumes on the indicated days after QR-32 inoculation were measured. Tumor incidences are shown upper right of each panel. (b) Gelatin sponge-infiltrating cells were collected from IL-17KO or WT mice 7 days after the implantation of gelatin sponge alone. Cells were transferred into IL-17 KO mice inoculated with QR-32 and gelatin sponge, and tumor volumes on the indicated days were measured. Tumor incidences are shown upper right of each panel.

Results

Critical role of IL-17A in the inflammation-induced cancer progression. To study the contribution of inflammation to cancer progression, we employed a unique *in vivo* model as described previously.[18,19] QR-32 murine fibrosarcoma cells are poorly tumorigenic and non-metastaic when injected into normal syngeneic C57BL/6 (B6) mice; however, they grow progressively under the *in vivo* inflammatory microenvironment induced by co-implantation with the inflammation initiator (gelatin sponge). More importantly, cell lines that are established from such growing tumors acquire not only greater tumorigenicity but also metastatic ability *in vivo* (Fig. S1).

By using this model, we first examined the role of inflammatory cytokines, specifically IFN-γ and IL-17A, in the process of inflammation-associated cancer malignant progression. While the benign QR-32 cells grow progressively in wild-type (WT) or IFN-γ-deficient (IFN-γKO) B6 mice by co-implantation with gelatin sponge (Fig. 1a, upper panels), such inflammation-induced progression was not observed in either IL-17A-deficient (IL-17KO) or IFN-γ/IL-17A-double deficient (IFN-γ/IL-17DKO) mice (Fig. 1a, lower panels), suggesting the critical role of IL-17A in this process. To further understand whether IL-17A needs to be produced locally or systemically, we injected QR-32 cells into IL-17KO mice together with the inflammatory cells that infiltrated into the gelatin sponge implanted in either WT or IL-17KO mice. As shown in Figure 1(b), the inflammatory cells from WT mice, but not IL-17KO mice, were able to support the progression of QR-32 cells; therefore, we concluded that IL-17A production within the local TME could be sufficient for the progression of QR-32 cells.

Host immunity is regarded as an important determinant for editing immunogenicity of cancer cells. To determine whether the inflammatory immune response triggered by IL-17A shapes the immunogenicity of QR-32 cells, we established the series of cell lines from *in vivo* growing QR-32 tumors in WT or IFN-γKO mice, and examined their metastatic ability and the expression of matrix metalloproteinases (MMP). Consistent with our previous studies,[18,19] cell lines of *in vivo* growing QR32 tumors established from WT mice (GS1-12) showed greater tumorigenicity and metastatic ability along with the upregulation of MMP-2 and MMP-9 expression (Fig. S2, upper panels). Importantly, the cell lines established from IFN-γKO mice (GKOGS1-6) also displayed greater *in vivo* metastatic ability and MMP expression (Fig. S2, lower panels), suggesting that the inflammatory microenvironment triggered by IL-17A could facilitate the cancer malignancy of QR-32 cells rather than selecting the immunogenic escape variants of them.

Importance of IL-17A for chronic inflammation leading to cancer progression. IL-17A is known as a key cytokine for chronic inflammation, which is believed to be an important component of the cancer-promoting microenvironment. By using

