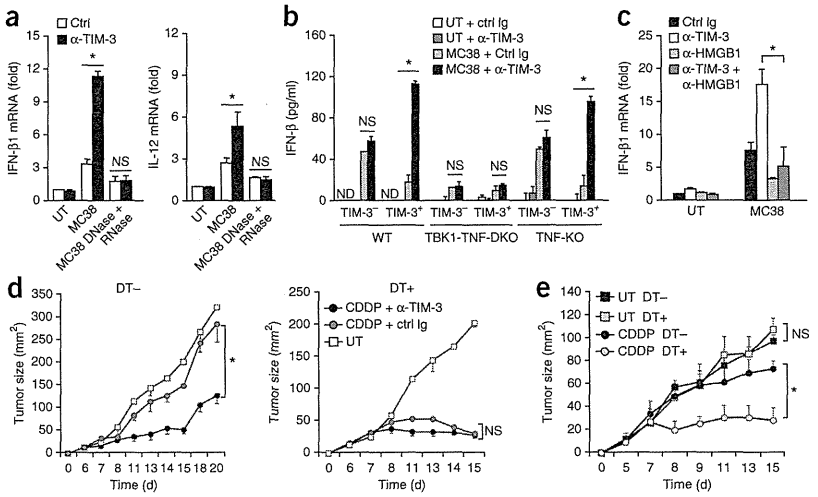

Figure 8 TIM-3 impedes the antitumor effects of chemotherapy. (**a**) Quantification of IFN-β1 and IL-12 mRNA in TIM-3$^+$ BMDCs cultured alone (UT) or together with apoptotic CDDP-treated MC38 cells with (MC38 DNase + RNase) or without (MC38) pretreatment with DNase and RNase in the presence of control immunoglobulin or mAb to TIM-3; results are presented relative to *Actb* expression. (**b**) ELISA of IFN-β1 in TIM-3$^-$ and TIM-3$^+$ wild-type BMDCs (WT), BMDCs deficient in TBK1 and TNF (TBK1-TNF-DKO) or TNF-deficient BMDCs (TNF-KO) cultured alone or together withdying MC38 cells in the presence of control immunoglobulin or mAb to TIM-3. (**c**) RT-PCR quantification of IFN-β1 and IL-12 mRNA in wild-type TIM-3$^+$ DCs cultured alone or together with dying MC38 cells in the presence of control immunoglobulin or mAb to TIM-3 and/or anti-HMGB1 (key); results are presented relative to *Actb* expression. (**d**) Tumor growth in CD11c-DTR mice given no diphtheria toxin or treated with diphtheria toxin, then inoculated subcutaneously with MC38 cells along with systemic CDDP in the presence of mAb to TIM-3 or control immunoglobulin, or no treatment. (**e**) Tumor growth in chimeras reconstituted with a mixture (1:1) of bone marrow cells from CD11c-DTR and TIM-3-deficient mice, then given no treatment with diphtheria toxin or treated with diphtheria toxin 2 d before inoculation with MC38 cells, followed by no treatment or CDDP on days 8, 10 and 12 after inoculation. $^*P < 0.05$ (paired Student's *t*-test). Data are representative of four experiments (**a**), five experiments (**b**) or three experiments (**c–e**; error bars, s.e.m.).

We further examined the involvement of HMGB1 in TIM-3-mediated suppression through the use of HMGB1-deficient MEFs. Transfection of HMGB1-deficient MEFs to express TIM-3 had little inhibitory effect on cytokine production after stimulation with B-DNA, but the addition of recombinant HMGB1 protein restored the TIM-3-mediated suppression (**Fig. 7f**). Furthermore, treatment with neutralizing antibody to HMGB1 abrogated the nucleic acid–mediated responses elicited by blockade of TIM-3 in TIM-3$^+$ DCs to the amount in cells treated with control immunoglobulin (**Fig. 7g**). Together these findings showed that TIM-3 may have negatively regulated HMGB1-mediated activation of the innate immune response to nucleic acids.

TIM-3 suppresses the antitumor effects of chemotherapy

Published studies have shown that some cytotoxic therapies may induce a form of immunogenic cell death in which the release of danger-associated molecules such as HMGB1 from dying tumor cells provides an endogenous adjuvant[36,37]. In addition, published evidence has shown that the immunogenicity of cancer vaccines is boosted by the simultaneous release of nucleic acids from dying tumors, which engenders the generation of protective antitumor immunity[38]. We therefore hypothesized that the TIM-3-mediated regulation of nucleic acid–sensing systems may modulate the antitumor immunity induced by chemotherapy-mediated 'immunogenic cell death'. To define the role of DC-derived TIM-3 in restraining cytotoxic agent–induced innate immune responses, we cultured TIM-3$^+$ DCs together with dying MC38 cells exposed a chemotherapeutic agent, in the presence of mAb to TIM-3. Treatment with mAb to TIM-3 resulted in more cytokine-encoding mRNA in TIM-3$^+$ DCs loaded with dying MC38 tumor cells treated with cisplatin (cis-diamminedichloroplatinum(II) (CDDP)) than did treatment with control immunoglobulin (**Fig. 8a**). The responses induced by mAb to TIM-3 were abrogated by the pretreatment of dying tumor cells with DNase and RNase (**Fig. 8a**), which indicated the involvement of nucleic acids. The responses triggered by treatment with supernatants of CDDP-treated MC38 cells, which contained free nucleic acids released from dying tumor cells, were also regulated through the nucleic acid–mediated innate sensing systems by TIM-3 (**Supplementary Fig. 7a**).

The kinase TBK1 serves as a critical regulator of nucleic acid–mediated innate immunity[7]. Consistent with the importance of TBK1 in this response, cytokine induction by dying tumors was suppressed in DCs deficient in both TBK1 and tumor-necrosis factor (TNF) relative to its induction in wild-type DCs or TNF-deficient DCs, and mAb to TIM-3 had little effect on IFN-β1 expression in DCs deficient in both TBK1 and TNF (**Fig. 8b**). These results indicated a critical contribution of TBK1 to these responses. Consistent with the role of TIM-3 in interfering with HMGB1-mediated nucleic acid–sensing pathways, blockade of TIM-3 in TIM-3$^+$ DCs cultured with CDDP-treated dying MC38 tumor cells did not result in more IFN-β in the presence of neutralizing antibody to HMGB1 (**Fig. 8c**).

In vivo treatment with mAb to TIM-3 had little additive effect on CDDP-induced antitumor activity against MC38 tumors in CD11c-DTR mice depleted of DCs (**Fig. 8d**). Thus, the *in vivo* antitumor effects elicited by the combination of TIM-3 blockade and chemotherapy resulted mainly from the suppression of endogenous DC activity. Notably, depletion of CD11c$^+$ cells alone substantially retarded the tumor growth elicited by treatment with CDDP (**Fig. 8d**), which indicated that TADCs served as the main suppressors of antitumor responses. Furthermore, the antitumor effects triggered by mAb to TIM-3 and CDDP were substantially impaired in CD11c-DTR mice not treated with diphtheria toxin by treatment with neutralizing antibody to IFN-IR and to IL-12 (**Supplementary Fig. 7b**), which suggested that type I interferon and IL-12 served as the main effectors of the execution of antitumor immunity by CDDP and mAb to TIM-3. Furthermore, CDDP was more effective against MC38 tumors in diphtheria toxin–treated TIM-3-deficient CD11c-DTR mice than in TIM-3-deficient CD11c-DTR mice not treated with diphtheria toxin (**Fig. 8e**), which indicated that TIM-3 on endogenous DCs served as a major repressor of the antitumor effects of chemotherapy. Thus, our results have indicated that DC-specific TIM-3 served as a negative regulator of chemotherapy-induced

antitumor responses by circumventing the nucleic acid–mediated innate immune pathways (**Supplementary Fig. 8**).

DISCUSSION

We have shown here that tumor-infiltrating DCs suppressed antitumor immune responses through TIM-3-mediated negative regulation of innate immune responses to nucleic acids. DC-derived TIM-3 interacted with HMGB1 to suppress the transport of nucleic acids into endosomal vesicles, thus attenuating the antitumor efficacy of DNA vaccines and cytotoxic chemotherapy by antagonizing nucleic acid–sensing systems. Our findings have demonstrated an additional mechanism by which DC-derived TIM-3 serves as a unique repressor of antitumor responses by targeting nucleic acids required for antitumor responses mediated by PRRs. More generally, whether myeloid cell–specific TIM-3 has a role in restraining innate immune responses to viral infection must be evaluated.

