Delay tumor progression with the introduction of engineered impactive cells

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Title

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Abstract

The complete eradication of tumor cells is extremely challenging. An alternative strategy is tumor containment, which utilizes the interaction of tumor cells and has achieved promising results. However, effective interaction and controllability are not guaranteed for a given tumor. Here, we proposed a novel strategy comprising an impactive cell population and a control system and realized it in leukemic mouse model. To identify impactive cells, we xenografted six leukemic cell lines together. We found NALM-6 cells strongly impact the infiltration of other leukemic cells. To control NALM-6 cells, we introduced the herpes simplex virus thymidine kinase/ganciclovir suicide system. We found they effectively controlled the infiltration of NALM-6 cells. To evaluate the effectiveness, we used this strategy in mice xenografted with REH cells. The survival time was significantly elongated. In summary, our strategy guaranteed the impact and controllability of effector cells, thereby extending the tumor containment strategy.

MAIN TEXT

Introduction

Tumor is a major cause of death all over the world, and the cumulative risk of contracting tumor in the whole life of contemporary people is close to 50% [1,2]. Although tumor has been recognized for at least 4500 years, it had been incurable for most time along the history [3-5]. In the middle and toward the end of the 19th century, surgery became practical and radiotherapy emerged, respectively [6,7]. Research of the effects of nitrogen mustard gas on proliferating cells during world War II inspired early chemotherapy for tumors; many drugs were developed in 1950s to 1970s, and some of them still serve as first-line drug nowadays [8]. Moreover, adjuvant chemotherapy remarkably improved the cure rate and survival of tumor patients since the 1960s [6,8]. With the progress in cell biology and biochemistry, the development of chemotherapy drugs gradually shifted focus to the molecular characteristics of tumor cells from the 1980s; one of the best examples is imatinib [9,10]. Since then, novel therapeutics have shown increasing dependence on the molecular characteristics of tumor cells [11], namely, the search for the “magic bullets” of tumor [12]. Existing tumor therapeutics can be

roughly classified into two groups: local therapy, with surgery and radiotherapy as representatives, and systemic therapy, with chemotherapy and cell therapy as representatives. Both achieve their effect via the elimination of tumor. However, the unit of elimination is tumor or even organ tissues for local approaches and individual tumor cells for systemic approaches. In general, the treatment outcome of non-solid tumors and advanced solid tumors is determined by systematic therapy. Despite continuous and extraordinary efforts and wealth, the control of advanced tumors is hardly satisfying and tumor heterogeneity was recognized as one of the largest obstacles [1,13,14].

Tumor heterogeneity is the summary of the differences of tumor at multiple levels. For more than 100 years, the recognition of tumor heterogeneity has underwent several phases, such as morphology, histology, cytokinetics, cell surface markers, and genetics [15-17]. Tumor heterogeneity has been widely accepted since the 1980s [18]. Many models have been proposed to elucidate the generation and maintenance of tumor heterogeneity and thereby to develop a coping strategy, and the most well-known are clonal evolution model and cancer stem cell (CSC) model [13,18,19]. The evolution model depicts the continuous divergence of the genetic material of tumor cells and emphasizes the generation and coexistence of tumor cell populations with highly diversified genome and clonal structures [1,13]. The CSC model depicts how tumor cell populations are maintained and emphasizes the varied tumor initiation capability of tumor cells [18,20]. According to the American Association for Cancer Research, CSCs represent only the cells capable of self-renewal in tumor tissues [21]. The clonal evolution model and CSC model are not mutually exclusive but unified [18,19,22]; the virtual units selected in clonal evolution ought to be cells with self-renewal capability, which can be provided by CSCs [13,23]. Accordingly, CSCs are the cellular foundation of the selection process underlying the clonal structure, disease progression, metastasis, recurrence, and drug resistance of tumor [24]. Current studies stated that a tumor is a complexed ecosystem composed of tumor cells and nontumor cells [25] and therefore possesses heterogeneity of additional levels. Nontumor cells mainly include macrophages, mast cells, neutrophils, T cells, B cells, and various myeloid progenitor cells [26,27] and also experience evolution along with progression [28-30]. The homotypic and heterotypic interactions between cells within the tumor augment the dimensions of tumor heterogeneity and also the layers of complexity to tumor therapies [14]. So, these phenomenon above explains our uncertain understanding of the internal dynamics of tumor despite the increasing knowledge.

