

Galectin-9 promotes immune persistence of tyrosine kinase inhibitor-tolerant cells in oncogene-driven non-small cell lung cancer

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Abstract

Despite of the dramatic antitumor effects of small-molecule tyrosine kinase inhibitors (TKIs), patients with oncogene-driven non-small cell lung cancer (driver-positive NSCLC) inevitably experience recurrence because of the existence of intrinsic drug-resistant subpopulation called drug-tolerant persisters (DTPs). We here examined whether innate immunity could eliminate DTPs in driver-positive NSCLC. In specific TKI-exposed driver-positive NSCLC cells, transcripts of galectin-9 (Gal-9), one of immune checkpoint molecules, were drastically upregulated via activation of interferon regulatory factor 1 in nucleus. Cytotoxicity assay by co-incubating cancer cells and natural killer (NK) leukemia (NKL) cells revealed that NK cell-mediated cytotoxicity was weakened by Gal-9 expression in cancer cells. Notably, while CRISPR/Cas9-mediated Gal-9-knockout (KO) cells were completely depleted by TKI exposure plus NKL mediated-cytotoxicity, scramble guide RNA-transfected cells repopulated, which suggested that NKL-mediated cytotoxicity reinforced by Gal-9 depletion could eliminate DTPs. Then, athymic nude mice inoculated with driver-positive NSCLC cells were treated with TKI. The regrowth of Gal-9-KO driver-positive NSCLC xenografts and allografts were significantly suppressed by NK cell-mediated cytotoxicity. Furthermore, Gal-9 expression was associated with worse progression free survival in human epidermal growth factor receptor mutation-positive NSCLC. These findings identified Gal-9 as an inducer of immune-evasion and elucidated its therapeutic targeting to eliminate DTPs.

Introduction

Lung cancer is currently the leading cause of cancer-related deaths worldwide¹. Most patients with lung cancer are diagnosed at a later stage when curative treatment is not applicable, and five-year survival rate tends to be below 20% in most countries^{1,2}. Therefore, it is indispensable to develop a novel medical treatment strategy that will improve the outcome of patients with lung cancer. Molecular targeted-therapies have been emerging for this purpose over the past two decades. Small molecule inhibitors have proved to exert dramatic antitumor effects and improve the prognosis of patients with non-small cell lung cancer (NSCLC) harboring oncogenic driver alterations such as epidermal growth factor receptor (*EGFR*) mutation, anaplastic lymphoma kinase (*ALK*) fusion, ROS proto-oncogene 1 fusion, B-Raf proto-oncogene mutation, Kirsten rat sarcoma viral oncogene homolog mutation, rearranged during transfection fusion, MET proto-oncogene mutation, neurotrophic tyrosine receptor kinase mutation, and human epidermal growth factor receptor 2^{3,4}. However, it is inevitable that those patients will eventually experience recurrence because of the existence of drug-tolerant persisters (DTPs), an intrinsic drug-resistant subpopulation⁵. Therefore, elimination of DTPs is crucial to acquire prolonged survival benefit in patients with oncogene-driven (driver-positive) NSCLC.

Cancer immunotherapy with immune checkpoint inhibitors (ICIs) revolutionized treatment of patients with metastatic NSCLC, which not only exhibits prolonging survival but also causes long-term durable response in a subset of patients⁶⁻⁸. However, several clinical studies demonstrated that ICIs did not exert satisfactory antitumor immunity against driver-positive NSCLC^{9,10}. Tumor-antigen presentation by

antigen presenting cells such as dendritic cells is essential for ICI-mediated adaptive immunity to show clinically meaningful antitumor activity¹¹. High tumor mutation burden (TMB) has been reported to be predictive of clinical benefit to ICI treatment in various types of cancer including lung cancer^{12,13}, however, regarding driver-positive NSCLC, TMB has generally been low¹⁴. This is supposed to be one of the main reasons that ICI-based therapy cannot bring about clinical benefit for patients with driver-positive lung cancer. Moreover, expression of major histocompatibility complex class I (MHC-I), one of key molecules that affect the interaction between tumor cells and immune cells, was reported to be downregulated in driver-positive NSCLC^{15,16}.

Based on the results from these previous studies, we assumed that effective utilization of innate immunity was critical to control driver-positive NSCLC because it can exert immune response regardless of antigen presentation and MHC-I expression by tumor cells. Although innate immunity alone may not be enough to reject all the driver-positive NSCLC cells, we expect that long-term remission will be achieved if innate immunity can destroy DTPs because most of the driver-positive NSCLC cells disappear by the treatment with small molecule inhibitor. Additionally, activating innate immunity launches the adaptive immunity against cancer cells and brings about durable response¹⁷.

Here, we investigated whether innate immunity could eliminate DTPs for durable remission in driver-positive NSCLC, and found that one of immune checkpoint molecules (ICMs), galectin-9 (Gal-9), was the promising therapeutic candidate molecule.

Results

Small molecule inhibitors induced upregulation of Gal-9 expression in driver-positive NSCLC cells.

Studies have shown that small molecule inhibitors exerted dramatic antiproliferative effects against driver-positive NSCLC cells but could not eradicate them at all due to the existence of DTPs^{5,18,19}. As shown in Fig. S1A and B, *EGFR* mutation-positive, HCC827 and PC-9 cells, and *ALK* fusion-positive, H3122 and H2228 cells, did not disappear even though they were exposed to the highest concentration of respective specific tyrosine kinase inhibitors (TKIs), osimertinib and alectinib. We, therefore, considered that it is highly necessary to eliminate these intrinsic drug resistant DTPs to achieve durable remission in driver-positive NSCLC, and made a plan to utilize antitumor immunity especially of the innate immune system for eliminating DTPs. To identify the candidate molecule which is involved in the antitumor innate immunity, DNA-microarray analysis was performed on transcripts extracted from HCC827 and H3122 cells which were exposed to either DMSO or respective specific TKI, 100 nM of osimertinib and 1000 nM of alectinib, for 24 hours. It has been reported that various immune checkpoint molecules (ICMs) participate in the regulation of antitumor innate and adaptive immunity^{20–22}. Among the ICMs listed in Table S1, DNA microarray analysis showed that *VTCN1*, *LGALS3* (gene name of Gal-3), *CD47*, *LGALS9* (gene name of Gal-9), *TNFRSF14* were the top five upregulated genes by TKI exposure (Fig. 1A and B, Table. S2). We then performed quantitative real-time polymerase chain reaction (qRT-PCR) for evaluation of the differential expression of individual ICMs involved in innate immunity, and

found that expression of galectins, especially galectin-9 (Gal-9), was notably upregulated in both HCC827 and H3122 cells (Fig. 1C). Public database analysis by Kaplan-Meier plotter (KM-plotter)²³ indicated that high Gal-9 expression was associated with poor overall survival (OS) in patients with lung adenocarcinoma and especially in patients who had never smoked. On the other hand, Gal-3 expression was not supposed to be associated with a poor outcome in patients with lung adenocarcinoma (Fig. 1D). Since the majority of the patients with driver-positive NSCLC never smoked²⁴, we presumed that Gal-9 expression had a negative impact on the outcome of the patients with driver-positive NSCLC. Moreover, specific TKI exposure increased the expression of Gal-9 transcripts in a concentration-dependent manner in both *EGFR* mutation-positive cells and *ALK* fusion-positive cells (Fig. 1E). To determine whether increase of Gal-9 expression was consistent in drug-resistant subpopulation, we established osimertinib-resistant HCC827OR cells and alectinib-resistant H3122AR cells by long-term exposure to each TKI (Fig. 1F). Increase of the expression level of Gal-9 transcripts and protein was observed in both drug-resistant cells compared with that of parental cells (Fig. 1G and H). These results suggested that DTPs in driver-positive NSCLC utilized Gal-9 to create immune protected state and evaded from anticancer immunity.

Interferon regulatory factor 1 (IRF-1) was the key regulator for Gal-9 induction by TKI stimulation in driver-positive NSCLC cells.

Several transcription factors such as IRF-1, transcription factor activator protein 4 and Smad3 have been reported as regulators of *LGALS9* transcription^{25,26}. Among them, we focused on the role of IRF-1 to clarify the mechanism of the induction of Gal-9 expression by TKI stimulation. Transcripts and protein expression level of IRF-1 increased in HCC827 cells and H3122 cells when they were exposed to osimertinib and alectinib, respectively (Fig. 2A and B). Additionally, immunofluorescence (IF) analysis proved that osimertinib upregulated IRF-1 expression mainly in nuclei in HCC827 cells (Fig. 2C). We then knocked down IRF-1 expression in HCC827 cells by transfecting IRF-1 targeting siRNAs (Fig. S2C). Induction of Gal-9 by osimertinib in HCC827 cells was significantly attenuated by IRF-1 knockdown (KD) (Fig. 2D). Moreover, osimertinib exposure inactivated β -catenin that was reported to cause degradation of IRF-1²⁷ (Fig. 2E), which might contribute to stabilization of IRF-1 protein. We further examined pharmacological inhibition of IRF-1 using toxoflavin which was recently proved to work as an inositol-requiring enzyme 1 α (IRE1 α) inhibitor²⁸. IRE1 α promotes forming spliced isoform of X-box binding protein 1 (XBP1s) which is translocated into nucleus and binds to the IRF1 upstream enhancer^{29,30}. Moreover, it was reported that a tolerable level of endoplasmic reticulum stress was preserved and IRE1 α -XBP1s signaling was conservatively activated in DTPs of PC-9 cells³¹. As expected, toxoflavin significantly suppressed the expression of Gal-9 transcripts induced by TKI via reducing IRF-1 expression in HCC827 cells and H3122 cells (Fig. 2F and G, Fig. S2D and E). These results suggested that IRF-1 was involved in the induction of Gal-9 expression by TKI stimulation in driver-positive NSCLC cells.