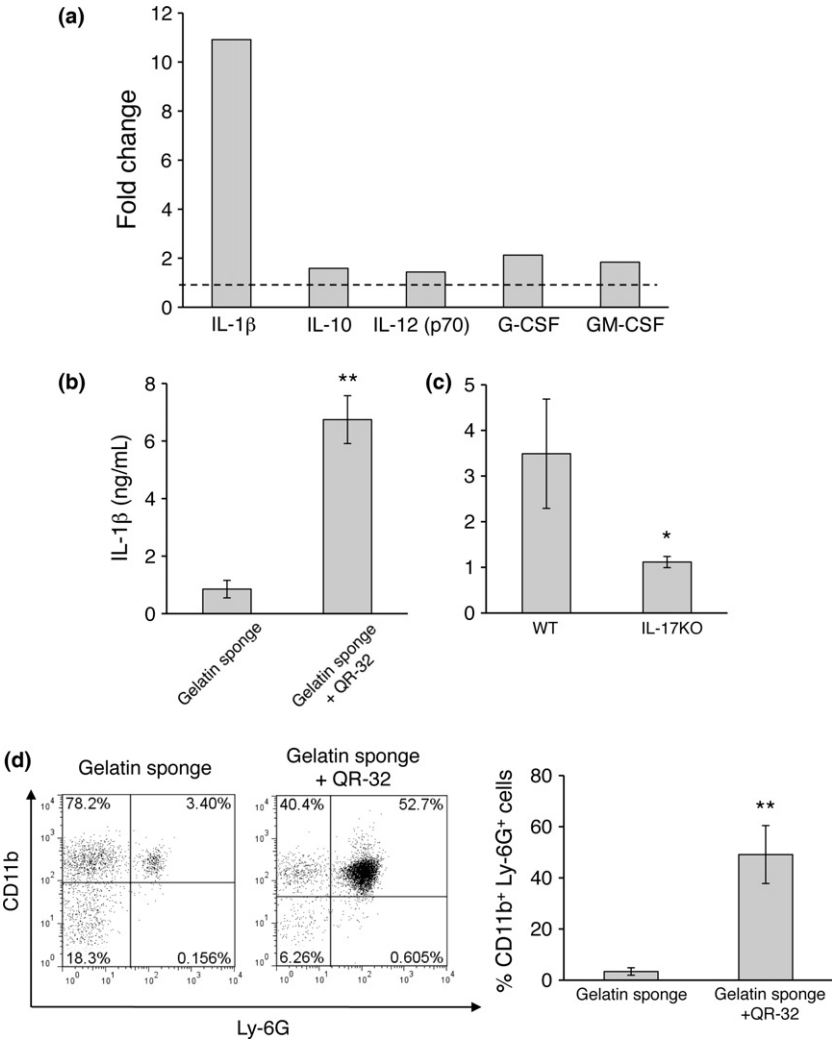

Fig. 2. Requirement of IL-17 for the amplification of inflammation leading to malignant progression of QR-32 cells. Gelatin sponge-infiltrating cells were collected 7 days after the implantation of gelatin sponge alone or with QR-32. (a) Cytokine concentration in culture supernatant of gelatin sponge-infiltrating cells was measured by Bio-Plex assay. Fold change = the average concentration when gelatin sponge and QR-32 were co-implanted/the average concentration when gelatin sponge alone was implanted. Cytokines of produced more than fold change >1 are shown. Dotted line represents where the fold change = 1. (b) IL-1β production into the culture supernatant of gelatin sponge-infiltrating cells were measured by ELISA. Data are shown as mean ± SEM. $^{**}P < 0.01$. (c) Gelatin sponge-infiltrating cells were collected from wild-type (WT) or IL-17 KO mice 7 days after the co-implantation of gelatin sponge and QR-32. IL-1β production into the culture supernatant of gelatin sponge-infiltrating cells were measured by ELISA. Data are shown as mean ± SEM. $^{*}P < 0.05$. (d) Gelatin sponge-infiltrating cells were collected 7 days after the implantation of gelatin sponge alone or with QR-32. The representative plots of flow cytometry analysis of inflammatory cells are shown (left) and the proportions of $CD11b^+$ $Ly\text{-}6G^+$ cells are shown as mean ± SEM (right). $^{**}P < 0.01$.