The gene encoding TIM-3 underwent substantial upregulation and reached higher expression and earlier expression in DCs than in $CD8^+$ T cells in tumor microenvironments. Indeed, TIM-3 expression on $CD8^+$ T cells increased gradually at later time points of tumor growth, in agreement with studies showing that TIM-3 expression reflects the phenotype of exhausted $CD8^+$ T cells at the chronic phase of tumors[16,39]. Our findings also showed high expression of TIM-3 by tumor-infiltrating myeloid cells other than DCs, such as tumor-associated macrophages. Whether TIM-3 regulates innate immune responses in different ways depending on the myeloid cell type should be explored in future studies.

The interaction between galectin-9 and TIM-3 on antigen-presenting cells has a positive role in DC maturation and the cross-priming of tumor-specific T cells[15,23]. In contrast, TIM-3 on DCs has a protumorigenic role and 'turns down' nucleic acid–mediated innate immune responses via a galectin-9-independent but HMGB1-dependent mechanism. We speculate that DC-derived TIM-3 serves as a dual regulator of innate and adaptive immunity depending on the microenvironment. In tumor microenvironments, which are characterized by impaired induction of galectin-9 and high expression of HMGB1, TIM-3 on DCs may suppress innate immune responses by interfering with HMGB1-mediated stimulation of nucleic acid sensing, whereas TIM-3 maintains the anergic status of $CD8^+$ T cells in a tumor antigen–specific manner. These coordinated actions of TIM-3 on innate and adaptive responses may incapacitate efficient tumor immunosurveillance during all phases of tumorigenesis. In contrast, whereas DC-derived TIM-3 facilitates efficient containment of infectious agents by 'preferentially' interacting with galectin-9-enriched infectious microenvironments, TIM-3 on $CD8^+$ T cells has evolved to suppress antigen-specific responses and thus prevents excess tissue inflammation during the recovery phase after infection. In this context, the functional relevance of TIM-3 may be very different in cancer versus infection. This raises the possibility that proper concentrations of galectin-9 in tumor microenvironments may be crucial for controlling tumorigenesis and for overriding the TIM-3-mediated suppression of innate immune responses. Indeed, studies have shown that treatment with exogenous galectin-9 elicits antitumor responses by expanding and activating plasmacytoid DC-like macrophage and natural killer cell populations[40].

We also found that TIM-3 may have disrupted the recruitment of nucleic acids into the endosomal pathway by interfering with HMGB1 function, which led to impaired TLR- and cytosolic sensor–mediated innate immune responses. Although tumor cells are mainly composed of mutated or normal 'self' nucleic acids, which are usually sequestered in the nucleus or mitochondria under physiological conditions, tumor microenvironments often acquire the ability to create an endogenous inflammatory milieu composed of damage-associated molecules such as HMGB proteins and uric acid[41]. Indeed, we found more HMGB1 in tumor tissues than their non-tumor counterparts. Such inflammatory signals could act together with tumor-derived nucleic acids to gain access to endosomal vesicles and activate innate immune responses[30,34]. Thus, abundant expression of TIM-3 on TADCs may enable evasion of innate immune responses in the tumor microenvironment.

Despite our finding that TIM-3 suppressed the recruitment of nucleic acids to endosomes, several scenarios may be considered as additional mechanisms whereby TIM-3 interferes with the activation of nucleic acid–mediated responses by HMGB1. First, TIM-3 may affect HMGB1-mediated recognition of nucleic acids by modulating the activity of endosomal endonucleases such as TREX1 or FEN1, which are critical for mediating the degradation of host DNA[42,43]. Alternatively, TIM-3 may inhibit the activity of other HMGB1-binding partners, such as RAGE and TLR4, in endosomal vesicles[28,37]. In addition to its role in nucleic acid–mediated innate immune systems, TIM-3 may also regulate other functions of HMGB1, including gene transcription, oxidative stress and autophagy[29,44].

An additional finding of our study was that blockade of TIM-3 augmented the antitumor efficacy of anticancer cytotoxic agents in part by augmenting HMGB1-mediated nucleic acid–sensing systems. Our observations are consistent with published reports showing that tumor-derived nucleic acids act together with danger signals to activate cells of the innate immune response through the activation of PRR-mediated pathways[38]. As TIM-3 also serves as a receptor for phosphatidylserine for the engulfment of apoptotic cells, blockade of TIM-3 may also manipulate the phagocytotic pathway in DCs, thus modifying mechanisms for the recognition of dying tumor cells. Further investigation should determine the signaling cascades by which TIM-3 on DCs controls innate immune pathways during encounters with dying tumor cells.

Several lines of evidence have emphasized the role of certain cytotoxic drugs in triggering immunogenic cell death that results in the enhancement of host antitumor immunity to tumor cells through multiple pattern-recognition systems[36]. CDDP has been perceived as a 'nonimmunogenic' chemotherapeutic agent because it is unable to induce the endoplasmic reticulum stress responses mandatory for triggering antigen-specific T cell responses mediated by danger-associated molecular patterns[37,45]. Instead, CDDP may compromise the antitumor innate responses of DCs by acting together with TIM-3-dependent inhibitory pathways. Thus, our findings may have provided additional evidence that the therapeutic efficacy of even 'nonimmunogenic' chemotherapy can be induced by the targeting of negative regulatory mechanisms that restrain nucleic acid–mediated innate immune responses.

In summary, we have presented evidence that TIM-3 serves as a negative regulator of nucleic acid–dependent innate immune responses in tumor microenvironments. Given published findings showing that TIM-3 serves as a functional marker of leukemia stem cells[46,47], we propose that TIM-3 is a major sentinel that promotes tumor progression by manipulating multiple pathways to support tumorigenic microenvironments. The many facets of TIM-3 in tumor biology position pharmacological targeting of TIM-3 as a promising strategy for treating patients who are refractory to the anticancer modalities available at present.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.J. and H.Ya. designed the experiments; S.C., M.B., H.A., H.Yo., Y.F. and M.J. prepared reagents and did the experiments; S.C., M.B., H.A., H.Yo., Y.F., Y.O., J.D.C., M.H., T.U., A.T., H.Ya. and M.J. analyzed and discussed the data; I.K. and H.D.-A. provided clinical samples; H.A., J.V.G., J.D.C., M.H. and H.Ya. developed new materials; and M.J. was responsible for the overall study design and writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6, NOD-SCID and CD11c-DTR mice were from SCL, Charles River and Jackson Laboratory respectively. Mice deficient in both TBK1 and TNF were provided by S. Akira. TIM-3-deficient mice were generated and used as described[48]. All experiments were done according to a protocol approved by the animal care committees of Hokkaido University.

Samples from humans. The clinical protocols for this project were approved by the Institutional Review Board of Hokkaido University Hospital (10-0114). Tumor tissues and peripheral blood were obtained from patients with stage IV NSCLC, colon carcinoma, gastric carcinoma or neuroendocrine tumors after written informed consent was obtained. Cells were isolated by Ficoll-Hypaque density centrifugation and were further purified as $CD11c^+$ DCs from tumors and peripheral blood.

Tumor cells and MEFs. MC38, B16-F10 and 3LL tumor cells were from American Type Culture Collection. MEFs were isolated from wild-type and HMGB1-deficient mice.

TIM-3 expression. TIM-3 expression on $CD11c^{lo}B220^+PDCA1^+$ plasmacytoid DCs, $CD11c^{hi}$ conventional DCs, $F4/80^+CD11b^+$ macrophages, $CD45^-gp38^+$ stromal cells, tumor cells in tumor tissues, tumor-draining lymph nodes, distal lymph nodes or spleens obtained from tumor-bearing mice were analyzed by flow cytometry with mAb to mouse TIM-3 (RMT3-23; prepared in-house[26]). For primary tumor infiltrates obtained from patients with advanced cancer or in peripheral mononuclear cells, TIM-3 in $CD11c^+$ DCs was also assessed by flow cytometry with mAb to human TIM-3 (344823; R&D Systems). The *in vitro* induction of TIM-3 in BMDCs was examined 24 h after coculture or treatment with supernatants of tumors cells with mAb to VEGF-R2 (DC101; Calbiochem), mAb to IL-10 (JES5-16E3; BioLegend), TGF-β-receptor inhibitor SB52334 (R&D Systems), anti-galectin-9 (RG9-35), inhibitor of arginiase I ((S)-(2-boronoethyl)-L-cysteine; Sigma-Aldrich) or 1-methyltryptophan (Sigma-Aldrich).