Gal-9 impaired the cytotoxicity of NK cells against DTPs in driver-positive NSCLC cells.

To determine the biological significance of Gal-9 upregulation by TKI stimulation, we evaluated if the cytotoxicity of innate immune cells was affected via Gal-9 expression in driver-positive NSCLC cells. We first generated Gal-9 overexpressing HCC827 (HCC827/LGALS9) cells by lentiviral transfection of human *LGALS9* gene (Fig. 3A), and performed cytotoxic assay using xCELLigence system by coincubation with natural killer leukemia (NKL) cells which express T-cell immunoglobulin mucin-3 (TIM-3) known as Gal-9 receptor (Fig. S3A)³². NKL-mediated cytotoxicity for HCC827/LGALS9 cells was significantly impaired compared with that for empty vector-transfected HCC827 cells at a 2:1 effector to target cell (E/T) ratio (Fig. 3B). Next, we examined whether anti Gal-9 function blocking antibody (α Gal-9 Ab) could enhance NKL-mediated cytotoxicity using parental HCC827 cells and HCC827OR cells. Before performing cytotoxicity assay, we confirmed that α Gal-9 Ab did not affect the proliferation of both cells regardless of the upregulation of Gal-9 expression in HCC827OR cells (Fig. 1G, 3C). Cytotoxicity assay revealed that NKL-mediated cell lysis was weakened in HCC827OR cells compared with that in HCC827 cells, however, α Gal-9 Ab reinforced NKL-mediated cytotoxicity against HCC827OR cells at a 2:1 E/T ratio (Fig. 3D). We further established CRISPR/Cas9-mediated *LGALS9* knockout (KO) (HCC827-sgLGALS9) cells in which Gal-9 expression was prevented from inducing by osimertinib (Fig. 3E). Compared with HCC827 scramble sgRNA-transfected (HCC827-scr) cells, HCC827-sgLGALS9 cells became slightly resistant to osimertinib, which was disadvantageous for us to eradicate HCC827-sgLGALS9 cells (Fig. 3F). However, long-term cytotoxicity assay performed by coincubation of HCC827-scr cells or HCC827-sgLGALS9 cells with NKL cells under the existence of 100 nM of osimertinib demonstrated that HCC827-sgLGALS9 cells were completely eliminated even at a low E/T ratio (1:1) while HCC827-scr cells finally repopulated (Fig. 3G and Fig. S3B). These results suggested that Gal-9 is involved in the impairment of NK cell-mediated cytotoxicity and Gal-9-targeted therapy is promising to eliminate DTPs in driver-positive NSCLC.

Gal-9-targeted therapy significantly inhibited the growth of subcutaneous EGFR mutation-positive cell xenografts and allografts in nude mice.

We then evaluated whether Gal-9 expression induced refractoriness to NK cell-mediated cytotoxicity against driver-positive NSCLC cells *in vivo* using nude mice which lack thymus and show T-cell deficiency but retain macrophages and NK cells. First, we subcutaneously inoculated HCC827-scr and HCC827-sgLGALS9 cells into the flank of nude mice. After confirming successful engraftment of xenografts, mice were divided into two groups and were orally treated with either dimethyl sulfoxide (DMSO) or 3mg/kg of osimertinib five times a week for 30 days. When mice were treated with DMSO, the growth speed of HCC827-scr xenografts was similar to that of HCC827-sgLGALS9 xenografts (Fig. 4A and B). In osimertinib-treated mice, tumors quickly shrank and temporarily disappeared in both HCC827-scr and HCC827-sgLGALS9 groups, however, xenografts regrew in considerable number of mice after cessation of osimertinib treatment. Despite of the higher sensitivity to osimertinib *in vitro* (Fig. 3F), the regrowth rate was also higher in HCC827-scr groups (6 out of 8 mice) than that in HCC827-sgLGALS9 group (3 out of 10 mice) (Fig. 4B), and median duration of response was contrarily longer in HCC827-sgLGALS9 group (not reached) compared with that in HCC827-scr group (52 days) (Fig. 4C). These results indicated that deletion of Gal-9 brought about antitumor immunity to eliminate osimertinib-resistant subpopulation of

cells. Although it was reported that human Gal-9 affected the activity of mouse immune cells³³, amino acid sequence of human Gal-9 did not completely correspond to that of mouse Gal-9³⁴. Therefore, we considered that establishment of allograft model was necessary to accurately evaluate the involvement of Gal-9 in antitumor immunity *in vivo*. We generated 3T3/EGFRmt cells by transfecting with human mutant *EGFR* into mouse fibroblast 3T3 cells (Fig. S4A). 3T3/EGFRmt cells became sensitive to osimertinib while control vector transected 3T3/ctrl cells did not (Fig. S4B). Additionally, 3T3/EGFRmt cells could grow when they were inoculated into nude mice, which suggested that 3T3/EGFRmt cells acquired malignant potential (Fig. S4C). We further established mouse *Lgals9*-deficient 3T3/EGFRmt cells (3T3/EGFRmt-sg*Lgals9*). Osimertinib induced expression of mouse Gal-9 in scramble sgRNA transfected cells (3T3/EGFRmt-scr) but not in 3T3/EGFRmt-sg*Lgals9* cells (Fig. 4E). After confirming that the sensitivity to osimertinib was similar in both cells (Fig. 4F), we subcutaneously implanted 3T3/EGFRmt-scr and 3T3/EGFRmt-sg*Lgals9* cells into nude mice and orally administered either DMSO, 3mg/kg of osimertinib or 12.5mg/kg of osimertinib five times a week. Osimertinib dose-dependently suppressed the growth of allografts of both 3T3/EGFRmt-scr and 3T3/EGFRmt-sg*Lgals9* cells, however, 3T3/EGFRmt-scr-derived allografts regrew earlier than 3T3/EGFRmt-sg*Lgals9*-derived allografts (Fig. 4F). These results showed the significance of Gal-9 in antitumor immunity and were consistent with the results achieved by using xenografts (Fig. 4B and C). For the clinical implication of Gal-9-targeted therapy in driver-positive NSCLC, we further evaluated the antitumor effect of αGal-9 Ab in subcutaneous 3T3/EGFRmt cell-transplanted allograft model. Mice were divided into 4 groups and received either DMSO plus rat IgG2b, DMSO plus αGal-9 Ab, osimertinib plus rat IgG2b or osimertinib plus αGal-9 Ab. In this experiment, 6mg/kg of rat IgG or αGal-9 Ab was intraperitoneally administered twice a week, and DMSO or 12.5mg/kg of osimertinib was orally given five times a week. Although αGal-9 Ab alone did not exert antitumor activity against 3T3/EGFRmt allografts, it significantly delayed the regrowth of 3T3/EGFRmt allografts when used in combination with osimertinib (Fig. 4G). Thus, Gal-9-targeted therapy was considered to be promising for improving the antitumor efficacy of small molecule inhibitors in driver-positive NSCLC.

Antitumor effect of Gal-9-targeted therapy against driver-positive NSCLC was exerted via activating NK cell-mediated cytotoxicity.

At the end of osimertinib treatment, we euthanized mice and collected 3T3/EGFRmt-scr allografts and 3T3/EGFRmt-sg*Lgals9* allografts. IHC analysis of those tumor samples showed that NK cell marker, Nkp46, staining-positive cells remarkably infiltrated in 3T3/EGFRmt-sg*Lgals9* allografts compared with that in 3T3/EGFRmt-scr allografts (Fig. 5A). This finding suggested that NK cells were still contributing to inhibit the growth of 3T3/EGFRmt-sg*Lgals9* allografts which survived from osimertinib treatment. Since increase of the expression of Gal-9 was observed within 24 hours after osimertinib exposure (Fig. 1A-C, E), we reckoned that Gal-9 inhibited NK cell-mediated antitumor effect from the early stage of treatment. We then performed immunohistochemistry (IHC) and flow cytometry analysis using 3T3/EGFRmt-scr and 3T3/EGFRmt-sg*Lgals9* subcutaneous tumor samples. Although the differences were not statistically significant, osimertinib treatment seemed to increase the number of tumor-infiltrating NK cells in

3T3/EGFRmt-sgLgals9 allograft compared with that of 3T3/EGFRmt-scr (Fig. 5B and C). Flow cytometry analysis revealed that the proportion of CD45-positive immune cells increased in 3T3/EGFRmt-sgLgals9 allografts. More importantly, the proportion of tumor-infiltrating NK cells in 3T3/EGFRmt-sgLgals9 allografts significantly increased compared with that in 3T3/EGFRmt-scr allografts when the tumor-harboring mice were treated with osimertinib (Fig. 5D). To verify that the antitumor effect of Gal-9 KO was mediated by NK cells, we performed NK cell-depletion experiment using α CD122 Ab in nude mice allograft model according to the experimental schedule shown in Fig. 5E. NK cells were successfully depleted by intraperitoneal administration of 5mg/kg of α CD122 Ab twice a week (Fig. 5F). In control rat IgG2b-administered mice, the growth of 3T3/EGFRmt-sgLgals9 allografts was slower than that of 3T3/EGFRmt-scr allografts. However, in α CD122 Ab-treated mice, the growth of 3T3/EGFRmt-sgLgals9 allografts became comparable with that of 3T3/EGFRmt-scr allografts (Fig. 5G). These results demonstrated that inhibition of Gal-9 suppressed tumor growth via NK cell-mediated cytotoxicity.