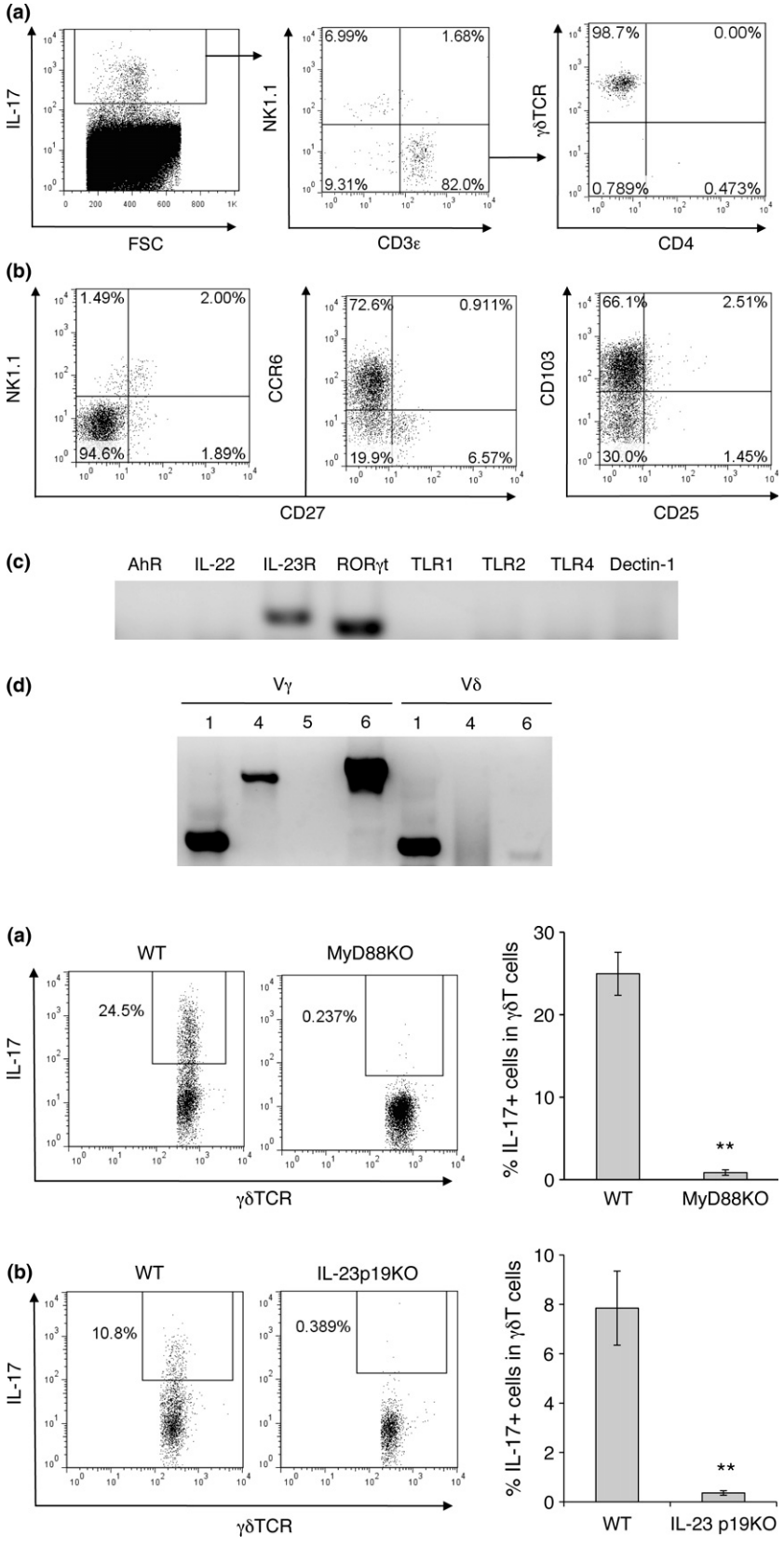

Fig. 3. Identification and characterization of γδT cells triggering malignant progression of QR-32 cells. (a) Gelatin sponge-infiltrating cells were collected 4 days after gelatin sponge implantation and incubated in the presence of GolgiStop without *in vitro* stimulation. IL-17-producing cells were determined by flow cytometry analysis. (b) Gelatin sponge-infiltrating cells were collected 4 days after gelatin sponge implantation. Expressions of the cell-surface markers related to IL-17-producing γδT cells were analyzed by flow cytometry. Plots gated on NK1.1$^-$ CD3$^+$ γδTCR$^+$ cells are shown. (c, d) NK1.1$^-$ CD3$^+$ γδTCR$^+$ cells were isolated from gelatin sponge-infiltrating cells by cell sorting. mRNA expressions of the lineage markers of IL-17-producing γδT cells (c) and γδT cell receptor repertoire (d) were determined by RT-PCR analysis.

