regulation of host immunosurveillance and tumor-associated inflammation. Immunosurveillance systems are directed towards pro-tumor or antitumor functions, and tumor-associated inflammation plays a critical role in generating pro-tumorigenic conditions in the privileged TMEs. Given the emerging evidence that TMEs regulate multiple properties of both immunosurveillance and inflammation, it is important to address the functional significance of therapy resistance on antitumor immunosurveillance, immunosuppression, and tumorigenic inflammation.

2 Immune regulation of therapy-resistant tumors

2.1 Chemotherapy

2.1.1 Molecular links between innate immune signals and chemoresistance

The multiple genetic and epigenetic alterations that follow cytotoxic chemotherapy treatment not only cause resistance to particular types of chemotherapeutic drugs but also manipulate the extrinsic environments including tumor-infiltrating non-transformed cells and extracellular matrices. In particular, tumor-associated immune cells frequently communicate with transformed cells, which have a significant impact on the immune cells' antitumor responses upon exposure to cytotoxic agents [17–19].

For example, gemcitabine and 5-fluorouracil activate innate immune responses in tumors by disrupting lysosomal integrity and release of cathepsin B in myeloid-derived suppressor cells (MDSCs) thereby stimulating nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing-3 (NLRP-3)-mediated IL-1β production. The MDSC-derived IL-1β results in the generation of pro-angiogenic IL-17-producing $CD4^+$ T cells, which contribute to pro-tumor inflammation and chemoresistance [20].

In addition to the specialized genetic mutations and epigenetic modifications in tumor cells imposed by cytotoxic drugs, tumor cells frequently acquire the expression of innate immune receptors and trigger pro-tumorigenic cascades in privileged TMEs. For example, Toll-like receptor (TLR) expression on tumor cells triggers innate immune signaling cascades, leading to the activation of pro-inflammatory responses by Myd88-NF-κB- and/or IRF3/7-mediated pathways [21, 22]. Tumor-derived cytokines and chemokines contribute to the amplification of inflammatory feed-forward loops by recruiting and stimulating myeloid cells and lymphoid cells within tumors [23, 24]. The activation of TLR-2, TLR-4, or TLR-7/8 also enhances tumor cell survival through the coordinated action of immune-mediated and cell-autonomous NF-κB- and Bcl2-dependent antiapoptotic programs [25–28]. TIM-3 is induced on tumor-infiltrating dendritic cells (DCs) by tumor-derived immunoregulatory mediators such as VEGF-A, IL-10, and arginase-I and is involved in the negative regulation of therapeutic responses to anticancer chemotherapy. TIM-3 acts by impeding innate immune signals mediated by nucleic acid-recognizing TLRs [29, 30]. Thus, TLR signals play a critical role in promoting tumor chemoresistance by creating inflammatory environments and protecting tumor cells from excess tissue damage by chemotherapy (Fig. 1).

2.1.2 Phagocytosis-mediated immune suppression and chemoresistance

Cytotoxic chemotherapy confers antigen-presenting cells (APCs) with the ability to capture and process tumor cells by inducing programmed tumor cell death and supporting the release of various chemoattractant factors including "eat-me" and/or "find-me" signals from TMEs [19]. Phagocytosis of tumor cells frequently mediates the immunosuppressive properties of tumor-associated APCs leading to the resistance of tumors to chemotherapy [31, 32]. Moreover, resistance to cytotoxic chemotherapy renders tumor cells capable of generating various growth factors, cytokines, and/or chemokines, several of which are responsible for recruiting APCs and promoting tolerogenic phagocytosis. For example, chemoresistant tumor cells produce several soluble mediators that serve as "eat-me" signals, such as milk fat globule-EGF factor VIII (MFG-E8), Gas 6, and ATP. These factors mediate suppression of antitumor immunity by facilitating phagocytosis of apoptotic tumor cells [33–35]. Moreover, phagocytosis regulated by CD47-calreticulin systems plays a critical role in controlling recognition and removal of tumorigenic cells, thereby greatly influencing the therapeutic potential of anticancer drugs [36–38]. In addition, TIM-4 expressed on TAMs contributes to immune tolerance and chemoresistance by triggering autophagy-mediated over-degradation of tumor-associated antigens derived from ingested tumor cells [39]. Collectively, these findings imply critical molecular links between tumor chemoresistance and myeloid cell-mediated removal of tumor cells. Thus, therapeutic targeting of phagocytosis-mediated immunoregulatory pathways may overcome cytotoxic chemotherapy resistance by increasing presentation of immunogenic tumor antigens and activating tumor-specific immunity.

Tumor cells also adopt their own strategy for manipulating APC phagocytic systems and creating immunosuppressive tumor microenvironments. Chemoresistant tumors express integrin αvβ3 at much higher levels than their treatment-naïve counterparts, and the chronic activation of ATM-dependent DNA damage signals is responsible for inducing integrin αvβ3 on chemoresistant cells. The integrin αvβ3 on chemoresistant tumors is engulfed by immature DCs through recognition of RGD motifs, which leads to impaired cross-

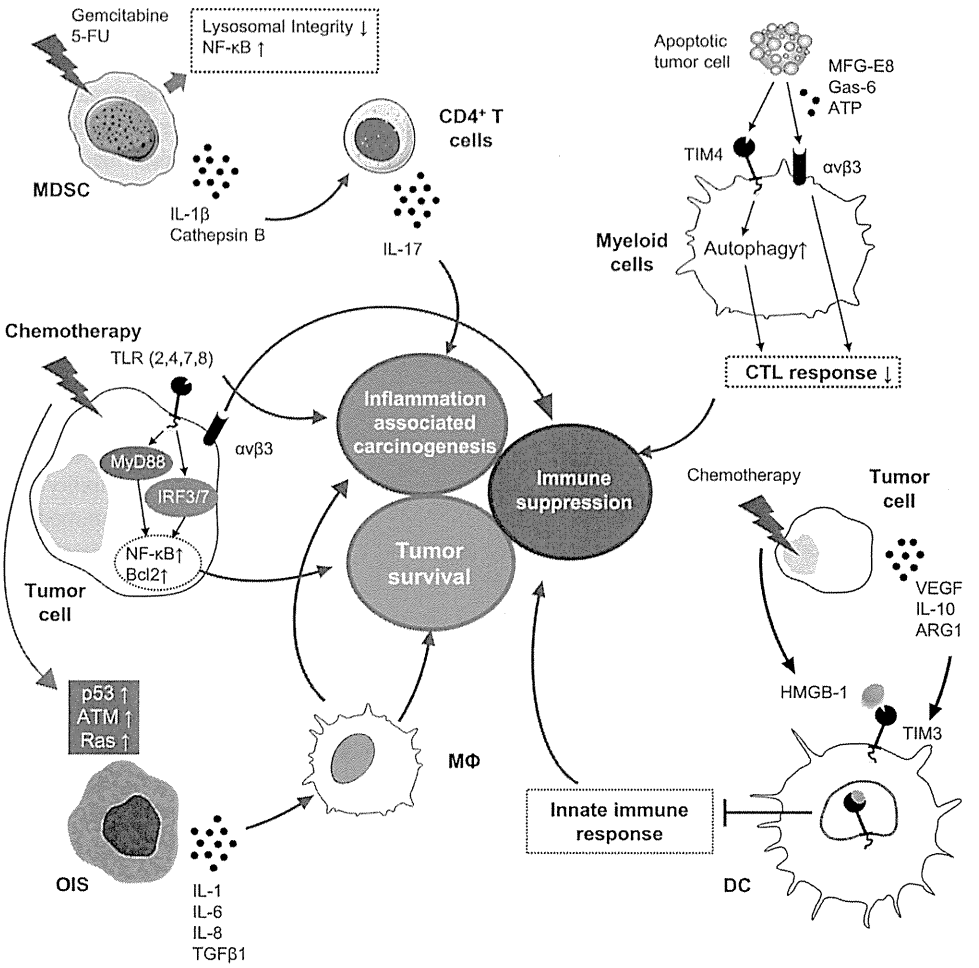

Fig. 1 Cross-talk between tumor and immune cells regulates the therapeutic responses to cytotoxic chemotherapy. The expression of immune regulators (TLR, integrin αvβ3, *etc.*) on tumor cells contributes to cellular survival and stimulates pro-inflammatory pathways, which mediate recruitment and activation of tumor-associated myeloid cells. On the other hand, tumor-associated myeloid cells expressing immunoregulatory factors (TIM-3, TIM-4, MFG-E8, Gas 6, *etc.*) counter the immunogenicity of dying tumor cells or the components they release (nucleic acid, DAMPs, *etc.*) by suppressing antigen presentation to antitumor CTLs or innate immune signals, respectively. The cellular senescence triggered by cytotoxic agents has a pro-tumorigenic effect by stimulating tumor-associated myeloid cells. These tumor- or myeloid cell-mediated machineries compromise sensitivities to cytotoxic chemotherapy

priming of antitumor CTLs [40]. Although it remains unclear how integrin αvβ3 regulates antigen processing and immune functions after tumor cell ingestion, these findings suggest that chemoresistance renders tumor cells capable of manipulating multiple pathways involving immunologic processes including phagocytic systems (Fig. 1).

2.1.3 Senescence-associated secretory phenotype and chemoresistance

Cytotoxic chemotherapy frequently triggers senescence of tumor cells, which serves as a cell-intrinsic tumor suppressive program for blocking the replicative potential of tumor cells upon oncogenic activation [41–43]. Senescence is not simply an arrest of cellular proliferation but also comprises the active processes of synthesizing various secretory forms of proteins, thereby affecting diverse sets of biological and immunological properties of the tissue microenvironment [44]. The senescence-associated secretory phenotype (SASP) triggered by cellular senescence regulates complex networks between tumor cells and surrounding non-transformed cells, thus affecting multiple processes involved in tumorigenesis and anticancer drug responses [45–47]. For example, SASP-mediated secretion of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, or osteopontin, *etc.*, from senescent tumor cells contributes to tumor growth and chemoresistance by supporting cell-autonomous oncogenic transformation as well as stimulating tumor-promoting inflammation in a paracrine fashion [47–49]. In addition, obesity-mediated disruption of gut microbial metabolites links DNA damage-mediated SASP in hepatic stellate cells with pro-tumorigenic inflammation and liver tumorigenesis upon exposure to chemical carcinogens [50]. Thus, it is plausible that SASP triggers resistance to anticancer therapeutics by creating tumorigenic and therapy-resistant networks formed by coordinated actions between transformed and non-transformed cells. However, the inflammatory mediators secreted by SASP also act as tumor suppressors and augment therapeutic responses to chemotherapy by activating innate effector cells such as NK cells and macrophages [46, 51–53]. Thus, SASP serves as a dual regulator of oncogenesis, and the tumorigenic vs suppressor activity may depend on the different profiles of genetic, epigenetic, and inflammatory repertoires in TMEs (Fig. 1).