Cytokine ELISA. For analysis of DC cytokine profiles, IFN-β, IFN-α, IL-6 and IL-12 in supernatants obtained from cultured cells were quantified by ELISA according to the manufacturer's instructions (BD Bioscience).

Quantification of cytokine mRNA. First, mRNA was isolated from cell lines, tumor-infiltrating DCs, tumor-draining lymph nodes or spleens after vaccination of tumor-bearing mice with plasmid DNA and treatment with mAb to TIM-3, or DCs from healthy donors or patients with NSCLC. Then, cytokine-encoding mRNA (IFN-β, IFN-α, IL-6 and IL-12) was quantified by real-time RT-PCR by SYBR Green Gene Expression Assay (Applied BioSystems).

***In vivo* antitumor effects of DNA adjuvants and mAb to TIM-3.** Wild-type or NOD-SCID mice were injected subcutaneously in the flank with 1×10^5 B16-F10 melanoma cells, and intratumor injection of plasmid encoding human gp100, TRP-2 or control DNA, or CpG-ODN (10 μg/mouse; InvivoGen), in the presence of mAb RMT3-23 to TIM-3 (250 μg/mouse) or control immunoglobulin, were done on days 8, 10 and 12 after cell injection.

***In vivo* antitumor effects of DNA adjuvants in CD11c-DTR mice.** CD11c-DTR mice were left untreated or treated with diphtheria toxin (4 ng per gram body weight, every 2 d from day 2 to day 22) for depletion of $CD11c^+$ cells. B16-F10 or MC38 tumor cells (1×10^5 cells per mouse) were injected into CD11c-DTR mice and mice were treated with control plasmid DNA (50 μg per mouse) and/or mAb RMT3-23 to TIM-3 (250 μg per mouse) in the presence or absence of antibody to the receptor for type I IFN-α2 (100 μg/mouse; MMHAR-2; BioLegend) and anti-IL-12p40 (250 μg per mouse; C17.8; eBioscience) on days 2, 5 and 8 after tumor inoculation.

Generation of mixed-bone marrow chimeras. The mixed-bone marrow chimeras were generated as described[49]. Bone marrow cells isolated from wild-type or TIM-3-deficient mice were mixed with those from CD11c -DTR mice at a ratio of 1:1. Bone marrow cells (1×10^6 cells per mouse) were transferred intravenously into lethally irradiated mice (15 Gy per mouse). The efficacy of donor-cell reconstitution was evaluated by the identification of $CD11c^{hi}$ major histocompatibility complex class II–positive populations in peripheral blood 4 weeks after the procedure.

TIM-3–HMGB1 interaction. Fusion proteins of Fc and wild-type TIM-3, Q62A mutant TIM-3 or Flt3L were used to coat plastic plates, which were then loaded with biotin-labeled HMGB1 at various concentrations. The binding of biotin-labeled HMGB1 to each fusion protein was measured by colorimetric analysis (absorbance at 450 nm). In some studies, mAb to TIM-3 was added with the HMGB-1 protein.

Generation of recombinant HMGB1 protein. A series of expression vectors with various deletions of HMGB1 (pET28b-HMGB1-Full, pET28b-HMGB1-ΔC-box, pIVEX2.4-HMGB1-ΔA-box, pIVEX2.4-HMGB1-ΔBΔC-box, and pET28b-GST) were introduced into BL21 competent cells, then proteins were induced and purified as described[50].

Immunofluorescence microscopy. BMDCs and MEFs were stimulated with biotin-labeled HMGB1 and X rhodamine–labeled B-DNA. After cells were fixed, early endosomes were probed by Alexa Fluor 488–labeled mAb to EEA1 (C45B10; Cell Signaling Technology), and internalized HMGB1 was probed by streptavidin–Brilliant Violet 421. Images were obtained with an FV-1000D laser confocal microscope (Olympus). Visualized DNA in endosomes was quantified with Meta-Morph software (Universal Imaging). Confocal sections (with a z-step of 0.42 μm) were acquired from the bottom to the top of the cells, and all sections were projected on one image plane. B-DNA^+ or $EEA1^+$ vesicles were extracted as regions with the 'granularity' module and those images were overlaid on the original B-DNA images, followed by quantification with the 'measure region' function.

Subcellular fractionation and detection of B-DNA. $TIM\text{-}3^+$ or $TIM\text{-}3^-$ DCs were incubated for 90 min with biotin-labeled B-DNA. Cells were homogenized with HB^+ (250 mM sucrose, 3 mM imidazole pH 7.4, 1 mM EDTA, 30 mM cycloheximide), and the postnuclear supernatants were subjected to sucrose-gradient ultracentrifugation at 150,000*g* for 1 h with step gradients of 40.6%, 35%, 25%, and 8%. After centrifugation, interfaces were collected (8%/25% late endosome; 25%/35% early endosome), and analyzed by immunoblot and DNA dot blot. For the preparation of heavy and light plasma membrane fraction, postnuclear supernatants were centrifuged for 30 min at 100,000*g* in 30% percoll gradient. For the detection of biotin-labeled B-DNA, each fraction was dotted onto a positively charged nylon membrane, which was probed with streptavidin–horseradish peroxidase.

Evaluation of chemotherapy-mediated antitumor responses. For *in vivo* tumor experiments, CD11c-DTR mice or bone marrow chimeras were challenged subcutaneously in the flank with MC38 cells (1×10^5), and CDDP (10 mg/kg), mAb RMT3-23 to TIM-3 (250 μg/mouse), anti-IFN-IR (100 μg per mouse; MMHAR-2; BioLegend) or anti-IL-12p40 (250 μg per mouse; C17.8; eBioscience) was injected intraperitoneally on days 8, 10 and 12. For *in vitro* assays, $TIM\text{-}3^+$ BMDCs were cultured together with apoptotic tumor cells pretreated with DNase and RNase (10 μg/ml; Invitrogen) in the presence of mAb to TIM-3, anti-HMGB1 (326052233; Shino-Test) or control immunoglobulin (731705; Beckman-Coulter), and cytokine-encoding mRNA was evaluated by RT-PCR.

Statistical analysis. A paired Student's *t*-test was used for statistical analyses, and a *P* value of less than 0.05 was considered statistically significant.

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Review Article

Regulation of cancer stem cell activities by tumor-associated macrophages

Masahisa Jinushi, Muhammad Baghdadi, Shigeki Chiba, Hironori Yoshiyama

Research Center for Infection-associated cancer, Institute for Genetic Medicine, Hokkaido University

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Abstract: Recent studies revealed that tumor-associated macrophages play a decisive role in the regulation of tumor progression by manipulating tumor oncogenesis, angiogenesis and immune functions within tumor microenvironments. However, the role of cancer stem cells in the tumorigenic activities of tumor-associated macrophages during the course of transformation and treatment remains largely unknown. Recent studies have clarified the functional aspects of tumor-associated macrophages in the regulation of the tumorigenic activities and anticancer drug responsiveness of cancer stem cells through complex networks formed by distinct sets of cytokines, chemokines and growth factors. In this article we discuss recent advances and future perspectives regarding the molecular interplay between cancer stem cells and tumor-associated macrophages and provide future perspective about the therapeutic implication against treatment-resistant variants of cancer.