Fig. 4. Requirement of MyD88-IL-23 axis for IL-17 prodution by Vδ1 T cells. Gelatin sponge-infiltrating cells were collected 4 days after gelatin sponge implantation from wild-type (WT) and MyD88KO (a) or IL-23 p19KO (b) B6 mice and were incubated in the presence of GolgiStop without any further *in vitro* stimulation. IL-17 production by γδT cells were analyzed by flow cytometry analysis. Plots gated on NK1.1$^-$ CD3$^+$ γδTCR$^+$ cells are shown. Data are shown as mean ± SEM. $^{**}P < 0.01$ as compared to WT.

bioluminescent imaging, we traced the exact behavior of QR32 cells during inflammation-induced progression and classified cell lines established from *in vivo* growing QR-32 tumors with different periods of exposure to inflammatory TME (Fig. S3a). While most of the cell lines (Fig. S3b, late progressors) acquired higher tumorigenicity, the cell lines established from rapidly growing QR-32 tumors (marked as E, Fig. S3a) showed very weak tumorigenicity (Fig. S3b, early progressor).

These results might implicate that the degree of exposure to inflammatory TME determines the degree of malignancy in QR32 cells.

We then next characterized the type of inflammatory TME triggered by IL-17A, which led to the progression of QR-32 cells, by profiling the expression of 17 different inflammatory cytokines and chemokines in the tumor samples. Among 5 cytokines whose expression was upregulated within an early inflammatory TME (Fig. 2a), we found that IL-1β production was highly amplified in the presence of QR-32 cells (Fig. 2b). Such amplification of IL-1β within TME was IL-17A-dependent (Fig. 2c), and associated with the massive infiltration of $CD11b^+$ $Ly\text{-}6G^+$ neutrophils (Fig. 2d). Collectively, these results suggest that IL-17A plays a dominant role in sustaining inflammatory TME, as seen in the amplification of IL-1β production and the infiltration of neutrophils.

Vδ1 T cells drive cancer-associated inflammation by producing IL-17A. To determine the source of IL-17A driving inflammation-induced cancer progression, we analyzed the gelatin sponge-infiltrating cells by flow cytometry. As shown in Figure 3(a), IL-17A-producing cells within the gelatin sponge-infiltrating cells were mostly γδT cells, but not $CD4^+$ Th17 cells. Such IL-17A-producing γδT (γδT17) cells were a $NK1.1^-$ $CD27^-$ γδT subset with heterogeneous expression of CCR6 and CD103 (Fig. 3b). We then further characterized the γδT17 subset in its lineage marker expression and γδ TCR repertoire by RT-PCR analysis. Given the γδT17 subset predominantly expressed RORγt and IL-23 receptor (Fig. 3c), and Vδ1 chain with skewed Vγ chain (Vγ1, 4 and 6, as shown in Fig. 3d), we concluded the semi-invariant Vδ1 T cells as a source of IL-17A within the gelatin sponge-infiltrating cells. Given the IL-17A production from gelatin-sponge infiltrating γδT cells was diminished in both IL-23-deficient and MyD88-deficient mice (Fig. 4), the MyD88-IL-23 axis is critically involved for Vδ1 T cells to produce IL-17A. IL-17A appeared not to be involved in either γδT cell infiltration into gelatin sponge (Fig. S4a) or the production of IL-1β from QR-32 cells (Fig. S4b). Collectively, these results implicate that Vδ1T cells are the innate-programmed γδT cell subset and trigger sterile inflammation by producing IL-17A in an MyD88/IL-23-dependent manner.