2.2 Radiotherapy

Accumulating evidence reveals that clinical responses to radiotherapy are well-correlated with local activation of both the innate and adaptive arms of antitumor immunosurveillance systems [54]. In particular, low-dose radiotherapy facilitates local production of type I IFN and differentiation of pro-immunogenic M1-type macrophages, Th1 helper T cells, and antigen-specific $CD8^+$ cytotoxic T cells. These populations coordinately organize effective innate and adaptive antitumor immune responses and tumor rejection [55–58]. In addition, the abscopal effect induced by radiotherapy, in which local treatment is associated with the regression of metastatic lesions at a distance from the irradiated site, manifests as the recruitment of effector lymphocytes sensitized by irradiated TMEs [59]. As a mechanism of action, radiotherapy triggers innate immune responses by inducing the release of endogenous PRRs, such as HMGB-1 and ATP, from damaged tumor cells and stimulating NF-κB-mediated inflammatory responses which further activate antigen-presenting cells and adaptive responses [60, 61]. Thus, local radiotherapy serves as a promising tool to coordinately stimulate antitumor immunity and tumor-intrinsic pro-apoptotic programs.

There are several lines of evidence that resistance to radiotherapy is associated with the deregulation of TMEs, which promotes macrophage-mediated neovascularization and innate immune-mediated inflammation [62, 63]. Thus, it is plausible that resistance to radiotherapy renders tumor cells able to modulate tumor-associated inflammation and generate "radioresistant niches," thereby contributing to tumor progression and dismal prognoses. Further studies are required to clarify whether resistance to radiotherapy creates a unique phenotype of tumor cells compared to other anticancer therapies in terms of effects on immune repertoires, interactions between resistant tumor cells and immune cells, immunological mechanisms of resistance, and dismal clinical courses.

2.3 Molecular-targeted therapy

Acquisition of genetic mutations following molecular-targeted therapies is not only associated with drug resistance developed by targeted tumor cells but also influences multiple biological properties of TMEs. The epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) serves as a major therapeutic tool for NSCLC patients, and it is well established that specific genetic subtypes have a great impact on the responses to EGFR-TKI in non-small cell lung carcinoma (NSCLC) [9, 64]. For example, mutation or substitution of leucine to arginine at position 858 (L858R) or deletion of exon 19 triggers autonomous activation of EGF signals, which is associated with sensitivity to EGFR-TKI [64, 65]. In contrast, the amino acid substitution of threonine to methionine at position 790 of EGFR exon 20 in the kinase domain (T790M) has been linked to therapeutic resistance to EGFR-TKI [64, 66]. Other oncogenic cascades, such as c-Met, NF-κB, and FAS, have been associated with reduced responses to EGFR-TKI in drug-sensitive NSCLC tumor cells [67, 68]. Accumulating evidence reveals that JAK2-Stat3 pathways greatly compromise the sensitivity to EGFR-TKI in NLSLC tumors [69]. In particular, inflammatory signals mediated by Stat3, which are mainly induced by IL-6 produced from myeloid cells and tumor cells, play a critical role in amplifying antiapoptotic programs, metastatic potential, angiogenesis, and immune tolerance, thereby efficiently generating inflammation-driven tumorigenic environments [70, 71]. Moreover, the activation of phagocytosis-related receptor Axl by Gas 6 in T790M-mutation-naive NSCLC cells triggers resistance to EGFR-TKI, suggesting that immune-mediated mechanisms may be involved in the resistance to EGFR-TKI [72].

BRAF kinase inhibitors (BRAF-I) have emerged as a mainstay for treating patients with advanced melanoma in which most tumors bear the B600V activating mutation [10, 73]. The RAF-MAP kinase cascade is also responsible for altering immune functions by communicating with multiple signals such as JAK2-STAT3 and PI3K, which deregulate the antitumor properties of tumor-resident myeloid cells and lymphocytes and promote tumorigenic inflammation [74, 75]. Interestingly, recent studies reveal that targeting immune suppression or activating host immune responses greatly improves therapeutic responses to BRAF-I, suggesting that BRAF activation may interfere with antitumor immune responses [76, 77]. Thus, it is critical to clarify which molecular cascades of the RAF-RAS-MAPK pathway have a role in regulating particular activities of innate and/or antigen-specific lymphocytes and antigen-presenting cells.

Inhibitors of mammalian target of rapamycin (mTOR) have emerged as potent antitumor agents against various types of malignancy including renal cell carcinoma [78]. Emerging evidence has revealed that the PI3K-mTOR pathway plays a critical role in the regulation of cancer and immune cell metabolism by activating aerobic glycolysis (Warburg effect) [79, 80]. Moreover, the antitumor efficacy of mTOR inhibitors may correlate with modulation of lymphocyte metabolic pathways in TMEs, and rewiring metabolic cascades in tumor-infiltrating $CD8^+$ T cells may influence responsiveness to mTOR inhibitors [81, 82]. Thus, the antitumor efficacy of mTOR inhibitors may derive from their modulation of metabolic cascades in intratumor immune cells.

The clinical efficacy of anti-HER2 mAb (trastuzumab) is associated with the intratumor infiltration of immune cells and tumor immunogenicity [83, 84]. Conversely, distinct immunological parameters, such as increased $CD68^+$ macrophages and reduced $CD8^+$ T cells in intra-and peritumor lesions, reflect poor prognoses for patients with breast cancer. In addition, blockade of colony-stimulating factor-1 receptor

significantly reduced tumor burden and prolonged tumor-free survival in murine mammary tumor models [84, 85]. Consistent with the role of antitumor immunogenicity in the therapeutic efficacy of anti-HER2 mAb, increased numbers of specific immune repertoires such as $CD4^+$ T cells and $CD1a^+$ dendritic cells in axial lymph nodes could be a sensitive marker with which to predict better clinical responses to trastuzumab and increase survival of patients with breast cancer [86]. These findings imply that distinct repertoires of intratumor lymphocytes have a tremendous impact on determining the therapeutic responses to HER2-targeted therapies against breast cancer.

Recent studies have unveiled the critical contribution of multiple myeloma (MM) microenvironments in the regulation of growth and anti-MM drug sensitivities. Host immune systems are critical components affecting therapeutic responses to various anti-MM drugs, including bortezomib and lenalidomide, which have emerged as new therapeutic options for curing patients with MM [87–89]. For example, interactions between plasmacytoid DCs and myeloma cells in bone marrow microenvironments promote the growth and drug resistance of MM [90]. Moreover, a proteasome inhibitor induces ligands for the NKG2D NK cell activating receptor on MM cells, and desensitization to this response may correlate with evasion of innate immune responses, tumor progression, and drug resistance [91]. On the other hand, lenalidomide may have a broad function in modulating antitumor immune responses, and resistance to lenalidomide may be associated with impaired immunosurveillance in the privileged MM microenvironments [92]. Thus, MM cells have a unique capacity to protect themselves from drug-mediated stress insults by manipulating innate and adaptive immunity.

Taken together, these findings demonstrate that the immunological microenvironment exerts significant influence on the regulation of responsiveness to various types of molecular-targeting therapeutics. Thus, targeting tumor-associated immune functions should be a feasible option for improving antitumor efficacies (Fig. 2).

3 Clinical implications

Different classes of anticancer therapeutics have distinct effects on modulating the tumorigenic and immune-modulatory properties of TMEs [18, 19, 31]. Thus, it is highly likely that

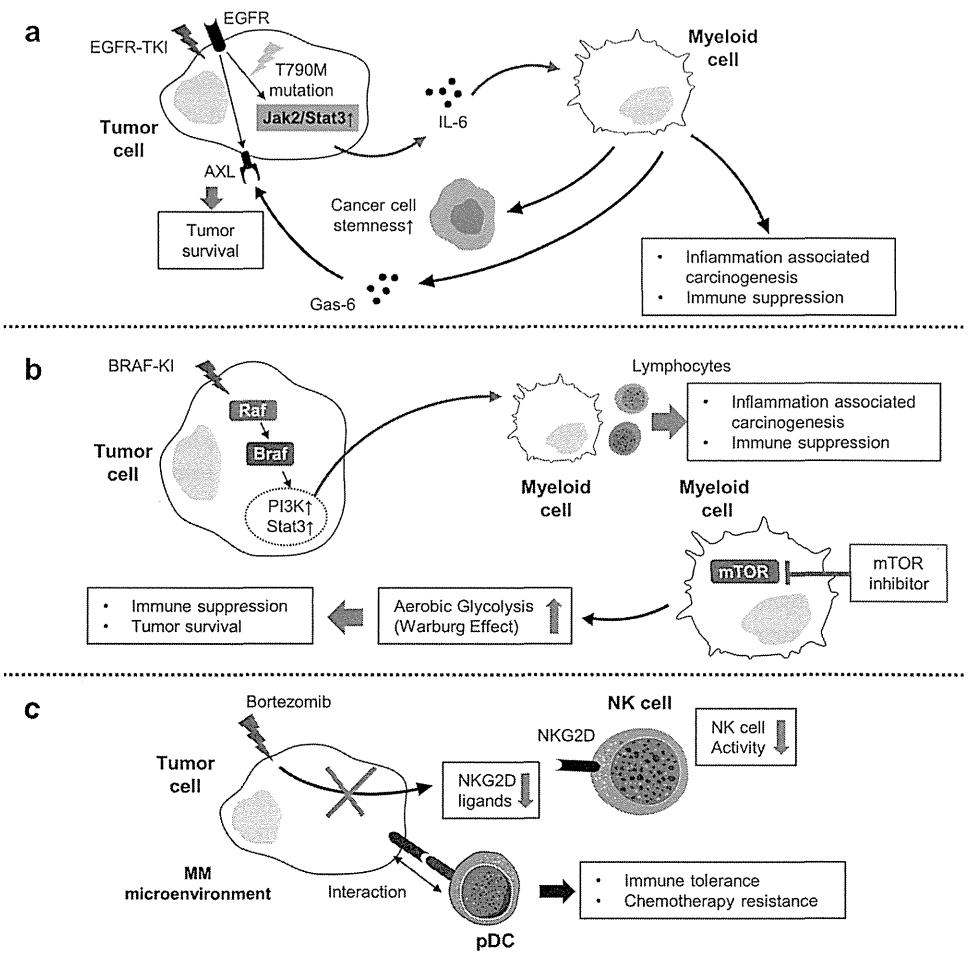

Fig. 2 Immune-mediated resistance to molecular-targeted therapies. **a** Potential pro-inflammatory and immune-modulating effects of FAS-NF-κB signals and Axin-Gas 6 pathways activated in NSCLC cells in the presence or absence of the T790M TKI-resistant mutation. **b** Interplay between the BRA-V600E mutation and PI3K and JAK-STAT signals has an impact on the immunoregulatory cascades in TMEs, whereas mTOR-mediated activation of glycolytic cascades modulates tumorigenic activities of tumor cells and immune cells. **c** Bortezomib-mediated modulation of plasmacytoid DC and NK cell activities in MM microenvironments. These oncogenic modulations resulting from the molecular-targeted therapies may greatly impact the repertoires and functions of tumor-associated immune cells leading to resistance to therapeutics

each anticancer drug has a unique ability to modulate the interaction between tumor cells and immune cells in a reciprocal fashion, creating unique molecular and cellular signals on subsets of tumor cells and tumor-infiltrating immune cells. In this regard, the immune-mediated components might be useful as potential biomarkers to predict the sensitivity and emergence of resistance to certain sets of anticancer drugs. For example, innate immune signals (TLR4, P2RX2, NLRP3, *etc.*) may be sensitive prognostic markers that distinguish patients who respond to particular types of anticancer drugs from those who do not [60, 93].