Gal-9 expression was associated with poor progression free survival in patients with EGFR mutation-harboring NSCLC who received osimertinib monotherapy.

To clarify the clinical implications of Gal-9 in patients with driver-positive NSCLC, immunoreactivity for Gal-9 expression was assessed by IHC on clinical samples obtained from 57 NSCLC patients who harbored exon19 deletion mutation (del19) or L858R point mutation in exon 21 (L858R) in *EGFR* and were treated with osimertinib monotherapy. The patients' clinical characteristics and *EGFR* mutation status are shown in Table 1. As representatively shown in Fig. 6A, Gal-9 expression in tumor cells was detected in 31 of 57 patients (54%). While objective response rate of osimertinib monotherapy was similar between Gal-9-negative and Gal-9-positive group (81% and 81%, respectively) (Table. 2), the median (95% confidence intervals [CI]) progression free survival (PFS) in Gal-9-negative group was significantly longer compared with that in Gal-9-positive group (45 [95% CI, 15–53] months vs 18 [95% CI, 13–24] months, respectively) (Fig. 6B). Consistent with the previous study³⁵, osimertinib brought about robust antitumor effect and longer PFS in patients with *EGFR* del19 mutant NSCLC than in patients with *EGFR* L858R mutant NSCLC (Fig. S5). Therefore, the number of residual cancer cells which could survive from osimertinib treatment were estimated to be lower in *EGFR* del19 mutant NSCLC than in *EGFR* L858R mutant NSCLC. We further examined whether the influence of Gal-9 expression on PFS differed between *EGFR* mutation types, and found that the difference of PFS between Gal-9-positive group and Gal-9-negative group was more prominent in patients with *EGFR* del19 mutant NSCLC than in *EGFR* L858R mutant NSCLC (Fig. 6C and D). Taken together, Gal-9 does not prevent the initial response to osimertinib but may facilitate survival and earlier regrowth of residual cancer cells. Moreover, IHC analysis on the samples obtained from the four patients before and after osimertinib treatment suggested that Gal-9 expression was upregulated by osimertinib exposure (Fig. 6E). Thus, also from a clinical point of view, we regarded Gal-9 as an attractive candidate molecule to achieve a long-term remission in driver-positive NSCLC.

Table 1
Clinical characteristics of the enrolled patients

Characteristics		number of patients (%)
(total = 57)		
Age		
Median	73	
Range	50–89	
	<75	30 (53)
	≥ 75	27 (47)
Sex		
	male	18 (32)
	female	39 (68)
Smoking status		
	Never	38 (67)
	Current	1 (1)
	Former	18 (32)
ECOG performance status		
	0	21 (37)
	1	25 (44)
	2	5 (9)
	3	4 (7)
	4	2 (3)
Disease classification		
	stage III	1 (1)
	stage IV	49 (86)
	recurrence	7 (13)
EGFR mutation type		
	Del19	32 (56)
	L858R	25 (44)

Table 2

Response to osimertinib monotherapy according to Gal-9 expression in EGFR mutation-positive NSCLC.

Gal-9	CR	PR	SD	PD	NE	DCR (%)
Negative	0	21	3	1	1	92.3%
Positive	4	21	5	0	1	96.8%
Total	4	42	8	1	2	94.7%

Abbreviation: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; DCR, disease control rate.

Discussion

Despite of the powerful antitumor effect of small molecule inhibitors against driver-positive NSCLC, patients inevitably experience relapse due to the acquisition of drug resistance. Recent studies have revealed that drug-resistance mechanism consists of on-target resistance mutation, bypass signaling activation or phenotypic change³⁶. To overcome drug resistance, novel therapeutic strategies with the use of molecular targeted agents such as an EGFR-MET bispecific antibody amivantamab plus chemotherapy and 3rd generation ALK-TKI lorlatinib have emerged^{37,38}. However, it is impractical to continue synthesizing a suitable small molecule inhibitor for individual resistant mechanisms. We therefore believe that utilization of antitumor immunity is essential for durable remission in driver-positive NSCLC. Unfortunately, antitumor efficacy of ICI-based adaptive T cell-immunity against driver-positive NSCLC was unsatisfactory due to the low TMB and MHC class I expression^{9,10,14–16}. Many clinical trials confirmed this when they showed a lack of antitumor activity or enhanced adverse effect in ICIs treatment of driver-positive NSCLC and its corresponding failure to bring about clinical benefit^{10,39}.

Conversely, a recent study suggested that EGFR-TKI resistant cells were more sensitive to chimeric antigen receptor (CAR) NK cells than CAR T cells⁴⁰. Therefore, we sought to utilize NK cells to eliminate DTPs for curative treatment in driver-positive NSCLC. However, because NK cells represented an exhausted phenotype and impaired its cytotoxic function in NSCLC, we needed to first reinforce the antitumor activity of tumor-infiltrating NK cells^{41,42}.

Gal-9 belongs to the tandem-repeat type subfamily of galectins containing two carbohydrate recognition domains (CRD) in the C- and N-terminal regions. It has been proved to have a wide range of immunomodulatory properties through interacting with various receptors such as TIM-3, PD-1, V-domain Ig-containing suppressor of T cell activation, 4-1BB, CD44 and DR3^{32,43}. In NK cells, which are fundamental components of innate immunity, Gal-9 has been reported to have bidirectional function. Under normal physiological conditions, interaction between Gal-9 and TIM3 on NK cells induces immune-stimulatory activity⁴⁴. On the contrary, binding of Gal-9 to TIM3 and CD44 on NK cells in tumor tissues induces exhaustion, which results in the suppression of NK cell-mediated cytotoxicity and proliferation^{45,46}. We also assume that induced and accumulated Gal-9 in cytoplasm of driver-positive

NSCLC cells by TKI exposure (Fig. 1A-C and E) will also suppress the cytolytic function of NK cells, because in TKI treatment, Gal-9 is released extracellularly as damage-associated molecular patterns from dying driver-positive NSCLC cells⁴⁷⁻⁵⁰. Moreover, Gal-9 was reported to be involved in augmenting of drug-resistance and tumor-initiating cell phenotype in lung adenocarcinoma⁵¹, which was consistent with our data showing the upregulation of Gal-9 in TKI-resistant cells (Fig. 1G and H). Thus, we considered that inhibition of Gal-9 was essential to activate innate immunity and eliminate DTPs in driver-positive NSCLC.

We demonstrated that Gal-9 expression was induced by activation of transcription factor IRF-1 in driver-positive NSCLC cells when exposed to specific TKI (Fig. 2A-C). Consideration of tumor microenvironment (TME) is also critical to maximizing the antitumor effect of cancer immunotherapy. Recently, it was reported that IRF-1 had an opposing role regarding antitumor immunity in TME. Namely, while its activation was required for immune cells to exert antitumor immunity, it conversely impaired the function of antitumor lymphocyte by inducing expression of immunosuppressive genes in tumor cells⁵². Our experimental results and the above previous studies suggested that driver-positive NSCLC cells exposed to TKIs built a favorable TME via IRF-1-mediated Gal-9 expression to evade from antitumor immunity.

The most widely studied pathway of Gal-9 involved in the antitumor immunity is Gal-9/TIM-3 signal. In the present study, we showed that induction of Gal-9 expression impaired NK cell-mediated cytotoxicity against DTPs in driver-positive NSCLC *in vitro* (Fig. 3B, D and G) and *in vivo* (Fig. 4B, C and F, Fig. 5A-C), and also that Gal-9 expression was associated with poor PFS in patients harboring *EGFR* mutation treated with osimertinib (Fig. 6A-C). Additionally, TIM-3, one of the receptors for Gal-9, was not only prominently expressed in NK cells in NSCLC tissue, but TIM-3 expression on peripheral NK cells was also independently correlated with shorter OS of the patients with lung adenocarcinoma^{53,54}. TIM-3-targeted therapy is usually conducted in combination with PD-1 blockade because PD-1 expression is observed in an earlier stage of T cell exhaustion, while TIM-3 expression is detected in terminally dysfunctional T cells^{55,56}. In NK cells on the other hand, TIM-3 expression is detected in the intermediate stage of exhaustion even prior to that of PD-1^{53,57}. Therefore, we expect that PD-1 blockade is not absolutely necessary for NK cells to reverse exhaustion.

Therefore, inhibition of Gal-9/TIM-3 pathway may be an attractive strategy to deplete DTPs by reactivating antitumor innate immunity. While taking into consideration the clinical application, we used α Gal-9 Ab to inhibit Gal-9/TIM-3 signal and demonstrated that α Gal-9 Ab significantly suppressed the growth of allografts harboring *EGFR* mutation (Fig. 5G). The reasons why we used α Gal-9 Ab but not α TIM-3 Ab are as follows. First, Gal-9 also interacts with CD44 which is commonly used to identify cancer stem cell in NSCLC and contributes to tumor initiation^{51,58}. In addition, NK cell exhaustion is induced by binding of Gal-9 to TIM3 and CD44 as mentioned above⁴⁵. Therefore, disruption of the interaction between Gal-9 and CD44 is not only able to activate NK cell-mediated cytotoxicity but also reduce the biological activity of DTPs. Second, we also considered that it was desirable to increase the

target cell specificity to maximize the antitumor activity of NK cells against DTPs. Phase I study to assess the safety and tolerability of α Gal-9 Ab (LYT-200) has been performed⁵⁹. If such Ab has a potential to exert antibody-dependent cellular cytotoxicity (ADCC), NK cell-mediated cytotoxicity will become more effective for eliminating DTPs because Gal-9 expression was found to be upregulated in DTPs (Fig. 1E, G and H). Moreover, Gal-9 was reported to polarize the phenotype of tumor-associated macrophages from antitumor M1 to protumor M2⁶⁰. Considering our flow cytometry analysis showing that the number of CD45⁺ immune cells infiltrated into Gal-9 KO EGFRmt allografts was larger than that into wild-type allografts (Fig. 5D), M1 macrophages might contribute to inhibiting the regrowth of Gal-9 KO EGFRmt allografts because they were reported to have a potential to activate NK cells⁶¹. We, thus, believe that innate immune cells including NK cells are essential to bring about better prognosis in patients with driver-positive NSCLC with low TMB because they can exert antitumor effect independently from antigen presentation. Meanwhile, recent study suggested that α Gal-9 Ab had a potential to enhance CD8-positive T cell response for *EGFR* mutation-positive lung cancer in mice⁶². If α Gal-9 Ab which not only induces the activation of NK cells and polarization of tumor-associated macrophage but also enhances T cell-mediated cytotoxicity will become available in the future, elimination of DTPs from driver-positive NSCLC by orchestrated-antitumor immunity can realistically be accomplished.