Keywords: Cancer stem cells, tumor associated macrophages, tumor microenvironments, MFG-E8, IL-6, TIM-3, M-CSF

Introduction

Genetic and epigenetic alterations in heterogeneous tumor cell populations regulate tumor initiation, progression and therapeutic responses. On the other hands, emerging evidence unveiled that heterogeneous tumor microenvironments composed of tumor cells as well as normal cells including mesenchymal stem cells, fibroblasts, endothelial cells and immune cells, have a large impact on the behavior of tumorigenic cells during the course of tumor progression [1-3]. In addition, tumor cells may modify the biological properties of stromal cells, endothelial cells and immune cells in their unique microenvironments, thereby contributing to further tumor progression and the emergence of drug-resistant tumor phenotypes [4, 5]. Thus, the interaction between tumor cells and their surrounding normal cellular components may have a determining role in the regulation of tumor initiation, progression, and responsiveness to anticancer therapeutics.

Recent advances molecular immunology and the clinical success of drugs targeting immune-regulatory circuits have emphasized the importance of tumor immune surveillance systems against nascent tumors [6-8]. On the other hand, tumor-infiltrating immune cells frequently promote tumor growth and incur invasive behavior through the coordinated activation of distinct inflammatory and angiogenic signals in the background of smoldering inflammation [9-11]. In particular, tumor-associated macrophages (TAM) have a critical role in modulating tumorigenic activities by activating oncogenic signals, angiogenesis, tissue/matrix remodeling and immune suppression [12-14].

In this review, we will describe recent advances in our understanding of the regulatory mechanisms whereby TAM impact cancer stem cell functions.

The role of tumor microenvironments in the regulation of cancer stem cell activities

Mesenchymal stem cells (MSC) are one of the critical components in tumor microenvironments. MSC enter the circulation into bloodstream from the bone marrow [15] or reside in

normal stromal tissues [16, 17]. Emergent evidence has unveiled the critical role of MSC in positively regulating the tumorigenic activities of cancer stem cells in several murine tumor models [17]. Furthermore, immunohistochemical analysis has confirmed the proximity of MSC and cancer stem cells in biopsies obtained from cancer patients, raising the possibility that MSC have an impact on the clinical course of human malignancies by modulating the cancer stem cell functions [18].

In the tumor microenvironments, MSCs have the ability to differentiate into stromal fibroblasts, which also interact with and influence tumor cells through paracrine signals and various soluble factors [19, 20]. SDF-1 produced by breast carcinoma-associated fibroblasts (but not normal fibroblasts) accelerates the growth and metastatic potential of breast cancers, which express high levels of the SDF-1 receptor CXCR4 [21, 22]. Hepatocyte growth factor (HGF) provides a co-stimulatory signal to the Wnt pathway during colon carcinogenesis [23]. Since niche activities regulated by Wnt-β-catenin cascades have a critical role in the survival and self-renewal of tissue and cancer stem cells, HGF released from stromal fibroblast may regulate cancer stem cell functions by stimulating Wnt-β-catenin pathways in a paracrine fashion [24]. Moreover, additional factors produced by stromal fibroblasts, which include NOS, PDGF, Notch ligand and Hedgehog ligands, are potential candidates to regulate cancer stem cell activities [25, 26]. In addition, the stromal signals serve as a prognostic factor in patients with breast cancer, suggesting that stromal microenvironments composed of MSC and stromal fibroblasts greatly impact the biological behavior of tumorigenic cells in actual clinical settings [27].

Endothelial cells may impact biological behaviors of cancer stem cells in tumor microenvironment by direct interaction with tumor cells as well as by their role in blood vessel formation. Endothelial cells constitute an important component of normal hematopoietic and neuronal stem cell niches, but tumor vasculature is different from normal vasculature, raising the possibility that tumor microenvironments produce defined factors that modulate the genetic and epigenetic profiles of endothelial cells. Indeed, more than 1,000 genes are differentially expressed comparing tumor *vs* normal endothelial cells, including FGF receptors, MMPs and NF-κB-regulated transcripts [28, 29]. In addition, cytokines produced by endothelial cells, which includes HGF, VEGF, PDGF and PIGF stimulate the self-renewal and survival of adjacent cancer stem cells [30, 31]. Interestingly, recent reports demonstrate that glioblastoma stem cells can trans-differentiate into endothelial cells to generate their own vasculature, thereby providing blood supply to adjacent tumor cells and further accelerating tumor progression [32, 33].

Together, the complex networks created by cancer stem cells and the surrounding normal cellular components contribute to the proliferation as well as the invasive activities of tumors in murine models as well as cancer patients (**Figure 1**).

The role of TAM in tumor progression and anticancer drug resistance

The immune system provides both inhibitory and stimulatory effectors in tumor initiation, promotion and metastasis, and the balance of these effects may be determined by different tumor microenvironments. Thus, the distinct modes of interplay between tumorigenic cells and host immunity may profoundly influence both the biological behaviors of tumors during the course of tumorigenicity and their responsiveness to anticancer modalities [6-11]. In particular, emerging evidences have unveiled the molecular mechanisms by which myeloid cells such as macrophages and myeloid-derived suppressor cells (MDSC), interact with tumor microenvironments to further accelerate tumor progression [12, 34].

Tumor-associated macrophages are characterized by distinct phenotypic polarization referred as "M1 and M2" subsets [14, 35]. The M1-polarized macrophages manifest high levels of proinflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, and promote Th1 responses, which contributes to tumoricidal activity and antitumor immunity. On the other hands, M2 macrophages serve as the main players facilitating parasite containment, tissue remodeling and immune tolerance, which may be linked with tumor progression [36, 37]. The M1 polarization in macrophages is mainly regulated by distinct transcriptional networks consisting of IRF-1/5, Stat-1/4 and NF-κB, whereas M2 polarization is regulated through other transcription factors such as IRF-4, Stat-

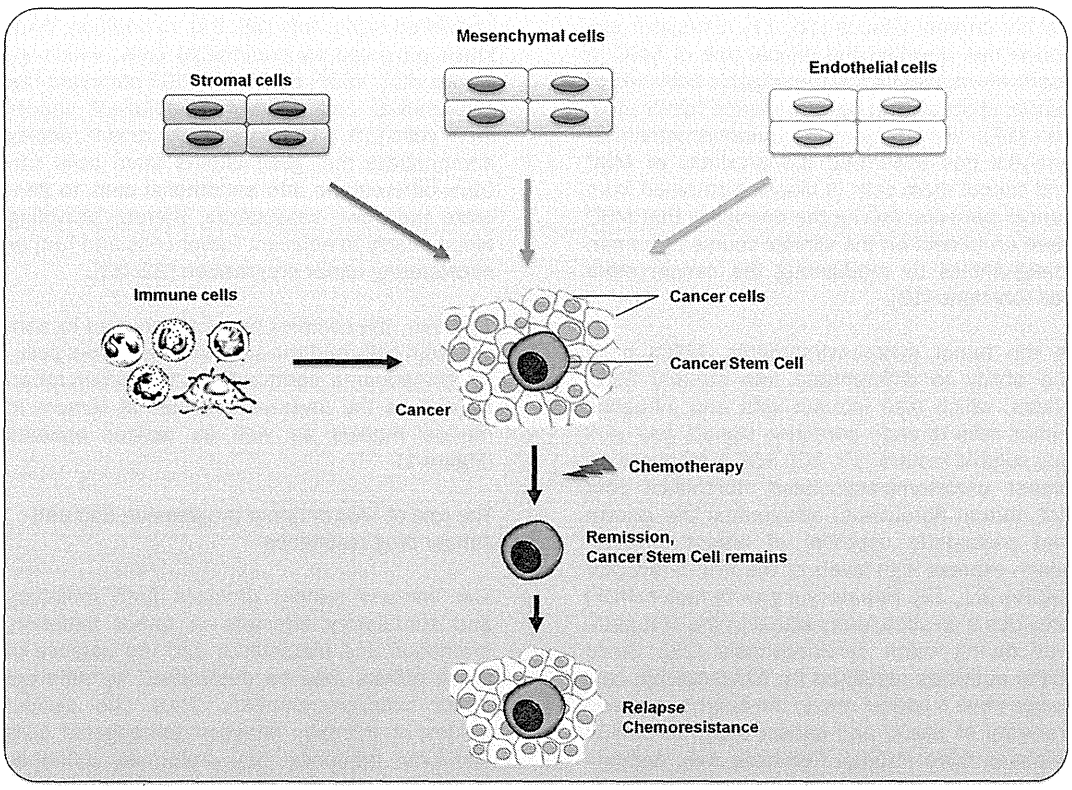

Figure 1. The complex networks created by cancer stem cells and their tumor microenvironments contribute to the tumorigenic and invasive activities of tumors.