In addition, the immune-mediated signals derived from chemoresistant TMEs may serve as potential therapeutic targets for improving clinical efficacy and overcoming poor responses to anticancer drugs against chemoresistant tumors. For example, integrin $\alpha v\beta 3$ expressed on various chemoresistant tumor cells of diverse origins may be suitable as a target to augment the antitumor responses to chemotherapy by harnessing host immune systems [40]. Moreover, targeting of myeloid cell-derived immunosuppressive and pro-tumorigenic factors induced by therapy-mediated inflammatory signals, such as TIM-3, TIM-4, or MFG-E8, *etc.*, may enhance the sensitivity to and durability of various anticancer therapeutics by inducing long-term formation of immunological memory in privileged tumor microenvironments [29, 33, 39].

The recent success of targeting immune checkpoint machineries encourages the further assessment of suitable combinations of immunotherapy and other conventional anticancer therapies. For example, the expression of PD-L1 on tumor cells is well-correlated with the oncogenic signals mediated by p38 MAPK [75, 94]. These findings raise the possibility that immune-stimulating antibodies might augment the antitumor activities of molecular-targeted therapies [69, 95]. Further investigations should clarify which immune-mediated factors should be targeted for improving the therapeutic responses of distinct subtypes of anticancer drugs in a comprehensive way.

4 Concluding remarks

We have presented a comprehensive overview and perspective as to how endogenous host immune systems impact therapeutic responses to anticancer modalities. Recent advances in identifying immune-mediated factors modified by anticancer therapeutics further confirm the importance of host immunity in clinical responses to anticancer drugs. However, it remains largely unknown which types of immune responses contribute to the pathogenesis of cancer patients who have reduced clinical responses to certain anticancer therapeutics. In this regard, it is necessary to evaluate the dynamism and kinetics of host immunity in mediating resistance to anticancer drugs in large numbers of patients in a prospective way. Finally, the detailed assessment of distinct profiles of immune-mediated signals in the natural course of cancer immunoediting processes may lead to the development of ideal prognostic markers for predicting the probability of when tumors develop resistance to anticancer drugs as well as therapeutic strategies for improving the clinical prognoses of cancer patients.

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Cancer Research

Cancer Stem-like Cells Derived from Chemoresistant Tumors Have a Unique Capacity to Prime Tumorigenic Myeloid Cells

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Abstract

Resistance to anticancer therapeutics greatly affects the phenotypic and functional properties of tumor cells, but how chemoresistance contributes to the tumorigenic activities of cancer stem-like cells remains unclear. In this study, we found that a characteristic of cancer stem-like cells from chemoresistant tumors (CSC-R) is the ability to produce a variety of proinflammatory cytokines and to generate M2-like immunoregulatory myeloid cells from $CD14^+$ monocytes. Furthermore, we identified the IFN-regulated transcription factor IRF5 as a CSC-R–specific factor critical for promoting M-CSF production and generating tumorigenic myeloid cells. Importantly, myeloid cells primed with $IRF5^+$ CSC-R facilitate the tumorigenic and stem cell activities of bulk tumors. Importantly, the activation of IRF5/M-CSF pathways in tumor cells were correlated with the number of tumor-associated CSF1 $receptor^+$ M2 macrophages in patients with non–small lung cancer. Collectively, our findings show how chemoresistance affects the properties of CSCs in their niche microenvironments. *Cancer Res; 74(10); 2698–709. ©2014 AACR.*

Introduction

Resistance to anticancer modalities poses serious obstacles that must be addressed to improve the clinical prognosis for patients with cancer. Recent studies have revealed that multiple mechanisms enable the development of resistance to anticancer therapies through genetic alterations and environmental modifications such as vascular and immunologic remodeling within tumor tissues (1–4). Coordinated intrinsic and extrinsic pressures force tumor cells to accommodate to stressful microenvironments and coopt multiple strategies for survival, invasion, and distant metastasis, further enhancing tumorigenicity and worsening clinical prognosis. Accumulating evidence has established that rare populations termed cancer stem cells (CSC) are indispensable as a main source of tumorigenicity and anticancer drug resistance (5). Although it remains largely unclear whether chemoresistance further modifies the phenotypic and functional manifestations of CSCs, previous studies have revealed that the low sensitivity to cytotoxic therapies of CSCs derives mainly from cellular quiescence and multidrug transporter activity (6).

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In this study, we elucidate an unexpected property of CSCs derived from chemoresistant tumors (CSC-R). Although such CSCs are identical to untreated CSCs in self-renewal and phenotypic properties, they have a unique ability to produce various proinflammatory mediators that act to generate tumorigenic myeloid cells. IFN regulatory factor-5 (IRF5) plays a critical role as a CSC-R–specific transcription factor that facilitates M-CSF production and promotes myeloid cell–mediated tumorigenic activities. Our findings provide novel mechanisms, whereby resistance to anticancer therapies changes the biologic character of CSCs in distinct niche microenvironments.

Materials and Methods

Mice and tumor cell lines

NOD–SCID (nonobese diabetic/severe combined immunodeficient) animals were purchased from Charles River Laboratories. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University (Sapporo, Japan).

Tumor cell lines (MDA-MB-231 and HCT116) were obtained from the American Tissue Culture Collection. All cell lines described above were obtained 1 year before being used in experiments and authenticated by the Central Institute for Experimental Animals (Kawasaki, Japan) for interspecies and mycoplasma contamination by PCR within 3 months before the experiments.

Patient samples

The clinical protocols for this study were approved by the committees in the Institutional Review Board of Hokkaido University Hospital (approval number, 10-0114). Pleural effusion cells were obtained from patients with stage IV non–small cell lung cancers (NSCLC) after written informed consents had

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been obtained. The cells were isolated by Ficoll-Hypaque density centrifugation, and further purified as $EpCAM^+$ epithelial cells and $CD14^+$ monocytes from tumor tissues or pleural effusion.

Generation of chemoresistant tumor cells

To generate chemoresistant tumor cells, MDA-MB-231 cells or HCT116 cells were treated with taxane or CDDP at low concentrations (1 μg/mL) for 96 hours, respectively. The cells were extensively washed three times and then treated with the identical drug at same concentrations for additional five passage times. The generation of chemoresistant cell variants of MDA-MB-231 or HCT116 cells was confirmed by the near-complete resistance to cell death (90% or more) by the treatment with taxane or CDDP, respectively.

Flow cytometry

The expression levels of CD44 and ALDH1 for CSC-R and CSC-N were evaluated with anti-human CD44 antibody (BD Biosciences) and ALDEFOUR reagents (VERITAS), respectively. For myeloid cell analysis, monocytes stimulated with M-CSF, with supernatants of CSC-R or with those of CSC-N were stained with anti-CD206 antibody and anti-CD68 antibody (BD Biosciences). The cell viability of tumor cells treated cytotoxic drugs was examined by staining with Annexin V/propidium iodide staining according to the manufacturer's instructions (BD Biosciences). The cells were subjected to flow cytometry using a FACSCaliber.

Sphere-forming assay

For sphere-forming assays, CSC-R, CSC-N, or bulk MDA-MB-231 cells primed with myeloid cells (1,000 cells/well) were cultured in ultra-low attachment culture dishes (Corning) in serum-free medium. Dulbecco's Modified Eagle Medium/F-12 serum-free medium was supplemented with 20 ng/mL epithelial growth factor and 10 ng/mL basic fibroblast growth factor (bFGF)-2 (PeproTech). Digestion and cell passage were performed every 3 days, and the sphere-forming colonies were counted after three serial passages.

Measurement of cytokine and chemokines

CSCs, non-CSC, or bulk cells from treatment-naïve or chemoresistant tumors (MDA-MB-231 or HCT116; 1 × 106/well at start point) were cultured for 48 hours, and the protein levels of cytokines and chemokines were quantified by ELISA using supernatants obtained from cultured CSC-R and CSC-N according to the manufacturer's instructions (BD Biosciences). Detection limit for cytokines interleukin (IL)-1β, 4 pg/mL; IL-6, 2 pg/mL; IL-8, 2 pg/mL; IL-10, 7.8 pg/mL; IL-12 p40, 15 pg/mL; TNF-α, 15 pg/mL; TGF-β, 19 pg/mL; GM–CSF (granulocyte macrophage colony–stimulating factor), 9.4 pg/mL; M-CSF, 16.2 pg/mL; IFN-α, 15 pg/mL; IFN-γ, 10 pg/mL; CCL2, 2,3 pg/mL; CCL19, 7.8 pg/mL; and CCL20, 7.8 pg/mL.

Quantification of cytokine mRNA by real-time PCR

The mRNA was isolated from CSC-R, CSC-N or these cells after transfection with control or IRF5 siRNA. The mRNAs of genes associated with myeloid cell differentiation [CSF1R (colony-stimulating factor-1 receptor), C/EBPα, and PU.1] or polarization (ARG-1, Relm-α, IL-4R) or effector functions (IL-10, IL-12p70, CXCL9, IFN-γ) were quantified by real-time PCR (RT-PCR) using SYBR Green Gene Expression Assays (Applied Biosystems).

Luciferase reporter assay for NF-κB and IFN-stimulated response element

CSC-R or CSC-N from MDA-MB-231 or HCT116 was treated with taxane or CDDP, respectively, and transfected with control or IRF5 siRNA (5′rGrArCUrCrCUrGUUrCrCrArArATT3′) for 24 hours. The cells were then transiently transfected with firefly luciferase reporter plasmid encoding NF-κB or IFN-stimulated response element (ISRE) and control *Renilla* luciferase plasmid (1 ng) for 20 hours. The luciferase activities of lysates from the cells were shown as a ratio of firefly reporter intensities to control *Renilla* intensities.

Immunoblotting

CSC-R or CSC-N from MDA-MB-231 or HCT116 was subjected to Western blotting to quantify the protein levels of IRF5 by using antibodies for human IRF5 antibody (Cell Signaling Technology). β-Actin was used as a loading control to check the integrity of each sample.