This study has several limitations. The clinical analysis was retrospective and restricted to patients with EGFR-mutant NSCLC treated with osimertinib. In addition, the experimental systems were designed to verify the mechanistic role of tumor-derived Gal-9 in NK cell evasion and do not establish the clinical efficacy of Gal-9-directed therapy. Nevertheless, the consistent findings across *in vitro* experiments using *EGFR* mutation- and *ALK* fusion-positive cells, genetic perturbation studies, *in vivo* models, and clinical specimens support Gal-9 as a biologically relevant mediator in DTPs.

Collectively, in the present study, we identified that Gal-9 played a key role for DTPs to evade from antitumor innate immunity, and showed that elimination of DTPs by Gal-9-targeted therapy had a potential to bring about long-term sustained response of specific TKIs for patients with driver-positive NSCLC (Fig. 7).

Methods

Cell lines and cell culture

Human NSCLC cell lines, HCC827, PC-9, H2228 and H3122, human embryonic kidney derived cell line HEK293T, and mouse fibroblast cell line 3T3 were obtained from American Type Culture Collection (Manassas, VA). The characterization of human natural killer leukemia cell line NKL was described previously⁶³. NSCLC cells and 3T3 cells were cultured in RPMI1640 and HEK293T cells were cultured in DMEM. Both culture media were supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). Interleukin-2 (100 units/ml), 1% sodium pyruvate and 1% non-essential amino acids were also added to RPMI1640 to culture NKL cells. All these cells were

routinely monitored for mycoplasma contamination by Mycoplasma Detection kit (Minerva Biolabs, Berlin, Germany).

Antibodies and reagents

Rabbit monoclonal Abs against human Gal-9 (#54330), IRF-1 (#8478), EGFR (#4267), β -catenin (#8480), non-phospho (active) β -catenin (#8814), mouse NKp46 (#71888) and GAPDH (#2118) were purchased from Cell Signaling Technology (CST, Danvers, MA). Rat Ab against mouse NKp46 (#137603), CD45 (#103111) were obtained from Biolegend (San Diego, CA). For *in vitro* functional blocking assay, anti-human Gal-9 Ab (clone 9M1-3, #348925) and its isotype control mouse IgG1 (clone MOPC-21, #400165) from Biolegend were used. For *in vivo* experiments, rat monoclonal Ab against mouse Gal-9 (clone RG9-1, #BE0218), CD122 (clone TM-Beta 1, #BE0298) and their isotype control rat IgG2b (clone LTF-2, #BE0090) were obtained from Bio X Cell (Lebanon, NH). Osimertinib for *in vivo* and *in vitro* experiments were purchased from MedChemExpress (Monmouth Junction, NJ) and Selleck (Houston, TX), respectively. Alectinib was also obtained from Selleck. Cell Counting Kit-8 (CCK-8) was available from Dojindo (Kumamoto, Japan).

Cell proliferation assay

To evaluate the antiproliferative effect of osimertinib and alectinib, human *EGFR* mutation-positive cells or *ALK* fusion-positive cells (5×10^3 /well) were plated onto 96-well tissue culture-treated plates (Corning, Corning, NY) and treated with serially diluted osimertinib or alectinib for up to 72 hours. The relative number of proliferating cells was quantified using CCK-8 following the instruction manual.

DNA microarray analysis

Total RNA was extracted from cells using Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA), and was subjected to whole transcriptome expression profiling using the Clariom-S human microarray platform (Filgen, Nagoya, Japan). Data were analyzed using Transcriptome Analysis Console 4.0 (Applied Biosystems, Waltham, MA).

qRT-PCR analysis

cDNA was synthesized from 100 ng of total RNA by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The quantification of real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on the Applied Biosystems QuantStudio 3 Real-Time PCR System (Applied Biosystems) with the following profile: 1 cycle at 94°C for 2 minutes; 40 cycles at 94°C for 15 seconds, at 60°C for 1 minute, and at 72°C for 1 minute. Data analyses were carried out by the ABI sequence detection software using relative quantification. The threshold cycle (Ct) is defined as the PCR cycle at

which fluorescence exceeds the threshold for the first time. The relative expression of each test gene transcripts was calculated by the $2^{-\Delta\Delta C_t}$ method. ΔC_t indicates the amount of the target gene transcripts relative to GAPDH transcripts, and $\Delta\Delta C_t$ means the difference of ΔC_t between transcripts from stimulated sample and control sample. Sequences of the primers used in this study are listed in Table S3.

Public database analysis

To evaluate the prognostic value of Gal-9 in patients with lung adenocarcinoma, OS data extracted from Gene Expression Omnibus, European Genome-phenome Archive and The Cancer Genome Atlas database was analyzed by KM-plotter²³. According to the transcripts per million (TPM) expression value, LGALS9 expression levels were divided into high and low expression group by median TPM values. Then, according to the survival data of patients in the databases, Kaplan–Meier survival analyses were performed and overall survival plots were generated. The difference between high gene expression and low gene expression was compared by Log-rank test.

Immunoblotting

Cells were lysed in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA). The whole cell lysates were separated in 5–20% gradient gel (Wako, Osaka, Japan) by SDS-PAGE, thereafter transferred to polyvinylidene difluoride membranes. The membranes were incubated with the proper primary Abs (diluted 1:500) overnight at 4°C followed by appropriate horseradish peroxidase-conjugated secondary Abs (diluted 1:1000, CST) for 1 hour at room temperature (RT). Immunoreactive bands were visualized using a chemiluminescent technique with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

IF analysis

HCC827 (3×10^4 /well) were seeded on the 8-well chamber slide (Thermo Fisher Scientific) and were treated with either DMSO or osimertinib for 24 hours. Thereafter, cells were fixed and permeabilized with ice-cold methanol (Wako) at -20°C for 10 minutes. After being washed thrice, cells were blocked with 1% bovine serum albumin for 60 minutes at RT, and were subsequently incubated with anti-IRF-1 Ab overnight at 4°C. For immunofluorescent detection of primary Abs cells were washed and incubated with a 1:1000 dilution of Alexa488-labeled goat anti-rabbit IgG (Thermo Fisher Scientific) for 45 minutes at 4°C. Actin filament was visualized by phalloidin staining. Images are photographed with BZ-X800 fluorescence microscope (Keyence, Osaka, Japan).

Gene KD analysis by siRNA

For IRF-1 KD analysis, HCC827 cells were grown in 12-well tissue culture-treated plates (Corning, New York, NY) and transfected with either 20 pmol/L of small interfering RNA (siRNA) targeted *IRF-1* (Silencer® Select, s7501 as #1 and s7502 as #2, Ambion, Austin, TX), or control siRNA (Silencer® Select, Negative Control #1) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). After 48 hours, cells were harvested and used for the individual experiments.

Generation of stable cell lines with gene overexpression or KO

To generate overexpressing cells, human *LGALS9* lentiviral vector (Applied Biological Materials, ABM, Richmond, BC, Canada, #26475061) and pHAGE-EGFR-L858R (a gift from Gordon Mills and Kenneth Scot, Addgene plasmid #11626) were used⁶⁴. To establish KO cells, human or mouse *LGALS9* single-guide RNAs (sg*LGALS9* or sg*Lgals9*) or scrambled single-guide RNAs inserted into CRISPR/Cas9 All-in-One lentiviral vector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro were purchased from Applied Biological Materials. The sgRNA target sequence for human *LGALS9* was GCAGGACAGTGCCTGAC, and sgRNA target sequence for mouse *Lgals9* was CGGGTTAATGTATGGAGACT. Lentiviral particles were generated by co-transfection of HEK293T cells with each sgRNA containing lentiCRISPR constructs and packaging plasmids encoding HIV Gag-Pol, Rev, Tat and VSV-G.

Cytotoxicity assay

To evaluate NK cell-mediated cytotoxicity against HCC827 and its derived cells, xCELLigence system (Agilent Technologies, Santa Clara, CA), a real-time cell electronic sensing system, was used. This system uses microplates coated with gold electrodes (E-plates) on the bottom of each well. The impedance of current flow in each well is dependent on size, shape and the number of adherent cells attaching to the surface of each well bottom, and is converted into unitless parameter termed cell index (CI). When cells were killed, CI decreases as a result of rounding and detaching of cells from the surface. HCC827 cells and NKL cells were used as target cells and effector cells, respectively. Target HCC827 and its derived cells were seeded at the density of 1×10^4 cells/well for 24 hours, then effector NKL cells were added at the different E/T ratio of 2 : 1 or 1:1 in the presence or absence of antibodies and successively cocultured for another 24 hours. To determine the cytotoxicity of NKL cells against DTPs, 1×10^4 cells/well of HCC827 cells were incubated under the existence of 100 nM of osimertinib prior to adding effector NKL cells. After confirming re proliferation of target HCC827 cells, NKL cells were added at the E/T ratio of 2:1 or 1:1. Thereafter, effector and target cells were coincubated for up to 14 days after the initial target cell seeding.