3/6, PPAR-γ, KLF-4 and histone demethylase such as Jmjd3 [38-42]. In several murine models of carcinogenesis, tumor progression is frequently associated with a phenotypic switch from M1 to M2 in TAM [43]. Furthermore, M1-polarized macrophages mediate elimination of senescent hepatocytes, which drive subsequent carcinogenesis [44]. It is therefore possible that classically activated M1 macrophages contribute to the tumor elimination and equilibrium phases during tumor progression *via* T cell-mediated mechanisms [6].

However, there is a growing appreciation that TAM are composed of several different populations that bear sufficient plasticity and flexibility to enable them to cause dynamic changes between the M1 and M2 phenotypes depending on the different tumor microenvironments [46, 47]. Furthermore, recent genetic profiling and phenotypic analyses have also revealed that tumor cells, through distinct sets of signaling molecules, transcription factors, and epigenetic modifiers, manipulate tumor-infiltrating myeloid cells to differentiate them into peculiar subsets with tumor-promoting capacities [48, 49]. In this regard, tumor microenvironments characterized by smoldering inflammation and/or modulated by anticancer drug-mediated stress responses may serve as driving forces to alter the genetic and phenotypic profiles of tumor cells, thus promoting tumorigenic activities of myeloid cells in an autocrine and paracrine fashion.

Recent comprehensive analysis revealed that the numbers and activities of tumor-associated macrophages may influence the prognosis of patients with Hodgkin's lymphoma and breast cancer [49, 50]. Furthermore, treatment with M-CSF kinase inhibitors had significant antitumor activity against patients-derived primary tumors arising in immunodeficient mice when com-

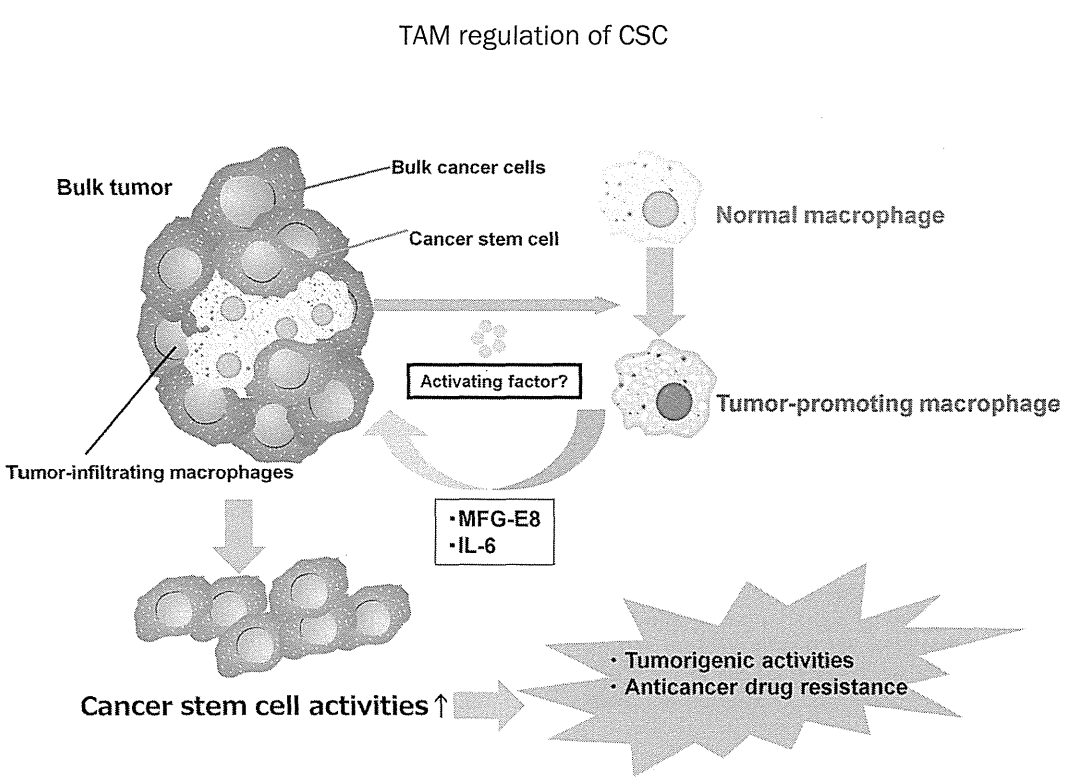

Figure 2. TAM serves as the major components of niche microenvironments regulating cancer stem cell functions. MFG-E8 and IL-6 predominantly produced from TAM in a cancer stem cell-specific fashion trigger tumorigenesis and anticancer drug resistance in cancer stem cells through the coordinated activation of the Stat3 and Hedgehog pathways. The interplay between cancer stem cells and TAM accelerates tumor progression and drug resistance through autocrine positive-feedback mechanisms.

bined with conventional cytotoxic chemotherapy [50].

These findings suggest that different tumor microenvironments may have a distinct impact on the ability of TAM on tumor growth and therapeutic responses to chemotherapy. The identification of the various cellular and molecular pathways and their downstream factors that participate in the interaction between tumorigenic cells and tumor-infiltrating myeloid cells in various human cancers will translate our understanding of cancer-related inflammation to meaningful therapeutic advances.

The interplay between cancer stem cells and TAM in the regulation of tumorigenicity and anticancer drug responses

Recent studies have clarified the importance of TAM as major contributors in the regulation of both self-renewal and anticancer drug responses of cancer stem cells through distinct networks of cytokines, chemokines and growth factors. In these processes, TAM interact with and promote the tumorigenicity of cancer stem cells *via* production of milk-fat globule-epidermal growth factor-VIII (MFG-E8) and IL-6 through coordinated activation of the STAT3 and sonic hedgehog pathways [51]. Interestingly, cancer stem cells are the major subset promoting the production of MFG-E8 and IL-6 from macrophages, implying that mediators specifically regulated by cancer stem cells render macrophages with the ability to facilitate the production of tumorigenic factors such as MFG-E8 and IL-6. In this sense, TAM might serve as a component of the "immunological niche", by which cancer stem cell activities are maintained and amplified within tumor microenvironments (Figure 2).

Cancer stem cells have unique characteristics that manipulate complex signaling cascades which regulate oncogenesis, embryogenesis and self-renewal. In turn, these amplification loop lead to oncogenic addiction, stem cell maintenance, angiogenesis, and immune modu-

lation within tumor microenvironments [52, 53]. Several oncogenic pathways, including Wnt/β-catenin, Notch, TGF-β/FOXO cascades, support self-renewal capacity and anticancer drug resistance in cancer stem cells [54, 55]. In addition, recent studies have revealed the indispensable role of the IL-6-Stat3 signal cascade in stimulating cancer stem cell activities in coordination with NF-kB-dependent inflammatory signals derived from tumor cells and their microenvironment [56, 57]. Moreover, Hedgehog signals have been identified as sentinels linking oncogenic aberration with the developmental program of normal and cancer stem cells [58]. In this regards, our findings that MFG-E8 and IL-6 derived from TAM mediate self-renewal and anticancer drug resistance through activation of Stat3 and Hedgehog signals provide additional evidences that TAM play a critical role in activating distinct signals that are crucial to the maintenance of the stem cell properties of tumor cells.

Recent studies in human leukemia and lymphoma have suggested that tumor cells express the antigen CD47, which serves as a "don't eat me" signal to tumor-associated macrophages by engaging their cognate receptor SIRT-1. Administration of a blocking antibody to CD47 induced macrophage phagocytosis of AML stem cells *in vitro* and in mouse models [59-61]. These findings provide the first evidence that macrophage phagocytosis serves as a critical mediator of tumor immunosurveillance against leukemia stem cells.

On the other hand, T cell immunoglobulin-mucin domain protein-3 (TIM-3), which is involved in apoptotic cell phagocytosis *via* recognition of phosphatidylserine, has been identified as a functional marker for dissecting acute leukemia stem cells from bulk tumor cells [62-64]. Furthermore, AML stem cells were eradicated by the administration of a TIM-3-depleting mAb [63]. Since TIM-3 expression is detectable in macrophages and dendritic cells upon stimulation with toll-like receptor ligands such as LPS, it is of great interest to examine whether TIM-3 is detected in tumor-associated myeloid cells and to determine the functional role of myeloid cell-derived TIM-3 and its phagocytic activity in the regulation of cancer stem cell functions.