RNA interference

The siRNA-mediated knockdown was performed by On-target plus SMART pool reagents designed to target human IRF5 (Thermo-Dharmacon). The human IRF5 or control siRNAs were transfected into tumor cells by Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Myeloid cell–mediated tumorigenic activities

CSC-R, CSC-N, or bulk tumor populations from MDA-MB-231 cells were transfected with control or IRF5 siRNA for 48 hours, and then cocultured with $CD14^+$ monocytes for 96 hours. The myeloid cells primed with CSC-R were isolated from cocultured cells and further cultured for 24 hours to obtain culture supernatant. Bulk MDA-MB-231 or HCT116 cells treated with the supernatant of CSC-R–primed myeloid cells were subjected to chemotherapy-induced apoptosis and sphere formation analysis.

In vivo tumorigenic activities of CSCs mediated by human myeloid cells

After depletion of endogenous myeloid cells by clodronate liposome (200 μg/mouse) intraperitoneally administered twice a week, the CSC-N or CSC-R isolated from HCT-116 cells were injected subcutaneously into NOD–SCID mice at small doses (1×10^2/mice) in conjunction with intravenous administration of $CD68^+$ macrophage (1×10^6/mice) isolated from the peripheral blood of the healthy donor. The tumor growth was measured on the indicated days, and numbers of human macrophages were evaluated in tumors from each mice.

In vivo tumorigenic activities of primary NSCLC tumors

$EpCAM^+CD133^+$ CSCs obtained from primary patients with NSCLC were transfected with control or IR5 siRNA for 48 hours, and then cocultured with $CD14^+$ monocytes isolated from

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autologous peripheral blood leukocytes (PBL) for 96 hours. $CD11b^+$ myeloid cells were isolated from the cocultured cells for *in vivo* tumor growth assays. For the *in vivo* primary tumor experiments, bulk NSCLC tumor cells (1×10^3/mouse: $n = 5$) were inoculated subcutaneously into NOD–SCID mice in combination with intravenous transfer of autologous $CD11b^+$ myeloid cells. Two days before tumor challenge, NOD–SCID mice were treated with clodronate intravenously to remove endogenous macrophages. Tumor growth was measured on the indicated days.

Statistical analysis

Statistical analysis was performed using the paired Student *t* test, and the degree of statistical correlation was evaluated from the Pearson correlation coefficients analysis. A *P* value of <0.05 was considered as statistically significant. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

Results

Characterization of CSCs derived from chemoresistant tumors

Cytotoxic chemotherapy manipulates multiple signaling cascades, including DNA damage pathways, cell death machineries, oncogenic signaling, and chromatin remodeling–associated events (1, 7). CSCs serve as the apex of cellular hierarchy responsible for tumor initiation and progression, but whether biologic alterations linked with chemoresistance further modulate the tumorigenic activities of CSCs remains largely unknown. To define this, we generated chemoresistant variants of MDA-MB-231 breast cancer cells and HCT116 colon cancer cells through repetitive exposure to low doses of taxane and cisplatin, respectively (8). CSCs were isolated from these chemoresistant and sensitive variants based on their identical CSC marker expression (Fig. 1A). CSCs derived from chemoresistant variants (CSC-R) were refractory to cytotoxic therapies compared with CSCs from untreated tumor cells (CSC-N). However, the frequencies of CSCs were nearly identical with similar expression levels of CSC markers (ALDH1 and CD44) on naïve and chemoresistant tumor cells (Fig. 1B), and there were little differences between CSC-N and CSC-R on the self-renewal activities as shown by similar levels of sphere-forming activities (Fig. 1C). Importantly, the chemoresistant cell variants generated from the treatment-naïve $CD44^-$ non-CSC subsets did not exhibit the phenotypic and functional properties of CSCs (Supplementary Fig. S1). Thus, chemoresistance does not render non-CSC with plasticity to undergo transdifferentiate into CSCs.

Together, our findings potentially suggest that CSC-N undergoes intrinsic genetic and/or epigenetic alternation to transform into the CSC-R upon chronic exposure with cytotoxic drugs, but further studies should be required for defining this possibility by comprehensive genetic analysis.

Proinflammatory profile of CSCs derived from chemoresistant tumor cells

Inflammatory signals play a critical role in the regulation of the stem cell characteristics and tumorigenic activities of CSCs through autocrine and paracrine-mediated mechanisms (9–11). We, therefore, next evaluated the possibility that chemoresistance modifies inflammatory profiles in CSCs from MDA-MB-231. To our surprise, we found that mRNA levels of various cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-12p40, TNF-α, M-CSF, and CCL2 were produced in CSC-R subsets at higher amounts compared with the bulk tumor cell counterparts, although bulk chemoresistant cells could produce proinflammatory mediators at higher levels than CSC-N or treatment-naïve bulk tumor cells (Fig. 2A and Supplementary Fig. S2A). We also confirmed the higher protein levels of several cytokines (M-CSF, IL-1β, IL-6, and TNFα) in CSC-R compared with bulk or non-CSC populations (Fig. 2B and data not shown). Consistent with its proinflammatory activities, the transcriptional activity of NF-κB was higher in CSC-R compared with CSC-N (Fig. 2C). We observed similar trends in CSC-R from HCT-116 (Supplementary Fig. S2A). Furthermore, the chemotherapy-naïve CSCs from MDA-MB-231 cells did not produce proinflammatory cytokines such as M-CSF when they were treated with cytotoxic drug taxane for a brief period, suggesting that acute stress responses changes evoked by exposure of cytotoxic drugs had little effects in modifying inflammatory profiles of CSCs (Supplementary Fig. S3A). Taken together, our findings suggest that chemoresistance rendered CSCs with the ability to triggers autocrine activation of inflammatory signals, which subsequently resulted in the production of proinflammatory mediators.

CSC-R prime monocytes to differentiate into M2 macrophages

Given the characteristic proinflammatory cytokine profiles of CSC-R, we focused our study on the effects of CSC-R–mediated inflammatory signals on phenotypic and functional properties of macrophages, because myeloid cells have a tremendous impact on the regulation of tumorigenic activities and anticancer drug resistance (12, 13). Macrophages express several immunoregulatory genes that are closely linked with impaired tumor immunosurveillance (14). We found that CSC-R culture supernatants induced several factors characteristic of M2 macrophages, such as arginase-I, CSF1R, IL-4R, Relm-α and IL-10, C/EBPα, and PU.1 (Fig. 3A and Supplementary Fig. S2B). In contrast, there was little difference between CSC-R and CSC-N in the expression of IL-12p70, IFN-γ, and CXCL9, which are associated with M1 phenotypes (Fig. 3B). Furthermore, the treatment with CSC-R–derived soluble factors upregulated the M2 macrophage marker CD206 mannose receptor at comparable levels with the treatment with M-CSF. However, bulk chemoresistant cells could generate M2 macrophages at higher levels than CSC-N or treatment-naïve tumor counterparts (Fig. 3C). It is notable that bulk tumor cells are capable for differentiating the M2 macrophages from $CD14^+$ monocytes, but non-CSCs had little ability in differentiating into M2 macrophages compared with nontreatment control (Fig. 3C). Thus, it is highly likely that the M2 macrophage differentiation by the bulk tumors relies mainly on the CSCs contained within

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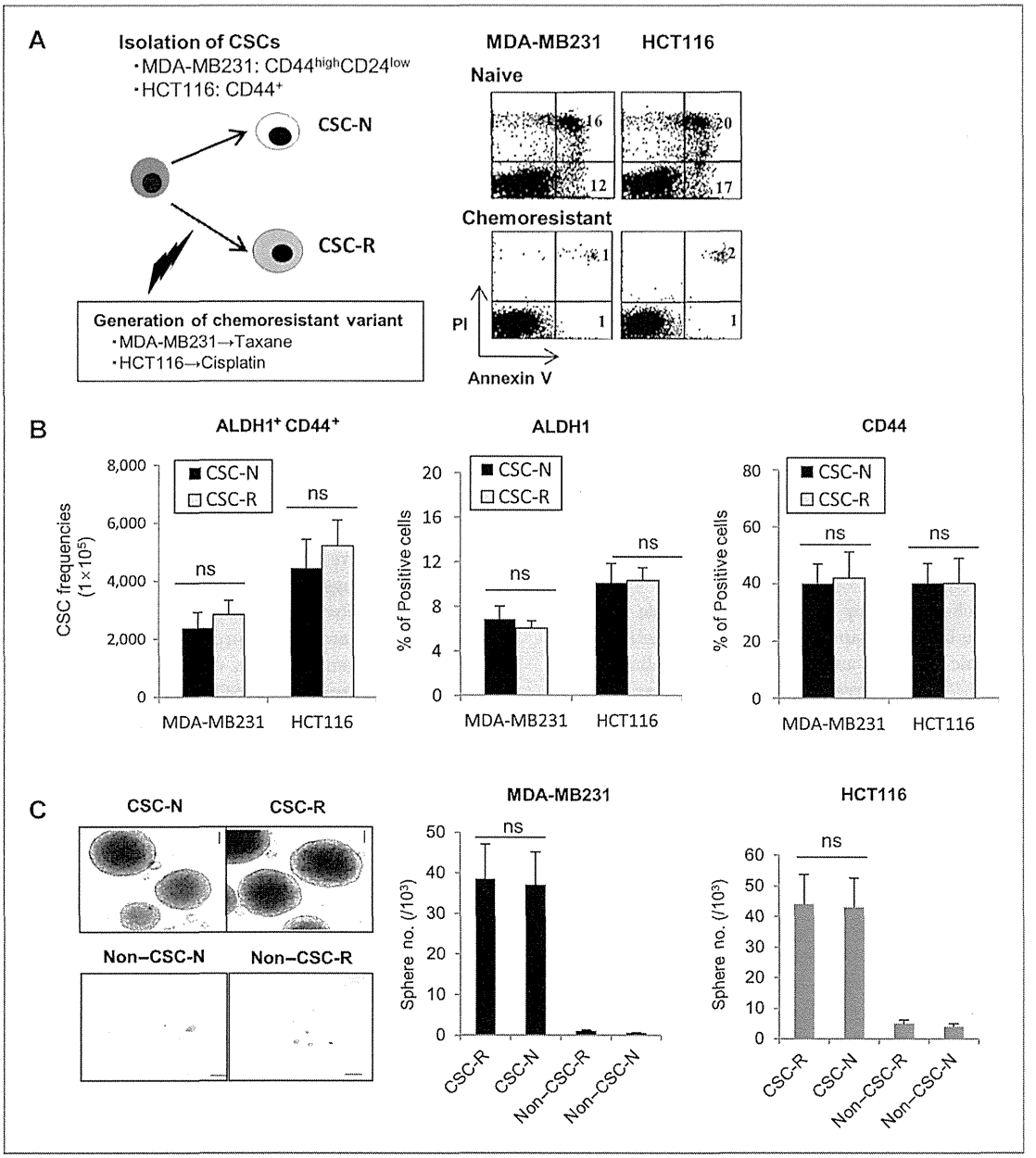