Animal experiments

Six-week-old female BALB/cAJcl-*nu/nu* mice were purchased from CLEA Japan (Tokyo, Japan). Genetically engineered HCC827-*scr* and HCC827-*sgLGALS9* cells (1×10^6), and also genetically engineered mouse 3T3/EGFRmt-*scr* and 3T3/EGFRmt-*sgLgals9* cells (5×10^5) suspended in RPMI1640 were inoculated subcutaneously in the flanks of mice. Tumor size was measured with a caliper two or three times per week, and tumor volume (V) was calculated using the formula $V = \text{length} \times (\text{width})^2 / 2$. When the tumor volume reached 100–200 mm³, mice were randomly assigned into four groups, corresponding to the following two or four different treatments according to the purpose of experiments: (A) vehicle (DMSO) + rat IgG2b; (B) vehicle + α Gal-9 Ab; (C) osimertinib + rat IgG2b; (D) osimertinib + α Gal-9 Ab. Then, mice were treated with the following schedule; 6mg/kg of rat IgG2b or α Gal-9 Ab was intraperitoneally administered twice a week, and 3 or 12.5mg/kg of osimertinib was orally given five times a week. For the NK-depletion experiments, mice were intraperitoneally administered with 5mg/kg of Rat IgG2b or α CD122 Ab twice a week three days before the inoculation of tumor cells. Mice were euthanized by intraperitoneal injection of the anesthetic mixture of medetomidine, midazolam and butorphanol once tumor volume exceeded 2000 mm³ or before mice became moribund. For some experiments, allografts were collected for flow cytometry or histological analyses.

All the animal experiments were performed under approval of the Committee on Animal Research of Hyogo Medical University (approval number: 23-102AG).

Immunohistochemistry (IHC)

Four- μ m thick sections were deparaffinized and incubated in Target Retrieval Solution pH 9.0 (Agilent, Santa Clara, CA) for 40 min at 96°C for antigen retrieval, after which endogenous peroxidase activity was blocked with Envision Flex Peroxidase Blocking (Agilent). The sections were allowed to react with diluted α NKp46 Ab or α Gal-9 Ab (1:100) overnight at 4°C. Then, the slides were incubated with a peroxidase-labeled polymer conjugated to secondary anti-mouse or rabbit Abs using EnVision™ FLEX/HRP (Agilent) and developed with 3,3'-diaminobenzidine as the chromogen. Regarding NKp46 staining, quantification was performed at least five fields per section excluding necrotic areas using imaging analysis software (KEYENCE X-800 Analyzer, Keyence, Osaka, Japan). Percentage of immunoreactive cells were quantified using Hybrid Cell Count software BZ-H3C (Keyence).

Clinical EGFR mutation-positive NSCLC samples

Clinical samples were collected from the patients diagnosed as EGFR mutation-positive NSCLC by Oncomine Dx Target Test (Thermo Fisher Scientific) or Amoy Dx Pan Lung Cancer panel (AmoyDx, Singapore, Singapore) and received osimertinib monotherapy at Hyogo Medical University Hospital (Nishinomiya, Japan) from October 2021 to March 2026. This study was approved by the institutional review board of Hyogo Medical University (Trial number: 3900). Written informed consent or opt-out method was applied so that the patients and families could refuse to participate in the study. Patients

were censored at the time of last follow-up. PFS was defined as time from the initiation of osimertinib to date of radiologic progression as per RECIST v1.1 or death.

Flow cytometry

To determine the role of Gal-9 in innate immune response, 3T3/EGFRmt-scr or 3T3/EGFRmt-sgLGals9 cells inoculated mice were treated with either DMSO or 12.5mg/kg of osimertinib for 3 days. Then, mice were euthanized and allografts were removed into cold RPMI1640 media. Allografts were minced into 2–4 mm fragments, and which were transferred into the tubes containing the enzymes included in Tumor Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Fragment samples were dissociated using gentleMACS™ Octo Dissociator with Heaters according to the instruction manual (Miltenyi Biotec). Dissociated cells achieved from allografts or NKL cells for *in vitro* experiments were incubated with properly diluted Abs at 1: 100–200 for 45 minutes at 4°C. After staining, cells were analyzed by BD LSRFortessa (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

All the *in vitro* studies for statistical evaluation were performed in triplicate in each experiment, repeated at least thrice, and mean $\pm$ standard error of the mean (SEM) values was calculated. For statistical analysis of *in vivo* experiments, data were obtained from 3–8 samples in each treatment group and mean $\pm$ standard deviation (SD) was estimated. The differences were evaluated by two-sided Student's *t* test for comparisons between two groups and one-way ANOVA with Dunnett's or Tukey's test for comparisons among multiple groups. $P < 0.05$ values were considered statistically significant.

Declarations

Competing interests

The authors declare no conflicts of interest associated with this manuscript.

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Author Contribution

T. Kondo and T. Minami contributed equally to this work as first authors. T.K., T.M., and T.K. conceived the study and designed experiments; T.K., T.M., H.K., Y.H., T.F., and T.I. carried out experiments; T.K., T.M., T.F., T.H., A.T., Y.N., T.O., K.M., R.T., D.F., A.N., M.H., S.F., and K.K. collected samples from patients; T.K., T.M., H.K., Y.H., T.F., K.Y., D.F., K.M., H.D., K.K., M.O., C.O., S.F., K.K., and T.K. analyzed the data; T.K., T.M., K.K., and T.K. wrote the manuscript, which was reviewed and edited by the other coauthors.

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Figures

the data from public databases, the correlation of overall survival of patients with lung adenocarcinoma with messenger RNA expression of *LGALS3* and *LGALS9* was analyzed by Kaplan-Meier plotter (<https://kmplot.com/analysis/>). The log-rank test was used to determine survival difference between groups. E, Expression of Gal-9 transcripts in *EGFR* mutation-positive (HCC827 and PC-9) and *ALK*-fusion-positive (H3122 and H2228) NSCLC cells increased when stimulated with either DMSO or specific TKI at various concentration for 24 hours. F, CCK-8 assay to confirm the establishment of osimertinib-resistant HCC827OR cells and alectinib-resistant H3122AR cells. G, Expression of Gal-9 transcripts in parental and drug-resistant driver-positive NSCLC cells. H, Representative image of immunoblotting of Gal-9 protein expression in parental and drug-resistant cells. Immunoblotting was performed twice with similar results. Results in 1C, E, F and G represent mean $\pm$ SEM of at least three independent experiments performed in triplicates. To evaluate statistical significance, one-way ANOVA with post hoc Dunnett's multiple comparison test was used in Fig. 1E, and two-sided Student's *t*-test was used in Fig. 1G. * $P < 0.05$ and ** $P < 0.01$.