CD30 as a key molecule of Vδ1 T cells to produce IL-17A for driving cancer progression. Given that Vδ1T cells are a key source of IL-17A to initiate cancer-promoting inflammation, we seek for the molecular mechanism by which regulate Vδ1T cell activation to develop a new therapeutic target for cancer progression. Contrary to the conventional splenic γδT cells, profound expression of CD30 on $CD27^-$Vδ1T cells was observed (Fig. 5a). Importantly, the functional blocking of CD153, a ligand of CD30, largely impaired the IL-17A and IL-1β production within TME (Fig. 5b). These data indicate the importance of CD30–CD153 pathway in the IL-17A production by Vδ1T cells. Indeed, the *in vivo* blockade of CD153 compromised the progression of QR-32 cells, as for IL-17KO mice (Fig. 6). Importantly, using the Prognoscan database, significant correlation was found between the expression of CD30 and CD153 and the disease progression in several different types of cancer patients (Fig. S5). Collectively, these results

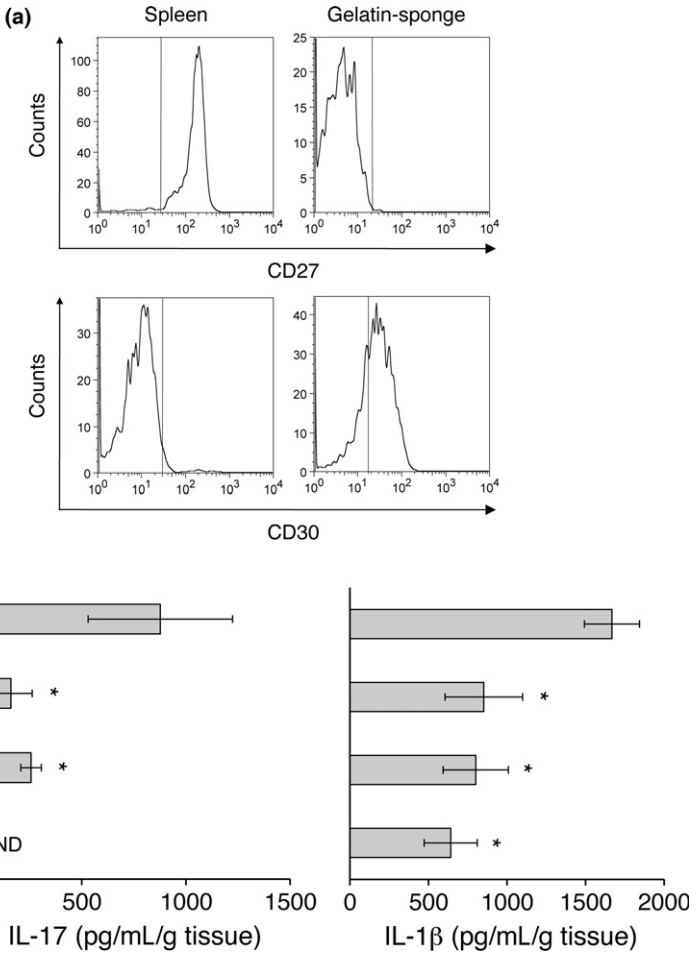

Fig. 5. Functional importance of CD30 on Vδ1 T cells. (a) Splenocytes or gelatin sponge-infiltrating cells were collected 4 days after gelatin sponge implantation. Expressions of CD27 or CD30 on γδT cells were analyzed by flow cytometry. Histograms gated on $NK1.1^-$ $CD3^+$ $\gamma\delta TCR^+$ cells are shown. (b) Groups of wild-type (WT) mice were treated with either anti-γδTCR (anti-γδTCR) or anti-CD153 (anti-CD153) mAb on day −1, 0 and 3. Gelatin sponge-infiltrating cells were collected 7 days after the co-implantation of gelatin sponge and QR-32 in WT or IL-17 KO mice and cultured for 24 h *in vitro*. IL-17 (left panel) or IL-1β (right panel) production in the culture supernatant of gelatin sponge-infiltrating cells were measured by ELISA. Data are shown as mean ± SEM. $*P < 0.05$ as compared to untreated WT mice. ND, not detectable.