In addition, MFG-E8 not only serves as a positive modulator of cancer stem cell activities, but also functions as an immunoregulatory factor within tumor microenvironments by promoting apoptotic cell phagocytosis and inducing $Foxp3^+$ infiltration into tumors [65, 66]. Moreover, TIM-4, expressed mainly on activated myeloid cells is also critically involved in the phagocytosis of apoptotic cells *via* the recognition of phosphatidylserine and the triggering of immune tolerance [67, 68]. The TAM receptor tyrosine kinase family composed of Axl, Mer-tyrosine kinase and Tyro-3, which serve as phagocytic receptors for apoptotic cells *via* recognition of Gas6, regulates innate immune responses and could be involved in the tumorigenic potentials of cancer cells [69, 70]. It is therefore likely that distinct sets of phagocytosis-associated molecules, such as CD47 / SIRT1, TIM-3, TIM-4, MFG-E8, Gas-6 *etc.* recognize distinct tumor subtypes including cancer stem cells, which arise from different backgrounds of oncogenic or epigenetic alterations and drug responsiveness. The identification and characterization of distinct sets of receptor / ligands on phagocytic macrophages may be an ideal strategy with which to investigate the interaction of cancer stem cells and TAM, and may lead to the exploration of new therapeutic targets against cancer stem cells.

Therapeutic implication for targeting the interaction of cancer stem cells and TAM

Comprehensive genetic approaches along with advances in the field of stem cell biology facilitated the identification of cancer stem cell-specific markers and multiple pathways potentially suitable for specifically targeting cancer stem cells [71, 72]. However, whether the target molecules identified from "pure" populations in cancer stem cells are actually effective against recurrent and multidrug-resistant variants of tumors remains largely uncharacterized. Thus, the development of drugs targeting the molecular networks between cancer stem cells and macrophages should provide useful tools with which to regulate cancer stem cell activities in coordination with those drugs targeting cancer stem cells and other factors derived from MSC, endothelial cells, fibroblasts and extracellular matrixes.

In addition, the targeting of TAM-derived downstream factors such as MFG-E8 and IL-6 may also be useful in repressing the emergence of chemoresistant tumors by controlling cancer

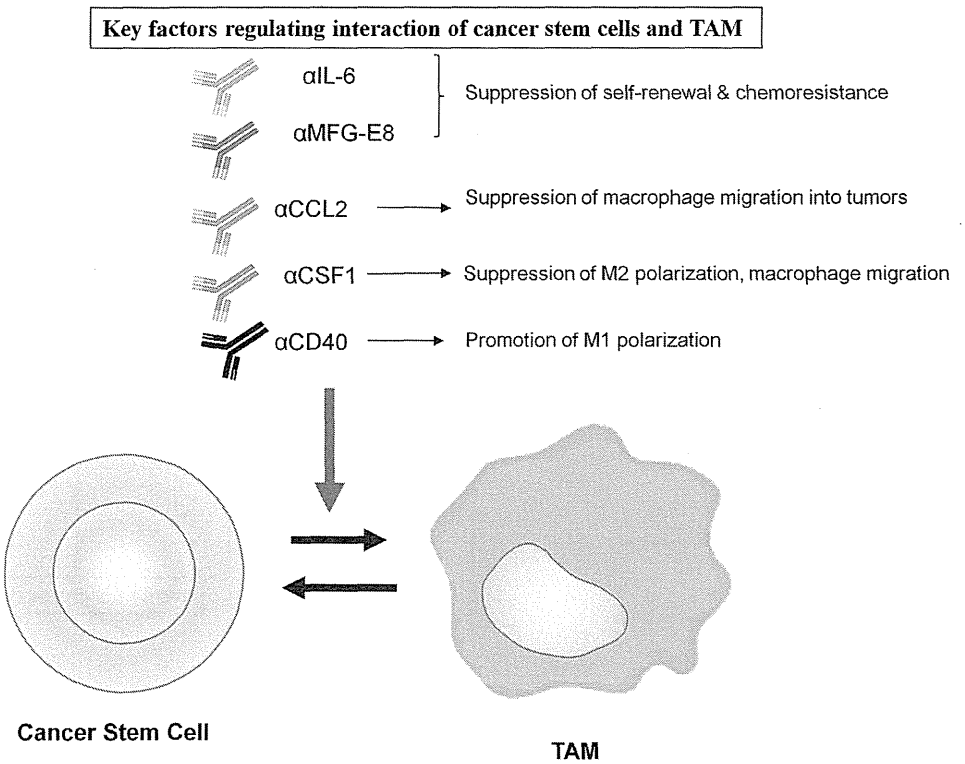

Figure 3. The strategy for targeting key mediators that influence the tumorigenic activities of TAM such as CCL2, M-CSF, CD40L and PPA has a potential to efficiently control cancer stem cell activities and might overcome the therapeutic limitations of conventional anticancer modalities.

stem cell activities [51, 73]. Moreover, therapeutic approach targeting TAM and their downstream effectors are now at the initial stages of clinical applications. Recruitment is a key determinant sustaining macrophage numbers at sites of inflammation and immunity. The CCL2/MCP-1 pathway has emerged as a new target for the prevention of myeloid cell recruitment to tumor microenvironments [74, 75]. CSF-1 receptor (c-fms) kinase inhibitors have been recently introduced into the clinical arena and exhibit antitumor and antiangiogenic activity in various murine and human tumor models [50, 76]. Furthermore, anti-CSF-1 antibodies augmented the antitumor effects of cytotoxic chemotherapy in a human breast cancer xenograft model [77].

The manipulation of macrophage polarization may serve as another strategy for controlling tumorigenicity. Consistent with this concept, CD40 agonist antibodies induce high expression of M1 markers in macrophages and augment antitumor responses to chemotherapy in pancreatic adenocarcinoma models [78]. Other therapeutic strategies that have been reported to affect macrophage polarization include PPAR-γ agonist and TLR ligands (Poly (I: C) and CpG) [79-81].

Together, these findings validate the potential applicability of targeting key mediators that influence the tumorigenic activities of TAM, and suggest that this may negatively regulate cancer stem cell activities and overcome the therapeutic limitations of conventional anticancer modalities (Figure 3).

Concluding remarks

We present the overview that TAM serve as a critical immunological niche in regulating cancer

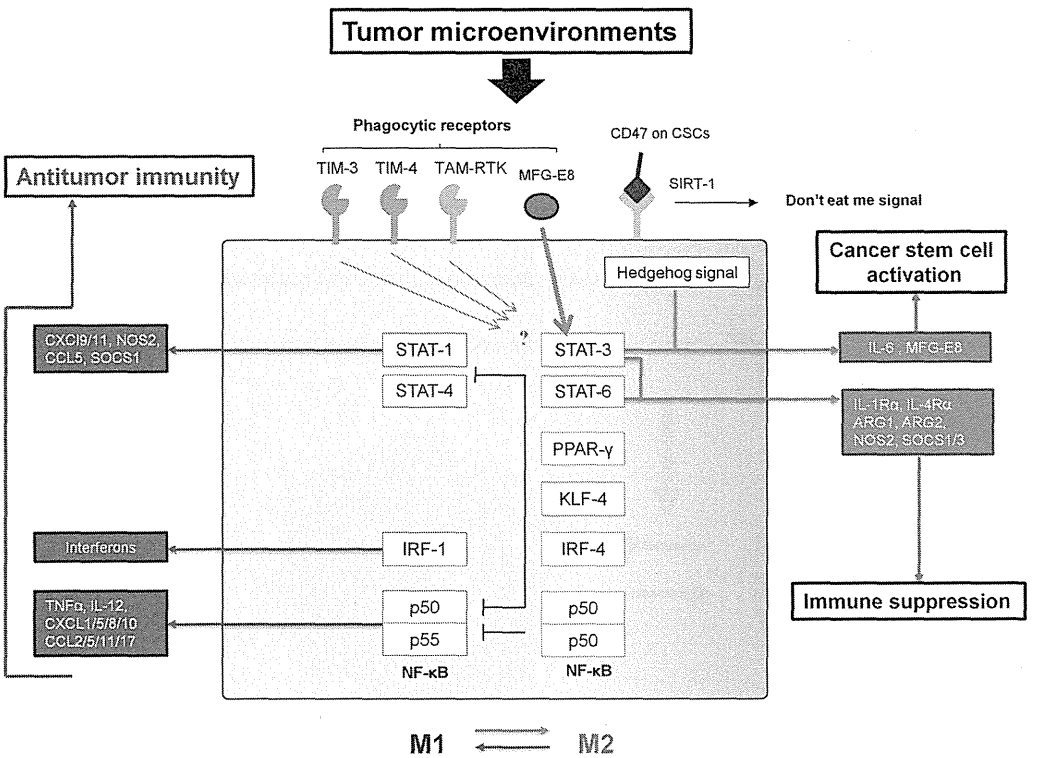

Figure 4. *Tumor microenvironments regulate distinct signal cascades that are critical for determining macrophage polarization and facilitating the expression of key molecules that control interactions with cancer stem cells.*