Fig. 2

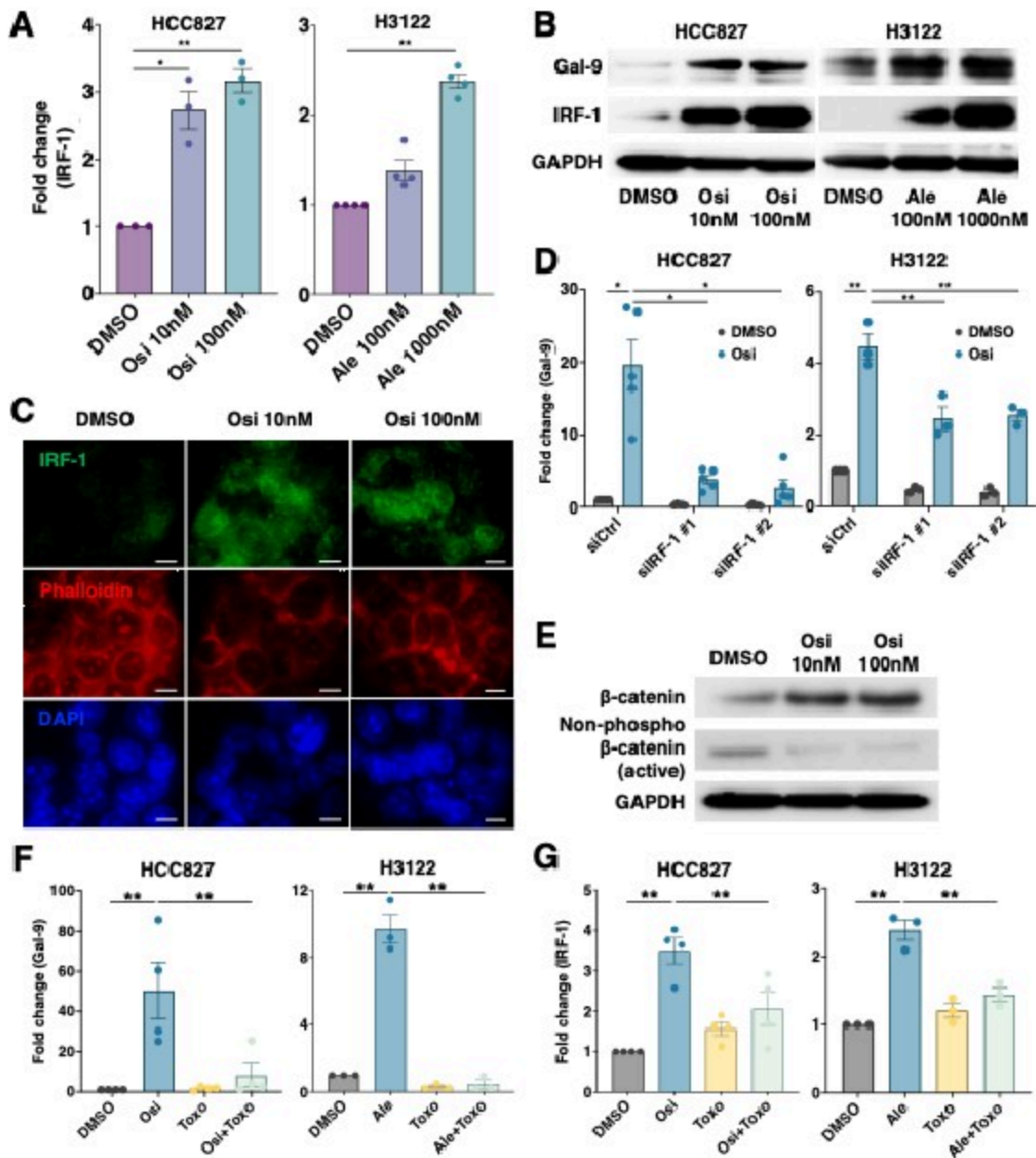


Figure 2

IRF-1 as a key regulator of Gal-9 expression induced by TKI stimulation in driver-positive NSCLC cells. A, Expression of IRF-1 transcripts in HCC827 cells and H3122 cells when exposed to osimertinib and alectinib, respectively. B, Both Gal-9 and IRF-1 protein expression in HCC827 cells and H3122 cells when exposed to osimertinib and alectinib, respectively. C, Representative IF images of IRF-1 expression in HCC827 cells exposed to DMSO, 10 nM of osimertinib or 100 nM of osimertinib. Scale bars, 10 μ m. D, Expression of Gal-9 transcripts in HCC827 cells and H3122 cells transfected with either control or IRF-1-targeting siRNAs (siIRF-1 #1 and siIRF-1 #2). E, Evaluation of the activity of β -catenin in HCC827 cells exposed to either DMSO, 10 nM of osimertinib or 100 nM of osimertinib. F, Changes in the expression levels of Gal-9 transcripts in HCC827 cells and H3122 cells when exposed to DMSO or osimertinib and

alectinib, respectively, with or without 1 μ M of toxoflavin. G, Expression of IRF-1 transcripts in HCC827 cells and H3122 cells when exposed to DMSO or osimertinib and alectinib, respectively, with or without 1 μ M of toxoflavin. Results in 2A, D, F and G represent mean $\pm$ SEM of at least three independent experiments performed in triplicates. To evaluate statistical significance, one-way ANOVA with post hoc Dunnett's multiple comparison test was used in Fig. 2A, and Tukey's multiple comparison test was used in Fig. 2D, F and G. * $P < 0.05$ and ** $P < 0.01$. For Fig. 2B, C and E, immunoblot and IF were performed twice with similar results.

Fig. 3

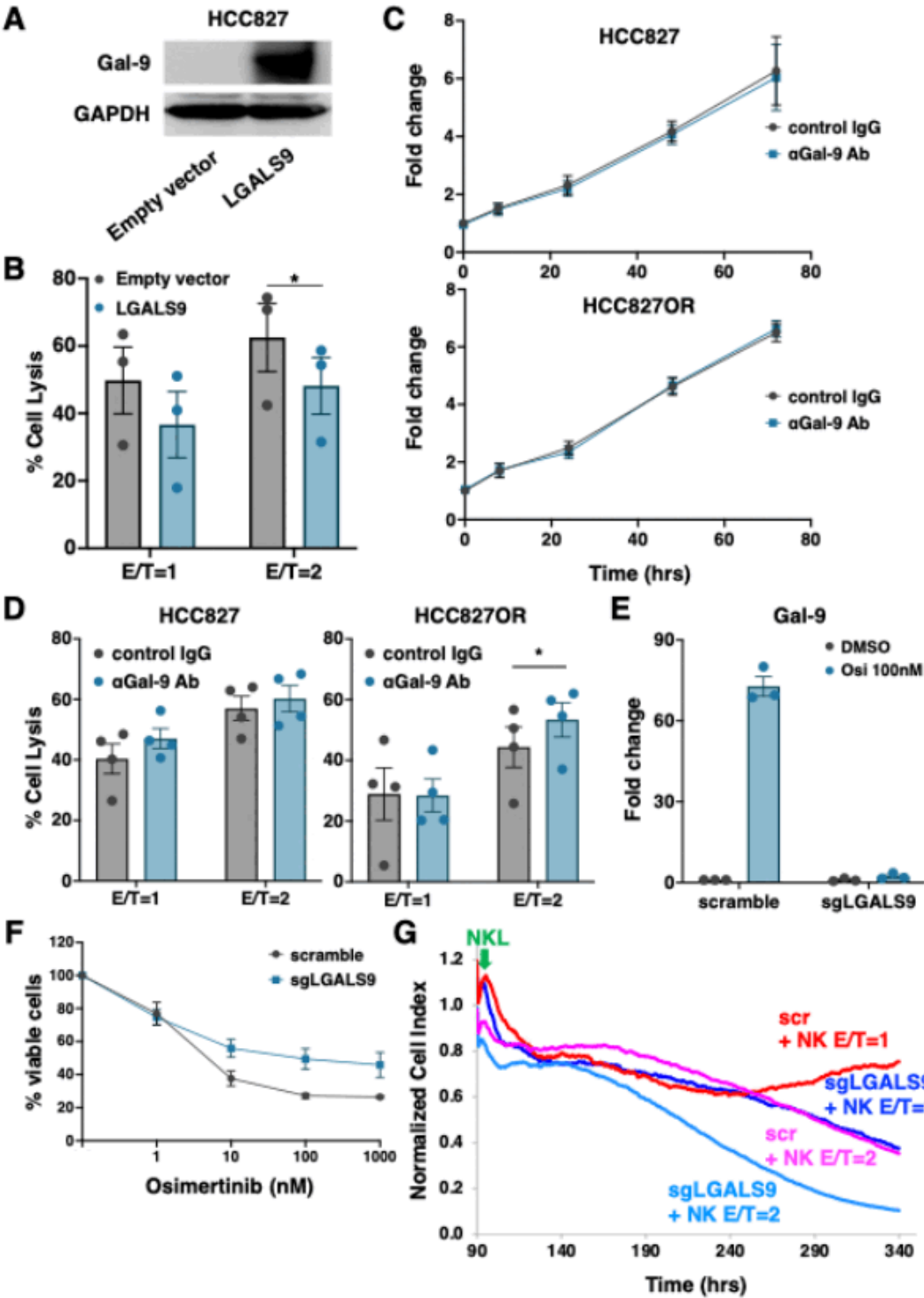


Figure 3

Gal-9 attenuated the cytotoxicity of NK cells against DTPs in driver-positive NSCLC cells. A, Generation of Gal-9-overexpressing HCC827/LGALS9 cells. Immunoblot was performed twice with similar results. B, NK-mediated cytotoxicity against HCC827/LGALS9 cells assessed by xCELLigence system. NK cells and HCC827/LGALS9 cells were used as effector (E) cells and target (T) cells, respectively. C, CCK-8 assay to evaluate antiproliferative effect of α Gal-9 Ab against HCC827 parental cells and HCC827 osimertinib-resistant (HCC827OR) cells. D, NK-mediated cytotoxicity assay against HCC827 cells and HCC827OR cells under the existence of 10 μ g/mL of control IgG or α Gal-9 Ab. E, Confirmation of the establishment of Gal-9 KO (HCC827-sgLGALS9) cells by qRT-PCR analysis. F, Osimertinib sensitivity in HCC827-scr and HCC827-sgLGALS9 cells was assessed by CCK-8. G, NK-mediated cytotoxicity against DTPs in HCC827-scr and HCC827-sgLGALS9 cells which survived from continuous exposure to osimertinib was determined by long-term xCELLigence analysis. Same result was confirmed by another experiment shown in Fig. S3B. Results in 3B, C, D, E and F represent mean $\pm$ SEM of at least three independent experiments performed in triplicates. In Fig. 3B and D, two-sided Student's *t*-test was used to assess the statistical significance. * *P* < 0.05.