Figure 1. Phenotypic and functional analysis of CSC-R. A, isolation of CSC populations from chemoresistant (CSC-R) or untreated MDA-MB-231 breast cancer cells or HCT-116 colon cancer cells (CSC-N). The cell viability of CSC-R and CSC-N was assessed upon cytotoxic chemotherapy treatment (taxane for MDA-MB-231 and cisplatin for HCT-116). B, CSC-R or CSC-N from MDA-MB-231 or HCT-116 cells were treated with cytotoxic drugs (taxane for MDA-MB-231 and cisplatin for HCT-116) for 24 hours, and the $ALDH1^+CD44^+$ CSC frequencies in bulk tumor cells, as well as expression of ALDH1 or CD44 was assessed by flow cytometry. C, the CSC-R, CSC-N, and their non-CSC counterparts from MDA-MB-231 and HCT116 cells were cultured in serum-free medium supplemented with EGF and bFGF in ultra-low attachment culture dishes for three passages, and the numbers of formed spheres generated per 1,000 cells were determined. We repeated three experiments with all similar results.

the whole populations, although it remains probable that chemoresistance itself differentiate M2 macrophage by CSC-independent fashion. Furthermore, supernatant of the chemotherapy-naïve MDA-MB-231 CSC-N treated with taxane overnight had little effect in generating $CD206^+$ M2 macrophages, suggesting that acute stress responses evoked by exposure of cytotoxic drugs do not render CSCs with the ability to induce macrophages with immunosuppressive phenotypes (Supplementary Fig. S3B).

The generation of therapy-resistant cell lines was largely dependent on the numbers of treatment with low-dose chemotherapy (more than four times). Therefore, we next setup the experiments by using CSC-N of HCT116 cells treated with low-dose CDDP for 72 hours on various

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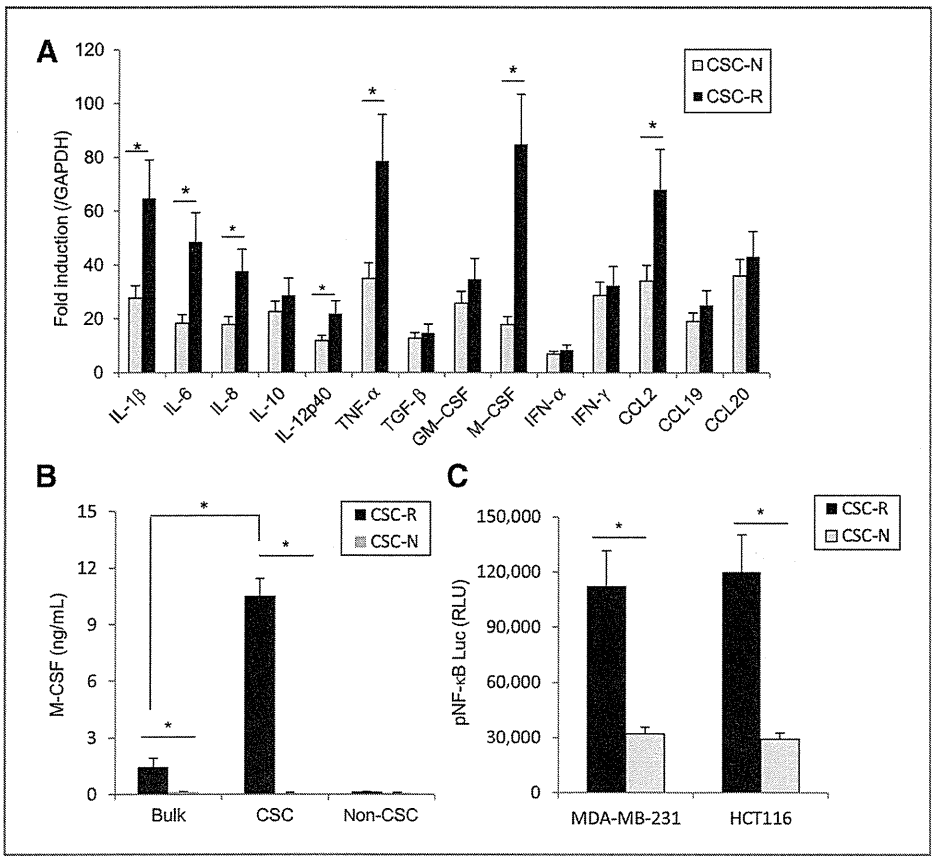

Figure 2. Proinflammatory profiles of CSC-R. A, the mRNA levels of cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p40, TNF-α, TGF-β, GM–CSF, M-CSF, IFN-α, and IFN-γ) or chemokines (CCL2, CXCL19, and CXCL20) in CSC-R or CSC-N from MDA-MB-231 were quantified by RT-PCR. B, the protein levels of M-CSF were measured in culture supernatants from bulk cells, CSC or non-CSC subsets of chemoresistant and treatment-naïve MDA-MB-231 cells. C, CSC-R and CSC-N (MDA-MB-231 or HCT-116) were transfected with a p-NF-κB reporter plasmid in the presence or absence of cytotoxic agents (taxane for MDA-MB-231, CDDP for HCT-116), and luciferase assays were performed to measure NF-κB activities in cell lysates. We repeated five experiments with all similar results. *, $P < 0.05$.

treatment/passage cycles. We found that the CSC-N generate M-CSF and prime M2 macrophages in concomitant with acquisition of chemoresistant phenotype (Supplementary Fig. S3C and S3D). To further address whether CSC-R differs from CSC-N in their unique activities of priming M2-macrophages *in vivo*, CSC-N or CSC-R isolated from HCT-116 cells were injected subcutaneously into NOD–SCID mice at small doses (1×10^2/mice) in conjunction with intravenous administration of $CD14^+$ monocytes (1×10^6/mice) isolated from the peripheral blood of the healthy donor, and the tumor formations were evaluated *in vivo*. In this experiment, NOD–SCID mice were pretreated with clodronate liposome to remove endogenous macrophages. Although the CSC-R–driven tumors were grown at greater levels than CSC-N tumors, their tumorigenicity was positively correlated with the presence of myeloid cells, because its tumor growth was accelerated by adoptive transfer of human monocytes. In marked contrast, the transfer of human monocytes had little effects on the CSC-N–derived tumor growth (Fig. 3D). More importantly, human $CD68^+$ macrophages expressing the putative M2 marker CD163 and CD206 were infiltrated into the tumor tissues of CSC-R at greater levels than those of CSC-N (Fig. 3E). In contrast, the frequencies of $CD163^{low}HLA\text{-}DR^{high}$ M1 macrophages were comparable in tumors from CSC-R and CSC-N (Fig. 3E). Together, these results further support our hypothesis that CSC-R specifically regulates infiltration and differentiation of immunoregulatory M2 macrophages.

Interestingly, the supernatants of CSC-R contributed to the induction of $CD33^+HLA\text{-}DR^{low}$ myeloid-derived suppressor cells (MDSC) at higher levels than those of CSC-N or non-CSC counterparts. In addition, the induced MDSCs were homogenously $CD14^+CD15^-$ monocytic subsets when treated with either CSC or non-CSCs from chemoresistant or treatment-naïve cells (Supplementary Fig. S4). Again, CSC-N acutely treated with chemotherapy did not induce M2-like macrophages, suggesting that CSCs could not acquire an ability to prime tolerogenic myeloid cells by chemotherapy-mediated acute stress responses (Supplementary Fig. S3B).

Together, these results suggested that soluble factors released from CSC-R may regulate the differentiation of immunosuppressive macrophages from monocyte precursors, which have a great impact on tumorigenicity.

Upregulation of IRF5 promotes M-CSF production from CSC-R

In addition to NF-κB–mediated inflammatory signals, transcriptional activities mediated by ISREs were detected in CSC-R at much higher levels than those in CSC-N (Fig. 4A). In contrast, type I IFN and transcriptional activities of IFN-γ activation sequences (GAS) were detected in CSC-R at levels similar to those in CSC-N upon chemotherapy (Fig. 4A).

Given that IFNs signal through Janus-activated kinase (JAK)/STAT pathways to induce IFN stimulation genes, which are under control of ISRE and GAS elements, we hypothesized that

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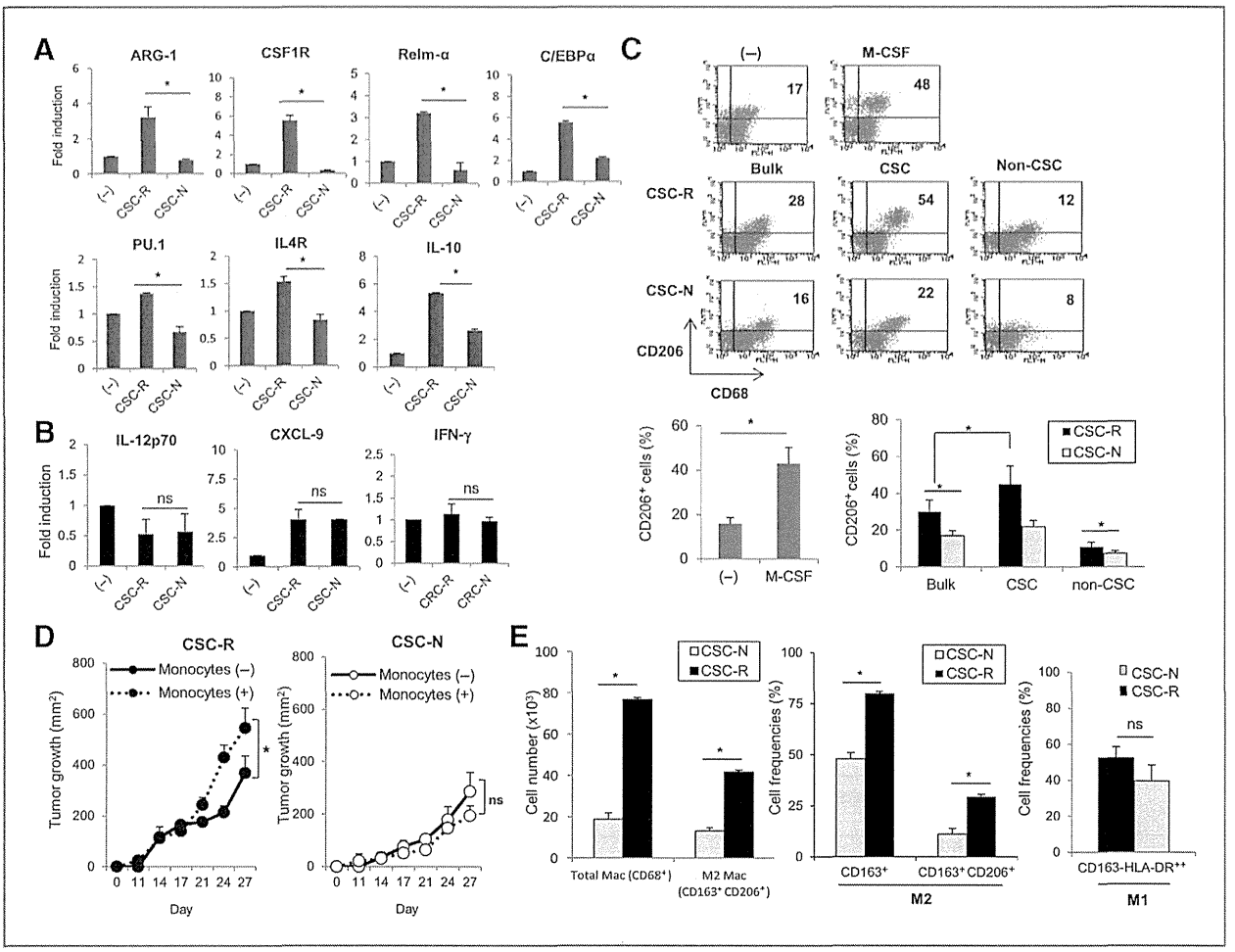