stem cell functions (**Figure 4**). Accumulating evidence reveals that the quality of tumor microenvironments may determine the direction of interplay of tumors and immune cells throughout the different stages of carcinogenesis. In addition, tumor-infiltrating immune cells other than TAM, such as MDSC, dendritic cell, granulocytes, NKT cells, B cells and $CD4^+$ T cells, also serves as positive regulators of tumor progression and metastasis, raising the possibility that various sets of immune cells interact with cancer stem cells to modulate their biological activities [10]. In this regard, comprehensive analysis of molecular intersection between intrinsic and immune-mediated pathways enriched in tumor microenvironments should provide useful insights into the regulatory mechanisms of cancer stem cell activities as well as provide new therapeutic approaches for targeting the components of immunological niche in the future.

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Address correspondence to: Dr. Masahisa Jinushi, Research Center for Infection-associated cancer, Institute for Genetic Medicine, Hokkaido University Tel: +81-11-706-6073; Fax: +81-11-706-6071; E-mail: jinushi@igm.hokudai.ac.jp (M.J.)

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Review

Epstein-Barr Virus (EBV)-associated Gastric Carcinoma

Hisashi Iizasa [1,†], Asuka Nanbo [2,†], Jun Nishikawa [3], Masahisa Jinushi [4] and Hironori Yoshiyama [4,*]

[1] Division of Stem Cell Biology, Institute for Genetic Medicine, Hokkaido University, N15 W7, Kita-ku, Sapporo 060-0815, Japan; E-Mail:iizasah@igm.hokudai.ac.jp

[2] Graduate School of Pharmaceutical Sciences, Hokkaido University, N12 W6, Kita-ku, Sapporo 060-0812, Japan; E-Mail:nanboa@pharm.hokudai.ac.jp

[3] Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Minami-Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan; E-Mail: junnis@yamaguchi-u.ac.jp

[4] Research Center for Infection-Associated Cancer, Institute for Genetic Medicine, Hokkaido University, N15 W7, Kita-ku, Sapporo 060-0815, Japan; E-Mails:jinushi@igm.hokudai.ac.jp (J.M.); hironori@igm.hokudai.ac.jp (H.Y.)

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: hironori@igm.hokudai.ac.jp; Tel.: +81-11-706-6073; Fax: +81-11-706-6071.

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Abstract: The ubiquitous Epstein-Barr virus (EBV) is associated with several human tumors, which include lymphoid and epithelial malignancies. It is known that EBV persistently infects the memory B cell pool of healthy individuals by activating growth and survival signaling pathways that can contribute to B cell lymphomagenesis. Although the monoclonal proliferation of EBV-infected cells can be observed in epithelial tumors, such as nasopharyngeal carcinoma and EBV-associated gastric carcinoma, the precise role of EBV in the carcinogenic progress is not fully understood. This review features characteristics and current understanding of EBV-associated gastric carcinoma. EBV-associated gastric carcinoma comprises almost 10% of all gastric carcinoma cases and expresses restricted EBV latent genes (Latency I). Firstly, definition, epidemiology, and clinical features are discussed. Then, the route of infection and carcinogenic role of viral genes are presented. Of particular interest, the association with frequent genomic CpG

methylation and role of miRNA for carcinogenesis are topically discussed. Finally, the possibility of therapies targeting EBV-associated gastric carcinoma is proposed.

Keywords: EBV; Carcinogenesis; EBV-associated gastric carcinoma; Epithelial; CD21; Methylation; miRNA

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpes virus with oncogenic activity. The EBV genome can be detected in malignancies of both lymphoid and epithelial cell origin, such as Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) [1,2]. In 1990, EBV genomes were detected in gastric carcinomas using polymerase chain reaction [3] and *in situ* hybridization (ISH) for EBV-encoded small ribonucleic acid 1 (EBER1). These findings indicated that EBV-associated gastric carcinomas (EBVaGC) comprise about 10% of all gastric carcinomas worldwide [4-6]. Since EBVaGC are monoclonal proliferations of a single cell persistently infected with EBV, EBV infection may be involved in the early stages of gastric carcinogenesis [7-9].

EBV spreads by the oral route [10].After primary infection, EBV establishes the lifelong virus carrier state, called latent infection, which expresses a limited set of viral genes required for viral episome maintenance, thereby conferring a survival advantage to the infected cell. BL, approximately half of the NPC, and EBVaGC belong to latency I, in which EBV nuclear antigen 1 (EBNA1), EBER1 and 2, and *Bam*HI-A rightward transcripts (BART) are expressed. Latency II neoplasm includes the remaining NPC and Hodgkin's lymphoma (HL) and is characterized by the expression of latent membrane protein 1 (LMP1) in addition to latency I transcripts. Latency III neoplasms, typified by lymphomas observed in immunosuppressed patients, additionally expresses EBNA2, 3A, 3B, 3C, and LP. Three promoters (Cp, Wp, and Qp) are utilized for EBNA transcription. The Cp- or Wp-initiated large transcript is differentially spliced into six EBNA mRNAs as observed in latency III cells, whereas Qp mediates selective expression of EBNA1 in Latency I or II cells [7]. The expression of latent genes is under a strict epigenetic regulation through DNA methylation. Cp and Wp are hypermethylated in latency I [11].

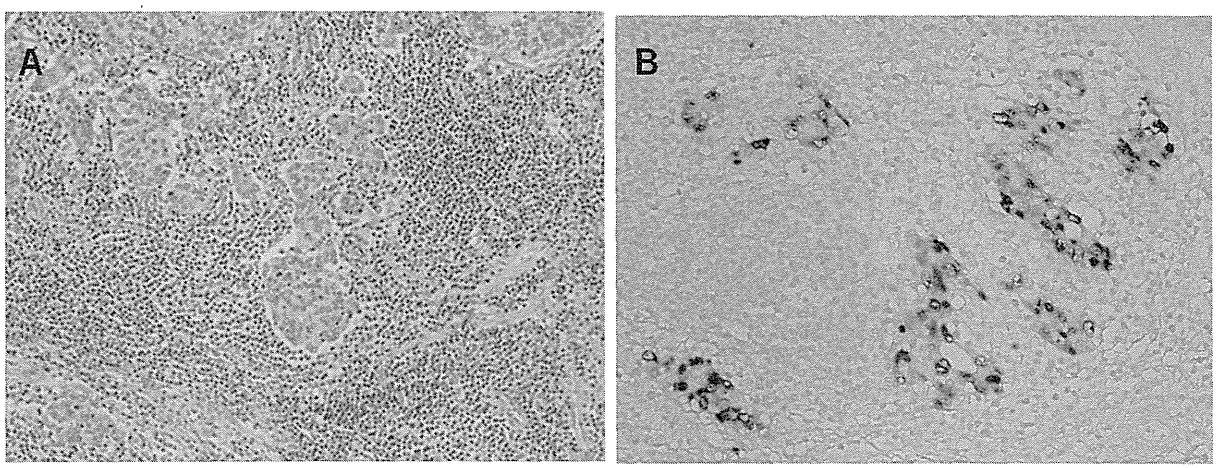
EBVaGC is a latency I neoplasm and expresses EBNA1, EBER, BART, and sometimes (40%) latent membrane protein 2A (LMP2A) [9]. Since EBVaGC does not express EBNA2 and LMP1, which are important for B cell immortalization and transformation [2,7], preexisting abnormalities may exist in gastric epithelial cells [12]. Not only viral gene, but also the host cell DNA methylation has been frequently observed in EBVaGC [13-15]. Promoter hypermethylation of tumor-related genes is known to cause down-regulation of their gene expression [16]. Target gene silencing by viral micro RNAs (miRNAs) [17] has also been observed in EBV infected cells. Both mechanisms may influence the tumor progression of EBVaGC.