Fig. 4

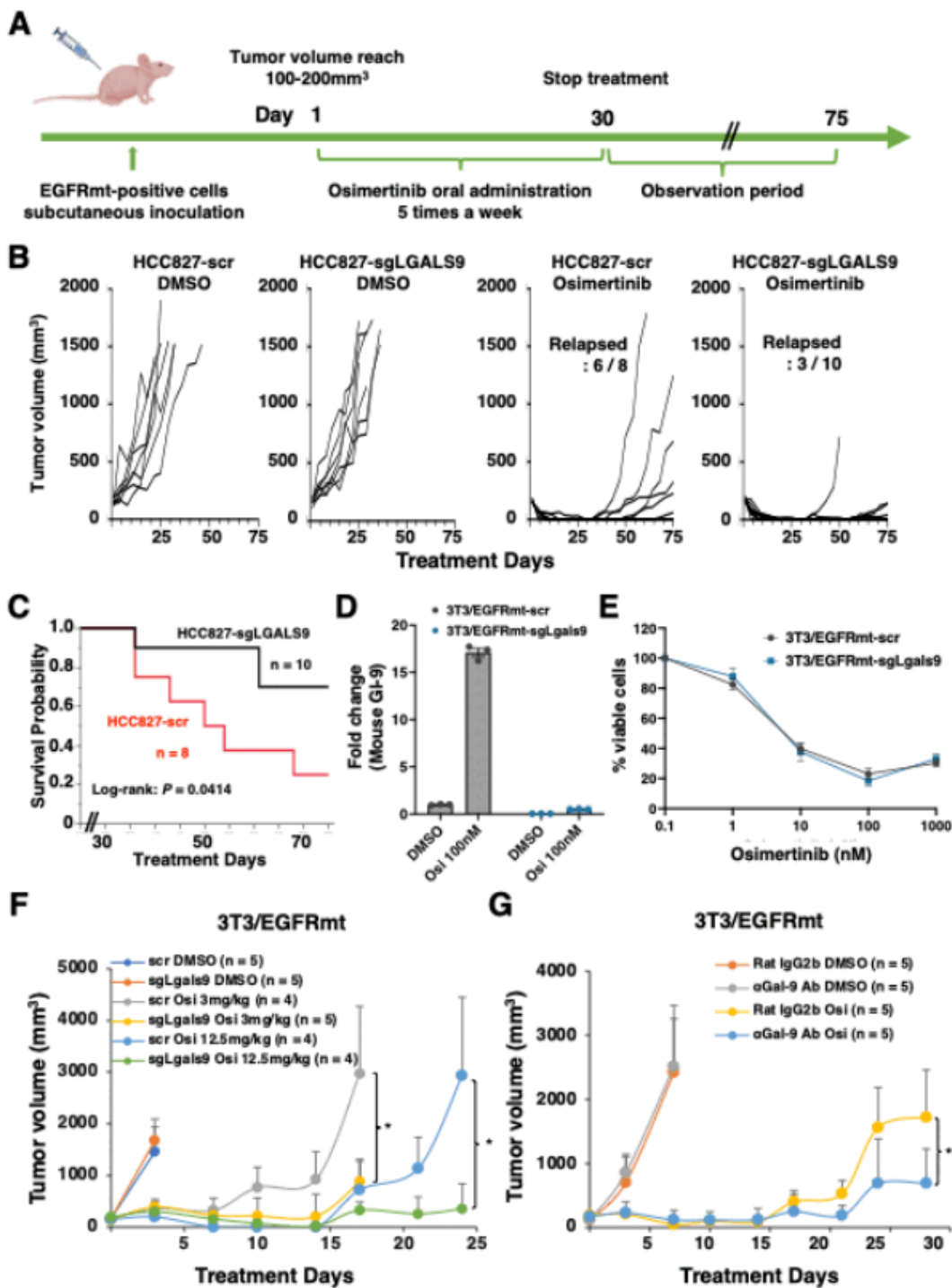


Figure 4

The growth of subcutaneous EGFR mutation-positive cell xenografts and allografts in nude mice was inhibited by Gal-9-targeted therapy. A, Schematic representation of the experimental design and treatment schedule for HCC827 xenografts and 3T3/EGFRmt allografts. B, The tumor volume curve of HCC827-scr and HCC827-sgLGALS9 subcutaneous xenografts treated with either DMSO or 3mg/kg of osimertinib. C, Kaplan-Meier plots showing the duration of response to osimertinib. Log-rank test was

used for statistical analysis. D, Confirmation of the establishment of mouse Gal-9 KO (3T3/EGFRmt-sgLgals9) cells by qRT-PCR analysis. E, Osimertinib sensitivity in 3T3/EGFRmt-scr cells and 3T3/EGFRmt-sgLgals9 cells was determined by CCK-8. For, Fig. 4D and E, experiments were repeated thrice in triplicates. F, The tumor volume curve of the 3T3/EGFRmt-scr and 3T3/EGFRmt-sgLgals9 tumor-bearing mice. Mice were orally treated either DMSO, 3mg/kg of osimertinib or 12.5mg/kg of osimertinib. G, The tumor volume curve of the 3T3/EGFRmt tumor-bearing mice. Mice were randomly assigned into four groups and treated with either Rat IgG2b plus DMSO, αGal-9 Ab plus DMSO, Rat IgG2b plus 12.5mg/kg of osimertinib and αGal-9 Ab plus 12.5mg/kg of osimertinib. In Fig. 4F and G, each plot point and bars indicate the mean tumor volume of each treatment group and SD, respectively. To evaluate statistical significance, two-sided Student's *t*-test was used in Fig. 4F and G. *, $P < 0.05$.

Fig. 5

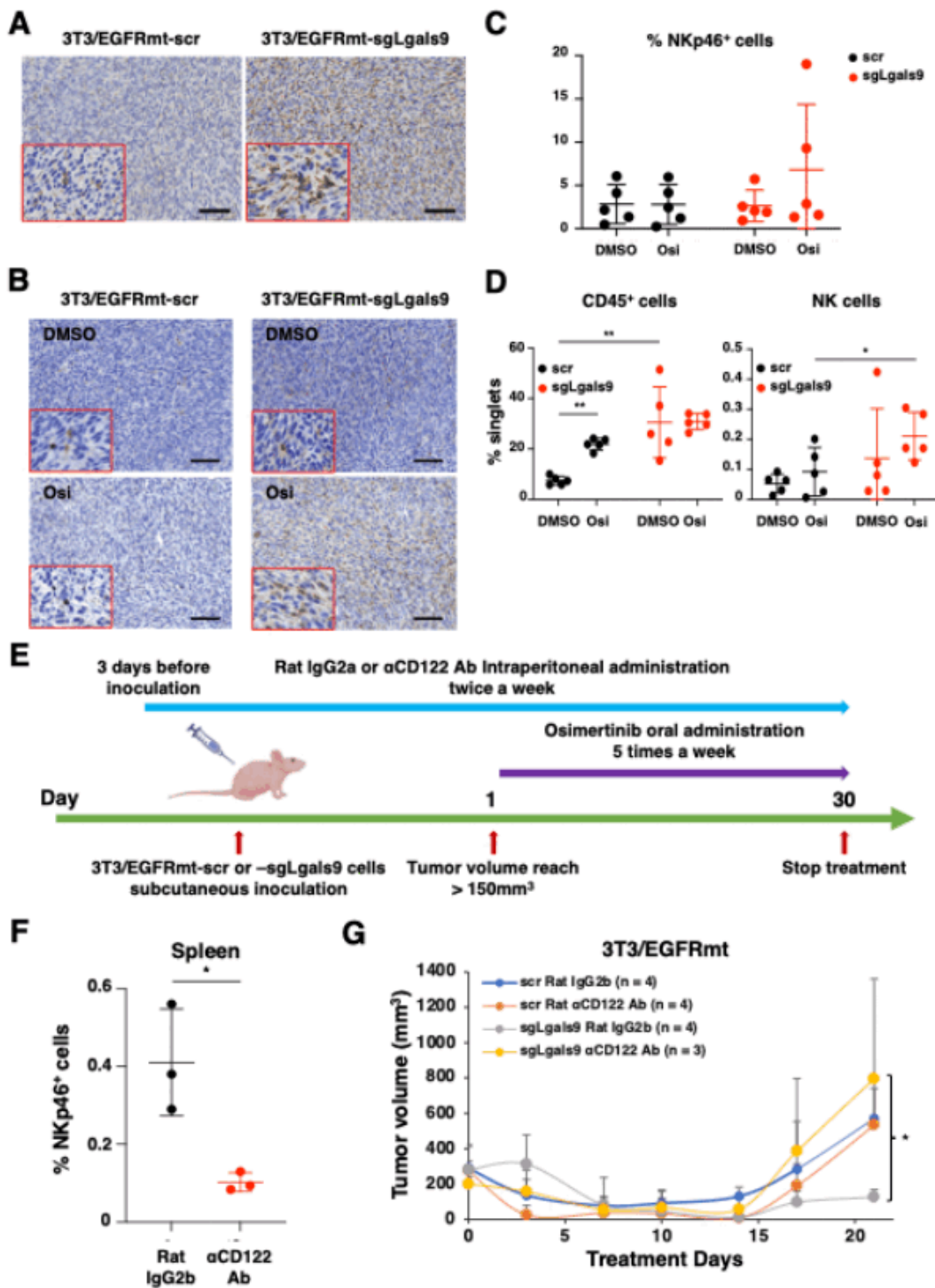


Figure 5

Antitumor effect of Gal-9-targeted therapy for *EGFR* mutation-positive NSCLC was exerted through NK cell-mediated cytotoxicity. A, Representative IHC image of NKp46 staining in tumor tissue obtained from osimertinib-treated 3T3/EGFRmt-scr and 3T3/EGFRmt-sgLgals9 allografts at the end of the experiment. Scale bar, 100 μ m. B, Representative IHC image for NKp46 staining of the tumor sample collected from 3T3/EGFRmt-scr and 3T3/EGFRmt-sgLgals9 allografts after treated with DMSO or osimertinib for 3 days.