Figure 3. CSC-R contributes to tumorigenic myeloid cell differentiation. A, $CD14^+$ monocytes were isolated from PBL and untreated (–) or treated for 48 hours with 20% culture supernatant of CSC-R or CSC-N from MDA-MB-231 cells. The mRNA levels of genes associated with polarization of immunoregulatory macrophages [arginase-I (ARG-I), CSF1R, IL-4 receptor (IL-4R), Relm-α, and IL-10] or with myeloid lineage differentiation (C/EBPα and PU.1) were measured by quantitative RT-PCR. Similar results were obtained from three experiments, and the means ± SEM are shown. B, the mRNA levels of genes associated with polarization of immunostimulatory macrophages (IL-12p70, CXCL9, and IFN-γ,) were measured by quantitative RT-PCR. C, $CD14^+$ monocytes were isolated from PBL and treated for 48 hours with recombinant M-CSF (100 ng/mL) or 20% culture supernatant of bulk cells, CSCs or non-CSC subsets of chemoresistant or treatment-naïve MDA-MB-231 cells. The expression levels of CD206 and CD68 were assessed by flow cytometry. Representative data (left) and statistical analysis are shown. We repeated four experiments with all similar results. D, CSC-N or CSC-R (1×10^2/mice) were inoculated into clodronate-pretreated NOD–SCID mice ($n = 3$/group) with or without $CD14^+$ monocytes obtained from the peripheral blood of the healthy donor (1×10^6/mice). The growth curves of each tumor are shown. E, total number ($\times 10^3$), as well as the frequencies (%) of $CD68^+$ total macrophages, $CD163^+CD206^+$ M2 macrophages, or $CD163^{low}HLA\text{-}DR^{high}$ M1 macrophages infiltrating into tumor tissues was evaluated by flow cytometry. *, $P < 0.05$.

CSC-R have a unique ability to stimulate ISRE-mediated transcriptional activities using autocrine IFN and/or JAK/STAT–independent mechanisms (15). IRFs serve upstream of ISREs, which have distinct and overlapping roles for IFN-γ/GAS–related signals. To evaluate the role of IRFs in the regulation of ISRE transcriptional activities, we used specific siRNA gene knockdown (KD) of each of the IRF genes (IRF1-9) in HCT-116 CSC-R and then treated the cells with cisplatin for 24 hours. In this analysis, we identified IRF5 as a critical factor, which is specifically expressed in CSC-R and regulate ISRE activities. The knockdown of IRF5, but not that of other IRF families, diminished the transcriptional activities of ISRE in CSC-R (Fig. 4B). In contrast, ISRE activities remained unchanged in CSC-N regardless to the inhibition of IRF members, including IRF5 (Fig. 4B). We also confirmed that the mRNA levels of IRF5 expression were detected in CSC-R at much higher levels than parental tumors or CSC-N, and the protein levels of IRF5 were also higher in CSC-R from MDA-MB-231 and HCT116 compared with the CSC-N counterparts (Fig. 4C and D).

To define the functional relevance of IRF5 to the immunemodulatory effects of CSC-R, we next examined whether IRF5 either regulates selective sets of cytokines or, instead, generally affects inflammatory signals. The siRNA knockdown of IRF5 suppressed M-CSF production from CSC-R but not non–CSC-R or CSC-N, whereas IRF5 had little impact on the regulation of other soluble mediators in CSC-R (Fig. 4E and data not shown).

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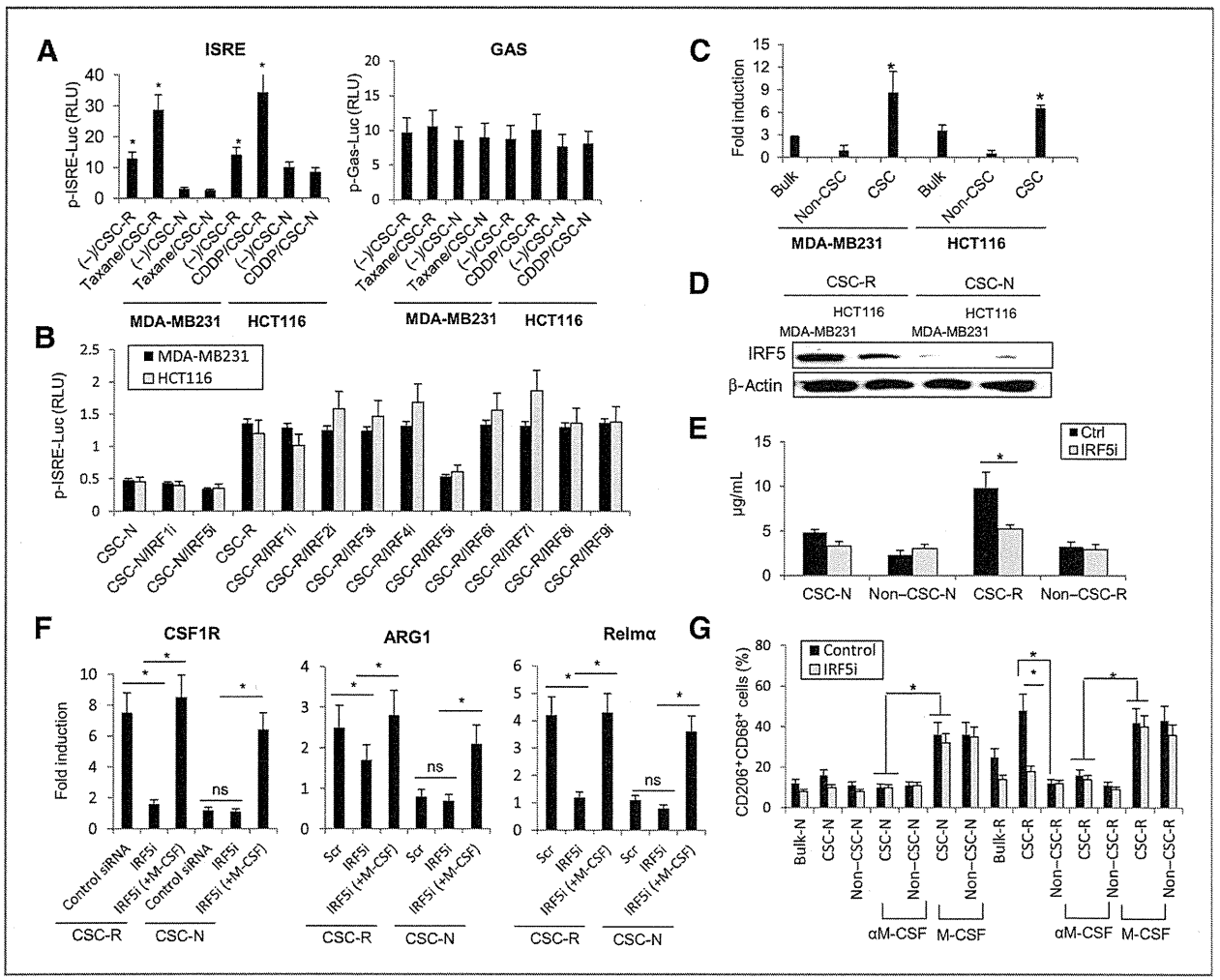

Figure 4. Selective activation of IRF5 in CSC-R. A, CSC-R and CSC-N (MDA-MB-231 or HCT-116) were transfected with p-ISRE or p-GAS reporter plasmids in the presence or absence of cytotoxic agents (taxane for MDA-MB-231, CDDP for HCT-116), and luciferase assays were used to measure the transcriptional activities using cell lysates. B, CSC-R and CSC-N (HCT-116) were transfected with control siRNA or siRNA for IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8, and IRF9 in the presence of a p-ISRE reporter plasmid for 48 hours, and then treated with CDDP for 24 hours. Luciferase assays were performed to measure transcriptional activities using the cell lysates. C and D, the mRNA (C) or protein levels (D) of IRF5 in CSC, non-CSC, or their bulk populations of chemoresistant or treatment-naïve cells were evaluated by RT-PCR or Western blot analysis, respectively. E, CSC-R, CSC-N, or their non-CSC counterparts were transfected with control or IRF5 siRNA for 48 hours. The protein levels of M-CSF in the culture media were measured. F, CSC-R or CSC-N was transfected with control or IRF5 siRNA for 48 hours. In addition, M-CSF (10 μg/mL) was added to the IRF5 siRNA-transfected cells. The mRNA levels of CSF1R, arginase-I (Argl) or Relm-α in macrophages primed with CSCs were measured by RT-PCR. G, CSC-R, CSC-N, or bulk tumor cells (HCT-116) were transfected with control or IRF5 siRNA for 48 hours and then treated with anti–M-CSF–neutralizing antibody (clone 26730), control Ig, or recombinant M-CSF for 24 hours. The 20% supernatant of each tumor subsets was used for treating with $CD14^+$ monocytes isolated from PBL for 48 hours. The CD206 expression on $CD68^+$ macrophages was determined by flow cytometry. We repeated three experiments with all similar results. *, $P < 0.05$.

Importantly, IRF5 inhibition substantially reduced the ability of CSC-R to induce several factors critical for M2 macrophage differentiation in $CD14^+$monocytes, but addition of M-CSF recovered the expression levels of M2 factors in macrophages primed with IRF5-KD CSC-R at comparable levels with those primed with control CSC-R, further indicating that IRF5-mediated M-CSF of CSC-R is critical for inducting M2 macrophage–associated factors (Fig. 4F).