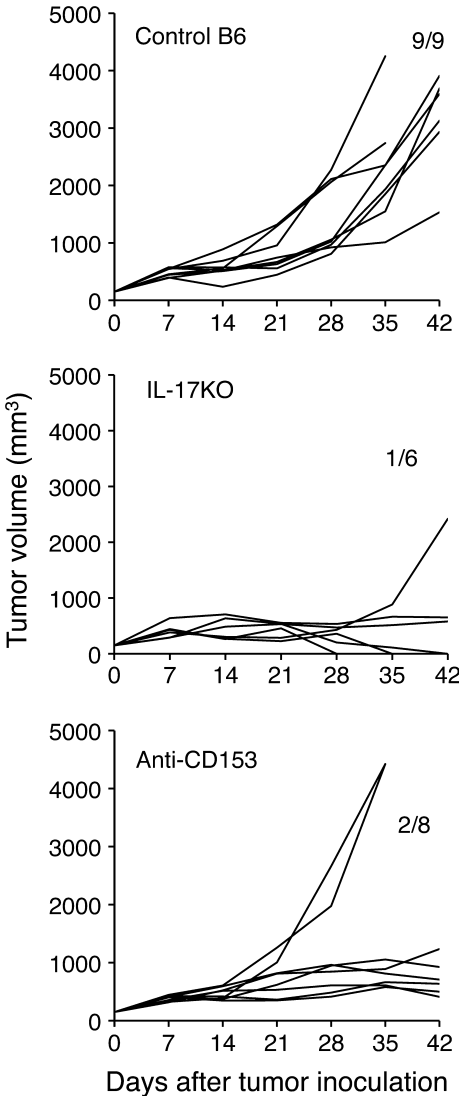

Fig. 6. CD30 as a regulatory molecule of Vδ1 T cells leading to cancer malignant progression. Wild-type (WT) B6 (Control B6) or IL-17 KO mice were inoculated with QR-32 and gelatin sponge. Group of mice were treated with anti-CD153 (Anti-CD153) mAb on day −1, 0 and subsequently every 3–4 days. Tumor volumes on the indicated days after QR-32 inoculation were measured. Tumor incidences are shown upper right of each panel.

indicate that CD30 could be a key molecule for triggering inflammation-induced cancer progression through regulating Vδ1T cell function.

Discussion

Inflammatory TME has been increasingly recognized as a key for survival, growth and metastatic dissemination of cancer cells.[15] Thus, the appropriate control of immunological TME can be a novel therapeutic strategy of cancer. In the present study, we have revealed that Vδ1 semi-invariant γδT cells trigger tumor-promoting inflammation by producing IL-17A, and CD30 on Vδ1T cells is a key regulatory molecule for the IL-17A production. IL-17A produced by $CD30^+$ Vδ1 T cells amplifies local inflammation at TME, as seen in the IL-1β production and neutrophil infiltration, which actively contributes to the progression of cancer cells (Fig. 7).

While the critical role of IFN-γ in cancer immunity has been established, the role of IL-17A, and whether it acts as a pro-cancer or anti-cancer cytokine, has been controversial. In general, inflammation is considered to be important for both elimination and escalation of malignancy in cancer disease. Given that the pro-tumor context of inflammation in our model was extensively studied previously,[16–19] we sought to clarify the role of IFN-γ and IL-17A in the inflammation-induced cancer progression. Importantly, we demonstrated the association between cancer cell malignancy and the degree of exposure to *in vivo* inflammation by using real-time imaging, which implies the importance of chronic local inflammation in achieving cancer progression. Together with the previous studies,[28] our present results clearly show that IL-17A is a critical player for establishing tumor-promoting inflammation.