2. Definition, Epidemiology, and Clinical Features

2.1. Definition

Almost 10% of the gastric carcinomas throughout the world are monoclonal proliferations of EBV-carrying tumor cells [4].A characteristic feature of EBVaGC is lymphoepithelioma-like carcinoma, which presents a diffuse-type histology with lymphoid infiltration. EBVaGC is defined by the presence of EBV in neoplastic cells. EBER1-*in situ* hybridization (ISH) is used to identify EBVaGC, because EBER1 is highly abundant (10 million copies per cell) in each infected cell. Typically, tumor cells, of which nuclei are positive for EBER1-ISH, are surrounded by lymphoid stroma (Figure 1). EBVaGC has distinct clinicopathological features, which predominantly arises in men and presents a generally diffuse histological type [18].

Figure 1. Lymphoepithelioma-like subtype of Epstein-Barr virus-associated gastric carcinomas (EBVaGC).**A.** Hematoxylin-Eosin Staining. **B.** EBV-encoded small ribonucleic acid-*in situ* hybridization (EBER1-ISH) demonstrates positive nuclei in the carcinoma cells, which are surrounded by infiltrating lymphocytes.



2.2. Epidemiology

Most studies did not show evident age dependence of EBVaGC frequency.Almost all studies have shown male predominance of EBVaGC, suggesting that risk from lifestyle or occupational factors may exist among males [19].An interview study in Japan showed that salty food intake and wood dust and/or iron filings exposure, which may induce mechanical injury to the gastric epithelia, are related to a higher EBVaGC risk [20].

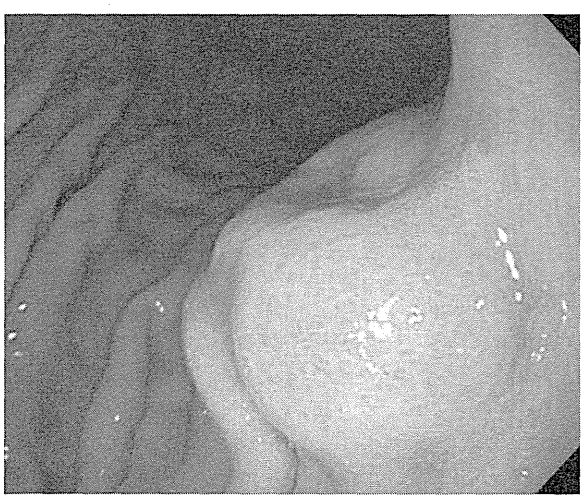
In contrast to BL and NPC, which are endemic in Equatorial Africa and Southeast Asia, respectively, EBVaGC is a non-endemic disease distributed throughout the world [6]. However, there are some regional differences in the incidence of EBVaGC. The incidence of EBVaGC in all cases of gastric cancer is distributed from highest (16-18%) in the USA and Germany to the lowest (4.3%) in China [6,21,22].A Japanese study investigated incidence of EBV-positive cases in all gastric cancers in

several areas. The study indicated that EBVaGC prevalence was inversely related to the GC incidence [23]. Prognosis of EBVaGC is relatively favorable.

2.3. Clinical Features

The most useful modality for the diagnosis of gastric carcinoma is endoscopy. By endoscopy, EBVaGC appears as superficial depressed (or ulcerated) lesions in the upper part of the stomach (Figure 2). Tumor locates predominantly in the non-antrum part of the stomach [19]. Because gastric cancer related to *Helicobacter pylori* (Hp), a causative agent of chronic gastritis, intestinal metaplasia, and cancer, locates predominantly in the antrum, these pathogens have been thought to cause gastric cancer by independent mechanisms [19]. Gastritis related to Hp frequently starts in the antrum. However, Yanai *et al.* reported that EBVaGC are frequently located near the mucosal atrophic border, where mild to moderate chronic atrophic gastritis (CAG) is common [24]. They also showed frequent detection of both EBV and Hp at the mucosa with moderate CAG, where inflammatory cell infiltration is abundant, and not at the mucosa with marked CAG, where inflammatory cell infiltration is scarce [25].

Figure 2. Endoscopic image of an early EBVaGC in the upper gastric body. The tumor shows protruded shape probably because of the abundant lymphocyte infiltration.



3. Route of Epithelial Infection

EBV infects human B lymphocytes and epithelial cells via different entry mechanisms. In case of B cells, the major outer envelope glycoprotein, gp350/220, is responsible for attachment of the virus with high affinity to CD21 or the human complement receptor type 2 (CR2) on B cell surface [26-29]. EBV is subsequently internalized into the cells via endocytic pathway. Fusion with viral envelope and endosomal membrane of B cells is triggered by the interaction of a second envelope glycoprotein, gp42, with HLA class II [30], and is thereafter mediated by the core fusion complex, gH/gL/gp42 [31,32].

In contrast, the mechanism by which EBV infects human epithelial cells remains unclear. Human epithelial cells are CD21-negative or express CD21 at low level in some epithelial cells in culture and highly resistant to cell-free EBV infection [33,34].At least three models have been proposed as mechanisms for the EBV attachment to epithelial cells independent of CD21. First, it has been demonstrated that EBV virions coated with immunoglobulin A (IgA) specific to gp350/220 can bind efficiently to the polymeric IgA receptor [35]. Polymeric IgA is commonly present in human saliva and binds to the secretory component (SC) protein, which is a transmembrane protein expressing on the basolateral surfaces of polarized epithelial cells.A complex of EBV/IgA/SC is internalized into epithelial cells via endocytic pathway. This may be relevant to infection through the basolateral surface of an epithelial cell, which presumably resembles the physiological environment that the virus encounters *in vivo* [36]. Second, a complex of gH and gL was proposed to serve as epithelial ligands in the absence of CD21. EBV derived from B cells binds with high affinity to CD21-negative epithelial cells, but recombinant viruses lacking gH/gL lose this ability [31,32], suggesting that there is an epithelial cell-specific receptor for gH/gL that serves in attachment of EBV. It has been also shown that the direct interaction between gH/gL and the integrins αVß6 and αVß8 can provide the trigger for fusion of EBV and plasma membrane of epithelial cells [37]. Finally, an interaction between an EBV-encoded membrane protein, BMRF2, and integrins on polarized epithelial cells has been proposed as a model for EBV attachment to the cell surface [38]. The tripeptide Arg-Gly-Asp (RGD) motif in the BMRF2 molecule is presented as a ligand for ß1, α5, α3, and αV integrins [39,40]. However, BMRF2 is not required for cell-to-cell fusion [41,42] and apparently very few BMRF2 molecules exist in the virion [43]. It remains unclear whether the interaction of BMRF2 with integrins is primarily responsible for attachment and/or post-attachment events.

Interestingly, EBV virions released from B cells are deficient in gp42 which renders them more efficient to infect epithelial cells, but less efficient to infect B cells [44,45]. In contrast, EBV released from infected epithelial cells possesses gp42 and efficiently infects B cells [44]. This change in cell type tropism for EBV infection suggests that EBV shuttles between epithelial cells and B cells in the host during infection cycle. This observation supports a model that pharyngeal epithelial cells are in lytic EBV infection and shed infectious EBV particles for transmission.

The fusion of EBV envelope with the plasma membrane of epithelial cells requires a trigger gH/gL complex [36,45-49]. The fusion of EBV with an epithelial cell is likely triggered by a direct interaction between gH/gL and unknown epithelial cell surface molecules, which might be the identical proteins that can serve as attachment receptors in the absence of CD21 [50].