The IRF5-dependent effects in priming M2 macrophage differentiation were largely dependent on M-CSF from CSC-R because the CSC-R–mediated induction of $CD206^+$ macrophages was substantially diminished by the treatment with anti–M-CSF–neutralizing antibody (Fig. 4G). Moreover, the treatment with recombinant M-CSF proteins increased CD206 expression on monocytes treated with CSC-N at similar levels to those with CSC-R, and abrogated the suppressive effect of IRF5 siRNA in priming M2-like macrophages by CSC-R (Fig. 4G). Collectively, these findings demonstrated that the IRF5–M-CSF pathway specifically regulated by CSC-R plays a critical role in priming immunoregulatory myeloid cells.

Colon CSCs have been defined as the cells expressing multiple cell-surface markers other than CD44, such as CD133 and

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CD166 (16). We found that the frequencies of $CD133^+CD44^+$ populations were similar on naïve tumor cells and their chemoresistant variants. Furthermore, $CD44^+CD133^+$HCT116-CSC-R displayed higher expression of IRF5, M-CSF production, and M2 macrophage differentiation, compared with $CD44^+$ $CD133^+$ CSC-N, further validating the importance of the IRF5–M-CSF pathway in defining CSC-R to activate immunosuppressive myeloid cells (Supplementary Fig. S5). Interestingly, M-CSF was detected in the CSC-N from HCT116 cells, unlike those from MDA-MB-231 cells. However, M-CSF was produced from HCT-116–derived CSC-N by an IRF5-independent manner, because the knockdown of IRF5 had a little impact on basal levels of M-CSF in HCT116-CSC-N (Supplementary Fig. S5). Therefore, the baseline levels of M-CSF were valuable among different cell lines, but chemoresistance conferred CSCs capable of selectively using IRF5 for M-CSF production.

Taken together, these results identified IRF5 as a critical transcriptional factor in CSC-R that suppresses the induction of tumorigenic and immunosuppressive macrophages by promoting M-CSF production.

CSC-R–derived IRF5 renders myeloid cells competent to promote tumorigenicity and chemoresistance

Several studies have revealed that IRF5 plays an antitumor role through the induction of cell death programs by cell-intrinsic and immune-mediated mechanisms (17–19). Our findings that IRF5 is selectively activated in CSC-R suggest that this transcription factor may potentially have unique functions distinct from those it has in chemosensitive tumors and immune cells. To define the functional significance of IRF5-regulated CSC-R/macrophage cross-talk, bulk HCT116 colon cancer cells were treated with supernatants from human macrophages prestimulated with CSC-R or IRF5-KD CSC-R, and then subjected to *in vitro* chemotherapy to induce apoptosis. The supernatant from macrophages primed with CSC-R suppressed apoptosis. In contrast, supernatant from macrophages stimulated by IRF5-KD CSC-R sensitized bulk HCT116 tumor cells to apoptotic cell death upon CDDP treatment at levels comparable with untreated cells (Fig. 5A). The importance of IRF5-regulated pathways in CSC/macrophage interaction was further confirmed using MDA-MB-231 breast cancer cells (data not shown).

We next evaluated the role of IRF5 in long-term tumor sphere-forming activity, which is a common characteristic of CSCs. The supernatant of CSC-R–primed macrophages increased sphere numbers and diameters in bulk MDA-MB-231 cells, but knockdown of IRF5 in CSC-R decreased the macrophage-mediated sphere-forming activities of MDA-MB-231 cells (Fig. 5B). In addition, treatment with anti-CSF1R–neutralizing antibodies completely suppressed sphere formation in HCT-116 cells primed by either control CSC-R or IRF5-KD CSC-R, suggesting that M-CSF produced by CSC-R–stimulated macrophages plays a critical role in inducing the stem cell activities of bulk tumor cells (Fig. 5B). Although the supernatants of CSC-N had a little ability in promoting sphere formation of bulk MDA-MB-231 cells, they support the sphere formation in the presence of recombinant M-CSF at similar extent with the CSC-R supernatants (Supplementary Fig. S6). These results further support our hypothesis that IRF5-mediated M-CSF of CSC-R plays an indispensable role in activating tumorigenic activities of bulk tumor cells by triggering CSC properties.

To further define the contribution of CSC-R–specific IRF5 to the regulation of *in vivo* tumorigenic activities in clinically relevant settings, we used clinical samples of patient refractory to anticancer therapies after extensive intervention by multiple rounds of chemotherapy and EGFR-TKI (tyrosine kinase inhibitor; gefitinib). The $CD14^+$ monocytes isolated from peripheral blood of these patients were cocultured with $EpCAM^+CD133^+$ autologous CSCs transfected with control or IRF5 siRNA for 48 hours. Interestingly, the CSC marker ALDH1 expression were substantially increased in bulk primary NSCLC cells stimulated by the CSC-primed monocytes, whereas CSC-primed monocytes had little effects on ALDH1 expression in the CSC-depleted bulk tumor cells (Fig. 5C). These results suggest that CSC-R–activated myeloid cells specifically expand the resident populations of CSCs, but they did not undergo the transdifferentiation from non-CSC to CSCs or increased tumorigenicity by CSC-independent manner.

We next evaluated whether the IRF5–M-CSF axis in CSCs promote *in vivo* tumorigenicity by using these primary NSCLC tumors. To do so, the bulk tumor populations were injected subcutaneously into NOD–SCID mice in low numbers (1×10^2/mice) in conjunction with the CSC-primed monocytes, and the *in vivo* tumor formations were evaluated at the indicated times. To exclude the involvement of endogenous macrophages, NOD–SCID mice were pretreated with clodronate liposomes to remove endogenous macrophages before all procedures. Cotransfer of CSC-primed monocytes resulted in larger tumor formation compared with those of non–CSC-primed monocytes or untreated tumor cells. Importantly, tumor growth was markedly suppressed by adoptive transfer of CSC–IRF5-KD–primed monocytes (Fig. 5D). Overall, these findings provide clear evidence that IRF5-mediated regulation of CSC–myeloid cell interactions serves as a critical pathway supporting tumorigenicity and chemoresistance.

Clinical significance of IRF5–M-CSF pathways

Finally, we sought to determine whether our observations could be verified in the clinically relevant settings. To do so, we obtained pleural effusion cells from patients with stage IV NSCLC to check the mRNA levels of IRF5 and M-CSF in $EpCAM^+$ epithelial cells. We also analyzed total numbers of CSF1R-positive macrophages in NSCLC tumors, which have been known as tumorigenic and immunosuppressive subsets in human cancers (20, 21). We found that mRNA levels of IRF5 were highly correlated with those of M-CSF in tumor tissues in patients with NSCLC (Fig. 6). Moreover, there were positive relationships between the number of $CSF1R^+$ $CD68^+$ macrophages and levels of IRF5 or M-CSF in NSCLC tumors, whereas the activation of IRF5–M-CSF pathways was not correlated with total numbers of $CD68^+$ macrophages (Fig. 6). Finally, the expression levels of CSC maker ALDH1 mRNA was also correlated with the those of IRF5, M-CSF, as well as $CSF1R^+$ cell numbers (Fig. 6). Together,

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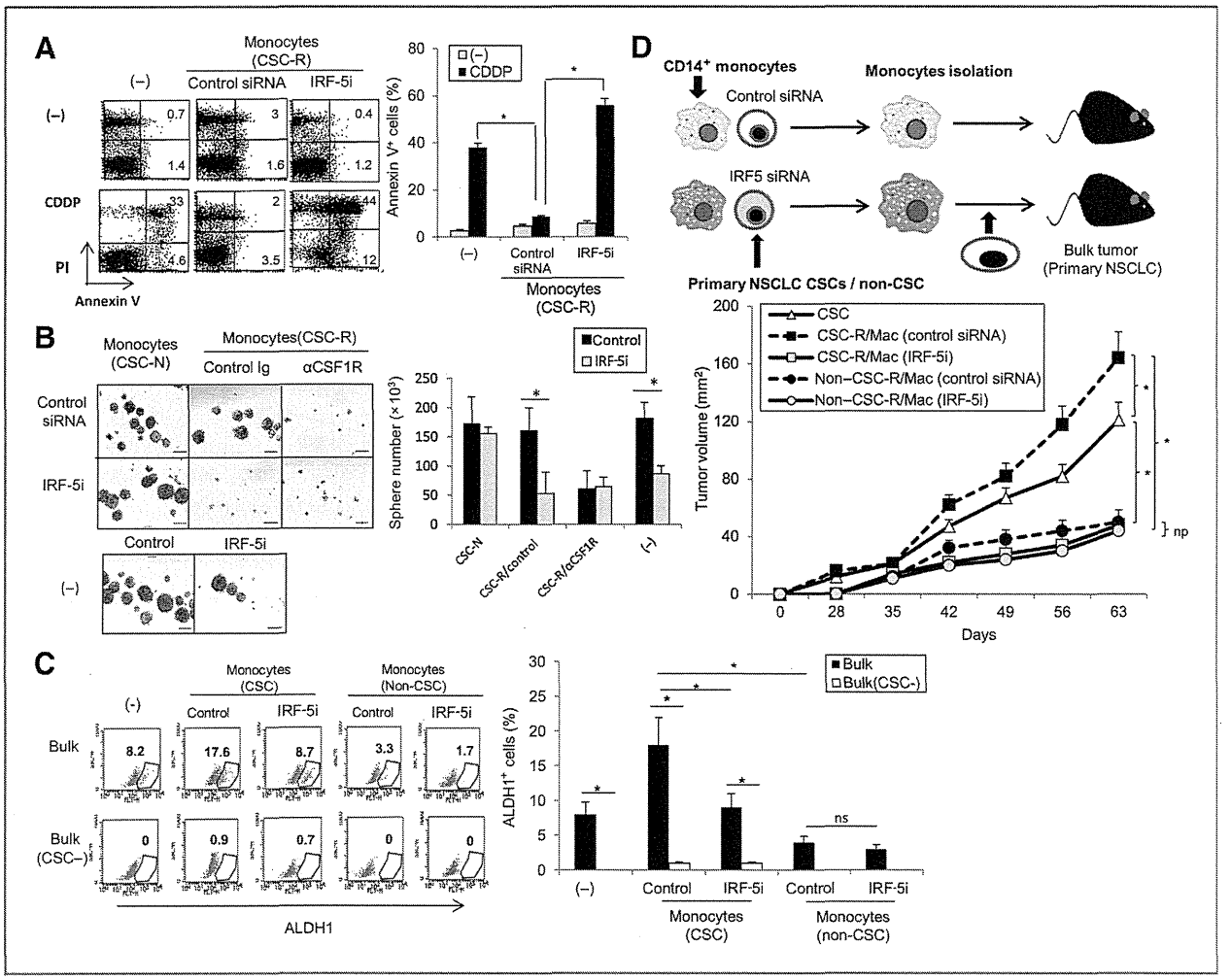