Scale bar, 100 μ m. C, NKp46-positive cell ratio was calculated by dividing the number of NKp46-positive cells by the total number of cells in randomly selected fields without necrotic areas. Dot plot shows staining positive cell ratio in individual allografts from each group (n = 5 each). D, Tumor -infiltrating CD45-positive immune cells and NKp46-positive NK cells in 3T3/EGFRmt-scr and 3T3/EGFRmt-sgLgals9 allografts treated with either DMSO or Osimertinib for 3 days were quantified by flow cytometry. Data represent mean $\pm$ SD. E, Schematic representation of the experimental design of NK cell depletion from athymic nude mice and treatment schedule for mice harboring 3T3/EGFRmt-scr or 3T3/EGFR-sgLgals9 allografts. F, Flow cytometry analysis for NKp46-positive cells out of CD45⁺ cells in spleen collected from mice either treated with control rat IgG2b or α CD122 Ab (n = 3 each). G, The tumor volume curve of the 3T3/EGFRmt-scr or 3T3/EGFRmt-sgLgals9 tumor-bearing mice treated with either rat IgG2b or α CD122 Ab. All mice were treated with 12.5mg/kg of osimertinib. To evaluate statistical significance, one-way ANOVA with post hoc Tukey's multiple comparison test was used in Fig. 5C, D, and G. * $P < 0.05$.

Fig. 6

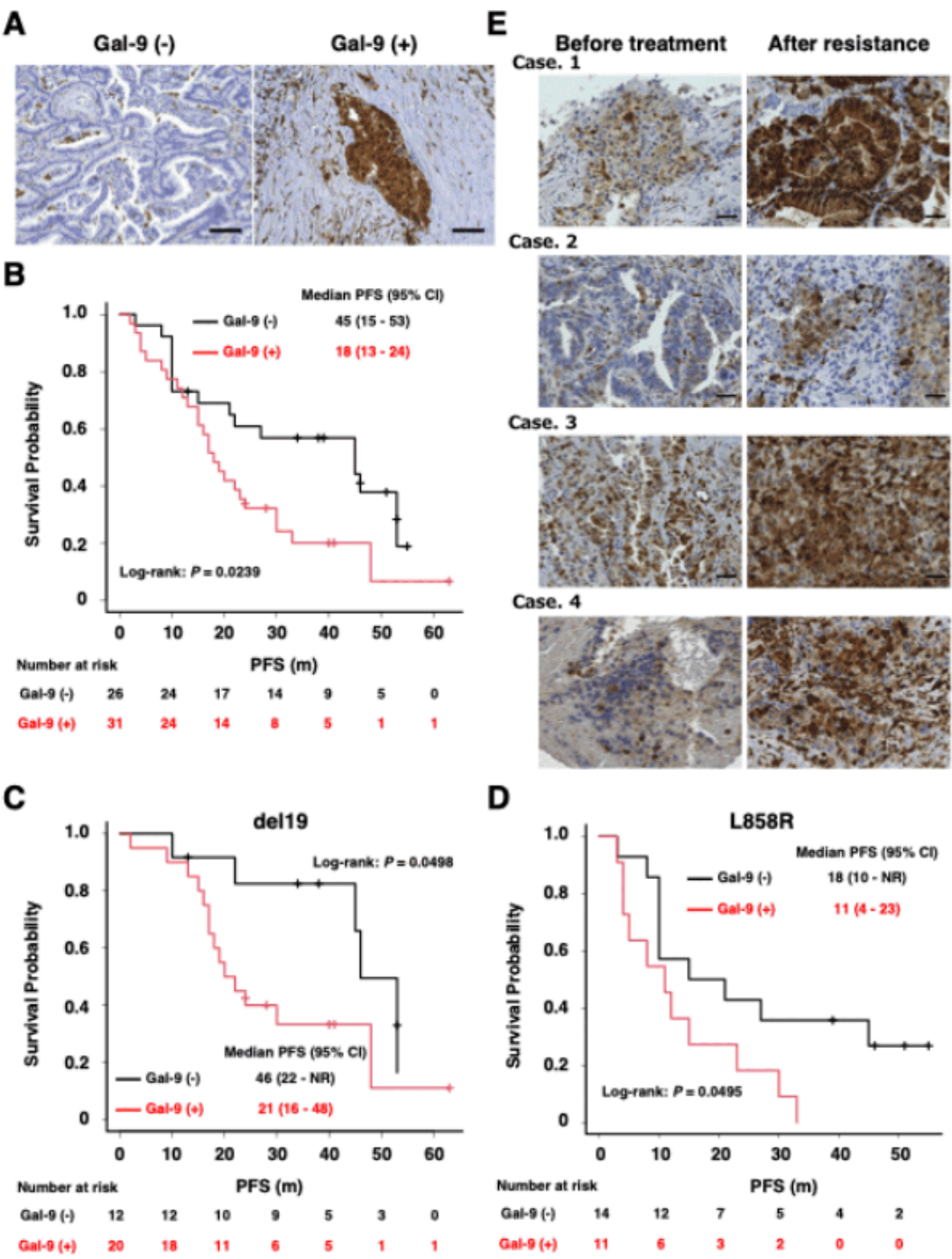


Figure 6

Correlation between Gal-9 expression and PFS in patients with EGFR mutation-positive NSCLC who received osimertinib monotherapy. A, Representative IHC image displaying Gal-9-negative and Gal-9-positive case. Each IHC sample was evaluated by two independent observers by light microscope. Samples which Gal-9 expression was detected in tumor cells and only in other cells in TME were regarded as Gal-9-positive and Gal-9 negative, respectively. Scale bar, 100 μ m. B, Kaplan-Meier curve

according to Gal-9 expression status for PFS in patients with EGFR mutation-positive NSCLC who received osimertinib monotherapy. C and D, Patients with EGFR mutation-positive NSCLC treated with osimertinib monotherapy were classified based on EGFR mutation subtypes (C, del19 and D, L858R). Kaplan-Meier analysis was carried out to estimate PFS according to Gal-9 expression status. Log-rank test was used to quantify differences between Gal-9-negative group and Gal-9-positive group. E, IHC analysis of Gal-9 expression in EGFR mutation-positive NSCLC samples obtained from four individual patients before and after osimertinib resistance. Scale bar, 50 μ m.

Fig. 7

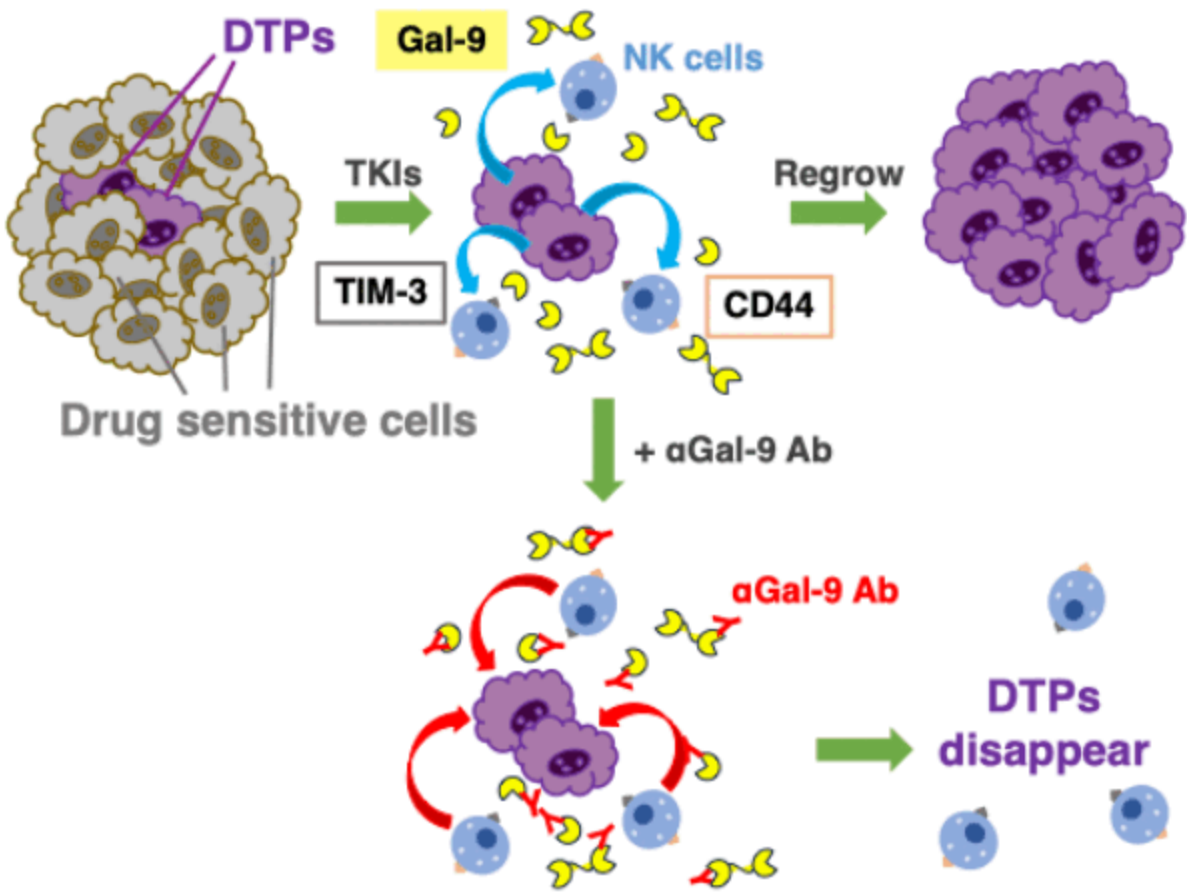


Figure 7

Gal-9 plays a key role for DTPs to evade from antitumor innate immunity in driver-positive NSCLC. In driver-positive NSCLC cells, exposure to specific TKI induces upregulation of Gal-9 expression in residual DTPs, which impairs the cytotoxicity of NK cells and enables DTPs to regrow. Gal-9-targeted therapy recovers the antitumor activity of NK cells and has a potential to bring about long-term durable response to TKI therapy by eliminating DTPs in patients with NSCLC.

Supplementary Files

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