The role of IL-17A in the cancer microenvironment has been extensively studied in regard to both its direct action on cancer cells and its indirect action on surrounding stromal cells. The typical mechanism of IL-17A to enhance tumor growth is known to induce IL-6 production by cancer cells and stromal cells, which, in turn, activates oncogenic STAT3 pathway to upregulate the proliferation and/or the expression of pro-survival and pro-angiogenic genes.[28] Considering there was no alteration in QR-32 cells cultured with rIL-17A *in vitro* (Fig. S4b and data not shown), it is less likely that IL-17A directly contributed to the QR-32 cell proliferation *in vivo*. Alternatively, it has been widely accepted that IL-17A contributes to both the acute and chronic inflammation by inducing the production of G-CSF, CXCL8 and IL-6 to amplify neutrophil infiltration and the release of other pro-inflammatory effectors such as IL-1.[4] Indeed, we observed the significant amplification of IL-β and G-CSF production, which are known to be important for neutrophil proliferation and survival, within TME (Fig. 2a) and such amplification of IL-1β was IL-17A-dependent (Fig. 2c). Although the neutrophil recruitment into TME was independent of IL-17 in our model (data not shown), the absence of IL-17 largely compromised IL-1β production in TME (Figs 2c,5b). Considering that neutrophil is a well-known source of IL-1β, we presume that Vδ1T cell-derived IL-17A may contribute to the qualitative change of neutrophils to produce IL-1β, rather than induce the recruitment of neutrophils into TME.

It has been known that much of the IL-17A released during an early inflammation is derived from T cell subsets except for Th17 and/or innate immune cells.[6] Among those, the IL-17-producing γδT (γδT17) cell subset is known as innate IL-17A-producing cells and plays an important role in tissue surveillance, mostly in the epithelial barrier, such as gut, lung and skin.[9,11,29] $CD27^-$ $NK1.1^-$ γδT cells were shown to constitutively express a transcription factor RORγt, known as a lineage marker of IL-17A-producing cells, and display a stable functionality in the periphery to produce IL-17.[7,30,31] In the context of γδ TCR usage of γδT17 cells, previous studies revealed that there is a skewed expression of Vδ and Vγ chains in the γδT17 cell subset.[32–34] In concert with those findings, the gelatin sponge-infiltrating γδT17 cells displayed $CD27^-$ $NK1.1^-$ phenotype and preferentially expressed Vδ1 chain and RORγt (Fig. 3c,d). Furthermore, the gelatin sponge-infiltrating Vδ1T cells also express IL-23 receptor and required MyD88-IL-23 axis to produce IL-17A (Figs 3d,4). This observation is consistent with the previous finding that γδT17 cells produce IL-17A in response to IL-23 alone, a cytokine known to expand and/or stabilize Th17 cells and produced by TLR-MyD88 pathway.[33] Given that the tissue-resident Vδ1T cells

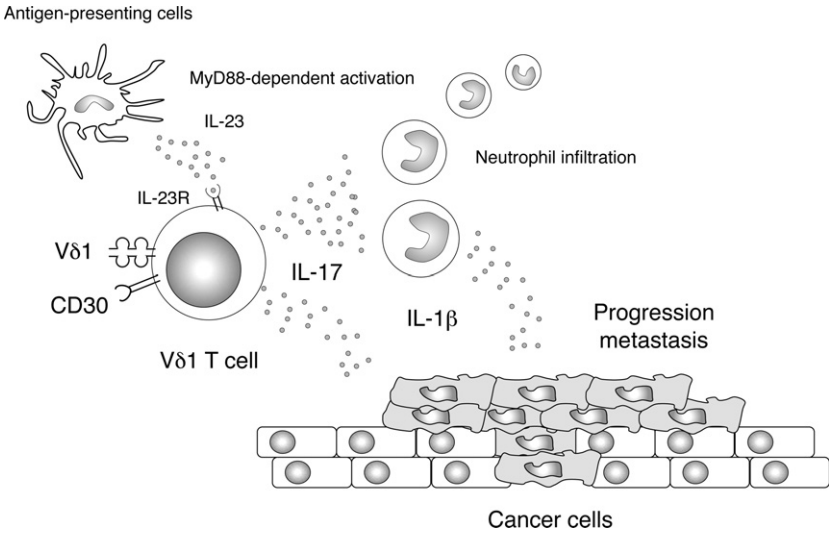

Fig. 7. Possible mechanism of inflammation-induced cancer progression driven by IL-17A-producing CD30$^+$ Vδ1 T cells. Schematic illustration of the possible mechanism that CD30$^+$ Vδ1 T cells drive the malignant progression of QR-32 cells by producing IL-17A in an MyD88/IL-23-dependent manner.

have been considered as an early source of IL-17A in murine infectious diseases through the IL-23-dependent mechanism to control subsequent neutrophil infiltration,[35] we therefore speculate that a similar mechanism is involved in triggering inflammation within TME for cancer progression.