Figure 5. CSC-R–derived IRF5 supports myeloid cell–mediated tumorigenic activities. A, CSC-R (MDA-MB-231) transfected with control or IRF5 siRNA were cocultured with monocytes obtained from PBL at 1:5 ratios for 96 hours. The $CD68^+$ myeloid cells were isolated from the cocultured cells and cultured for 24 hours to obtain supernatants. Bulk MDA-MB-231 cells were untreated (–) or treated with 20% supernatants of myeloid cells primed with control or IRF5-KD CSC-R in the presence of CDDP (20 μg/mL) for 24 hours. The cell viability was analyzed by quantifying Annexin V/propidium iodide–positive cells. B, the supernatants of myeloid cells primed with CSC-R (MDA-MB-231) were obtained as described above. Bulk MDA-MB-231 cells were untreated (–) or treated with 20% supernatants of the CSC-R–primed myeloid cells with control Ig or anti-CSF1R–neutralizing antibody (10 μg/mL) in ultra-low attachment plates, and the numbers of formed spheres generated per 1,000 cells were determined. C, the CSC or non-CSC obtained from chemoresistant NSCLC cells were transfected with control or IRF5 siRNA (IRF5i) for 48 hours. The bulk cells (Bulk) or those depleted of CSCs [Bulk(CSC-)] were untreated (–) or stimulated with 20% supernatants of the monocytes primed by the supernatant of myeloid cells primed with CSC [Monocytes (CSC)] or non-CSC [Monocytes (non-CSC)] for 24 hours. The expression of CSC markers ALDH1 was evaluated by ALDEFLOUR assays. D, $EpCAM^+CD133^+$ CSCs or non-CSCs obtained from patients with NSCLC were transfected with control or IRF5 siRNA for 48 hours, and then cocultured with autologous $CD14^+$ monocytes. The CSC or non-CSC-primed monocytes were isolated from the cocultured cells and used for intravenous transfer into clodronate-pretreated NOD–SCID mice ($n = 4$/group) with subcutaneous injections of autologous bulk NSCLC tumors. The tumor growth was measured on the indicated days. The experimental procedures (top) and tumor growth curve (bottom) are shown. Similar results were obtained in three independent experiments. *, $P < 0.05$.

these findings support the notion that the IRF5/M-CSF pathway positively regulates tumorigenic myeloid cells and CSC activities in patients with cancer.

Discussion

Although intrinsic genetic and epigenetic alterations serve as a mainstay, leading to generalized chemoresistance in transformed cells, it remains largely unclear whether these chemoresistant phenotypes further modulate the biologic properties of specialized subsets of tumorigenic populations. In particular, CSCs have emerged as the main tumor-initiating and -propagating cells and are responsible for acquiring chemoresistance through multidrug transporter activities and cellular quiescence. Therefore, it is critical to clarify whether altered drug sensitivities modulate other functional properties of CSCs. Here, we provide the first evidence that CSCs isolated from therapy-resistant tumors have unique tumorigenic properties compared with those from untreated counterparts. CSC-R promoted M-CSF production through an

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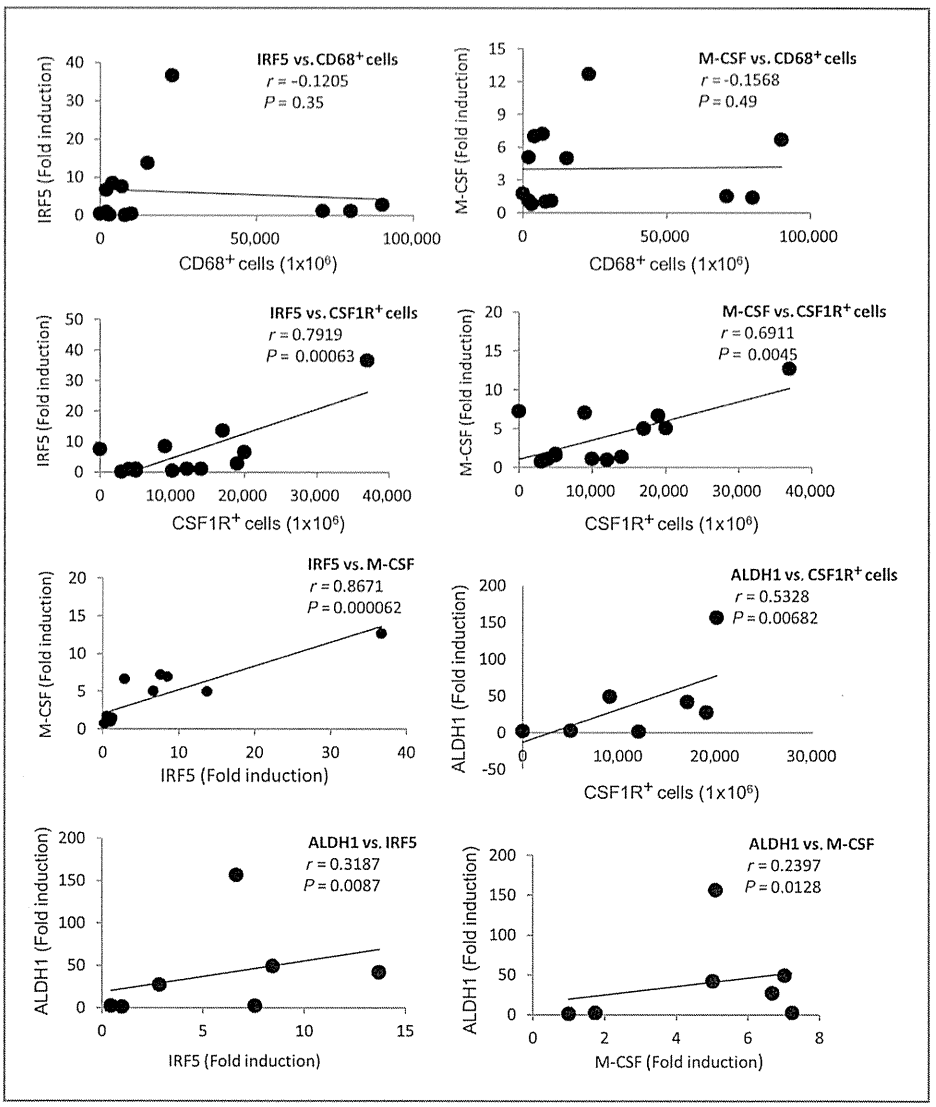

Figure 6. Clinical significance of IRF5/M-CSF regulation of tumorigenic myeloid cells. The $CD45^+$ immune or $EpCAM^+$ epithelial cell suspensions were isolated from bulk populations of pleural effusions obtained from patients with stage IV NSCLC ($n = 12$). The mRNA levels of IRF5 M-CSF and ALDH1 were quantified by RT-PCR and total frequencies of $CD68^+$ or CSF1R $(CD115)^+CD68^+$ macrophages were evaluated by flow cytometry, and the correlation coefficiencies of each parameter were analyzed.

IRF5-dependent mechanism, and IRF5-mediated M-CSF further facilitated the tumorigenicity of bulk tumor cells by activating tumorigenic myeloid cells. These findings offer a major advance in understanding the molecular machineries, whereby responses to anticancer therapeutics serve as a critical factor in regulating the functional plasticity of CSCs and may greatly affect prognosis and therapeutic responses in patients with cancer.

IRF5 has been considered a tumor-suppressive factor that activates apoptosis-related signaling pathways in transformed cells and creates antitumor inflammatory microenvironments through immune cells (17–19). Moreover, deletions of chromosome 7q32 are associated with downregulation of IRF5 function, disease progression, and poor prognosis in patients with marginal zone lymphoma. Genetic polymorphisms of IRF5 may serve as biomarkers to predict clinical responses to immunotherapy and chemotherapy in patients with melanoma and hematologic malignancies, respectively (22–24).

Previous study revealed that IRF5 serves as a lineage marker for M1-type macrophages, and macrophages promote the generation of immunogenic Th1 or Th17 subsets by an IRF5-dependent manner (25). In marked contrast, we demonstrate that IRF5 expressed on chemoresistant CSCs contributes to the differentiation of M2-like macrophages by M-CSF–mediated mechanisms. Thus, transcription factors that have been known as a proinflammatory mediator might be changed to an immune suppressor under specific conditions in which therapeutic interventions could reconstruct the complex genetic and epigenetic networks in tumor cells. Moreover, these "transformation" of cross-talks between tumor cells and immune cells may dramatically change phenotypic properties and biologic actions of identical genes and their pathways. Furthermore, chemoresistance may modify the tumor microenvironments and create a specialized chemoresistant niche that further amplifies the tumorigenic and therapy-resistant behaviors of tumor cells (1–4). Thus, it is essential to reexamine

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the molecular and functional dynamics of various regulators identified as "tumor suppressors" according to therapeutic interventions.

We demonstrated that IRF5-dependent induction of M-CSF is a key step in the generation of tumorigenic myeloid cells by CSC-R. Although the molecular mechanisms by which CSC-R regulate M-CSF production remain to be defined, several transcription factors, such as PU.1, C/EBP-α, and NF-κB, may coordinate with IRF5 to regulate specific sets of cytokines and growth factors. Moreover, PU.1 associates with various types of IRFs to amplify its transcriptional activities, which greatly modifies the immunologic functions of myeloid cells in multiple ways (26–29). Thus, it is tempting to speculate that IRF5 may cooperate with various myeloid cell differentiation factors in CSC-R and that such cooperation may be critically involved in the genetic and epigenetic control of M-CSF expression.

We also found that CSC-R have unique propensities to activate inflammatory signals, thus inducing multiple sets of cytokines and chemokines. Among them, TNF-α, IL-6, and IL-8 have emerged as tumorigenic mediators that link various inflammatory signals with oncogenic cascades to create tumorigenic microenvironments (30–32). In addition, CCL2 supports the recruitment of $CCR2^+$ inflammatory monocytes into tumor tissues, which serves as a key step in generating tumorigenic macrophages (33, 34). Thus, it is important to address how the distinct and coordinated actions of these inflammatory mediators affect the tumorigenicity and anticancer drug responses of CSCs within the privileged tumor microenvironments.

In conclusion, we provide a novel pathway whereby responsiveness to anticancer drugs determines the plasticity and biologic properties of CSCs in part through the paracrine regulation of tumor-associated nontransformed cells. Comprehensive analysis of CSC-niche interactions upon exposure to various types of anticancer agents should clarify the molecular and cellular pathways that govern CSC functions and reveal new therapeutic options in clinically relevant settings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Jinushi
Development of methodology: M. Jinushi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yamashina, M. Baghdadi, I. Kinoshita, H. Dosaka-Akita
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Yamashina, M. Baghdadi, M. Jinushi
Writing, review, and/or revision of the manuscript: T. Yamashina, I. Kinoshita, H. Dosaka-Akita, M. Jinushi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Yamashina, M. Baghdadi, A. Yoneda, S. Suzu, M. Jinushi
Study supervision: M. Jinushi

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