In addition to the TCR and lineage markers, the expressions of other cell surface markers are also known to distinguish γδT17 cells from other subsets of γδT cells, including chemokine receptor CCR6,[31] IL-2 receptor α chain CD25[36] and integrin αE chain CD103.[37] Although none of those clearly distinguish the gelatin sponge-infiltrating Vδ1T cells (Fig. 3), we found that CD30 was exclusively expressed on the Vδ1T cells and functionally important for the IL-17A and IL-1β production within TME (Fig. 5). CD30 is a member of the TNF receptor superfamily and was originally proposed as a marker for Th2 cells.[38] CD30 is also well recognized as a marker for hematological malignancies, including Hodgkin's lymphoma cells.[39] There are a few reports regarding the functional role of CD30 on γδT cells in both mouse and human models. The function of CD30 for γδT cell activation was originally presented in human γδT cell clone[40] and subsequently followed by a finding in murine mucosal Vγ6 γδT17 cells.[41] The interaction between CD30 and CD153, a ligand of CD30, is supposed to be important for the peripheral maintenance and activation of γδT cells.[41] Considering that the blockade of CD30 pathway by anti-CD153 mAb compromised the QR-32 progression (Fig. 6), we propose the importance of targeting CD30–CD153 interaction to control Vδ1 T cell-dependent cancer-promoting inflammation. Importantly, by using the Prognoscan database, we found that the expression of CD30 and CD153 in cancer tissues significantly correlated with the disease progression of patients in several cancer types (Fig. S5). Thus, we believe those data also support the clinical importance of our present findings.

There are some challenges remaining because our results are largely dependent on the unique inflammation-associated tumor progression model. However, ECM have been known to initiate tumor-associated inflammation as well as the wound healing process in many physiologically relevant animal models and even in clinical observations.[21,23] Indeed, cancer has often been regarded as a wound that never heals. Thus, we strongly believe our model is not totally non-physiological because gelatin sponge implantation may mimic such ECM-rich TME to initiate inflammation. In alignment with our presented data, recent work also highlights the importance of γδT cells as a source of IL-17A to promote breast cancer metastasis.[35] Nevertheless, our presented findings reveal the importance of IL-17A-producing CD30$^+$ Vδ1T cells in triggering inflammation and orchestrating a microenvironment leading to cancer progression.

Acknowledgments

We are grateful to Makoto Arita, Kaori Denda-Nagai and Nobuaki Higashi for valuable discussions, and to Satomi Yoshinaga, Setsuko Nakayama and Asuka Asami for technical assistance. We also thank Shizuo Akira for kindly providing mice, and Yasunobu Yoshikai and Kensuke Shibata for providing the reagent and advice. This work was supported by grants from a Grant-in-Aid for Scientific Research (C) 26430158, the MEXT (Y.H.) and a Research Grant of Tokyo Biochemical Research Foundation (TBRF).

Disclosure Statement

Daniel J. Cua is an employee of Merck & Co. All other authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Schematic illustration of *in vivo* malignant progression model by using QR32 murine fibrosarcoma.

Fig. S2. Low tumorigenic cell line QR-32 acquires highly malignant phenotype after exposure to IL-17-dependent inflammatory response.

Fig. S3. Higher tumorigenicity of late progressors compared to early progressors of QR-32 cells.

Fig. S4. No involvement of IL-17 in the recruitment of γδT cells into the inflammatory microenvironment associated with the malignant progression of QR-32.

Fig. S5. Clinical association of CD30 and CD30L in the progression of cancer patients.