With heterogeneity of multiple levels, almost infinite uncertainty is generated and thereby seriously hinders studies and clinical practices of tumor therapy. In studies, the tumor model is irreplaceable; however, the heterogeneity of tumor makes it impossible for a tumor model to completely recapitulate the feature of the original tumor [31]. Given their main focus on short-term responses, current drug development strategies tend to miss drugs that are effective to CSCs and eventually obtain drugs with poor long-term efficacy [15]. In clinical practices, the diagnosis and treatment of tumor can be seriously impeded by its heterogeneity. Given that an individual tumor cell is the unit of eradication for systemic therapies, all tumor cells ought to be accurately diagnosed to ensure the complete eradication of tumor. However, the heterogeneity of tumor makes it extremely challenging to obtain enough information that required to draw diagnosis with limited biopsy [32]. Therefore, blind spots constantly exist, and the tumor cells inside are constantly out of the coverage of the treatment. For tumor cells that are covered by the decided treatment, numerous mechanisms associated with heterogeneity render them resistant to treatment and thus extremely difficult to be eradicated. The resistance of tumor can be divided into de novo and acquired [33]. De novo resistance can be further

divided into intrinsic and extrinsic that are typically represented by the resistance to various treatments of CSCs [18,34] and environment-mediated drug resistance [11], respectively. Given that acquired resistance commonly develops over time as a result of sequential genetic changes, clonal evolution is the source of acquired resistance; the resistance of non-small cell lung cancer to gefitinib and the resistance of CML to imatinib are good examples [35,36]. However, the impediments imposed by tumor heterogeneity are far from being completely enumerated. Therefore, heterogeneity is a formidable opponent for existing therapeutics, and unsatisfactory result is almost inevitable when coping with the uncertainty of advanced tumor using largely rigid therapeutics [14,37].

Owing the high relapse rate of advanced tumor, the justification and rationale of extremely aggressive treatments disappear when a cure cannot be achieved [38]. The ultimate goal of tumor therapy is to maintain the survival of patient as long as possible and with sufficient quality of life; maximum eradication of tumor cells is the most commonly adopted approach to achieve this goal [38-40]. A few alternative strategies have been proposed and tested, and they typically shift the goal from "treatment for cure" to "treatment for stability" in attempt to transform advanced tumors into a chronic disease [13,38,41]. Some examples include using cytostatic drug to slow down the evolution of tumor and thereby delay the emergence of resistance [13], inducing the advantage of less malignant subclones over resistant clones to prevent recurrence [37], and maintaining the amount of tumor cells sensitive to chemotherapy to inhibit the proliferation of resistant tumor cells [40,42]. Containment strategies have been found to be superior to conventional treatment in preclinical studies [38], and some of them even achieve promising results in clinical trials [43-45].

In principle, tumor containment utilizes the interactions among tumor cell populations and is inspired by the understanding that tumor is an adaptive ecosystem [14,41]. Tumor cells have features that resemble invasive species [37]; the promising results of ecology-based approaches against invasive species, pest, and weed management have led to the idea that a similar strategy might be effective in cancer treatment [41,44]. In the tumoral ecosystem, cells experience various interactions mediated by the tumor microenvironment, and these interactions are similar to those observed in the populations of species in the natural ecosystem [37]. The clonal evolution of tumor is achieved through the continuous interaction between the cell populations within the tumor, and this interaction results in the variation of the cell number of different populations; some cell population thrive, and some decline and even become extinct [13,14]. All interactions in the ecosystem can be roughly divided into three categories: positive, negative, and neutral. Positive and negative interactions eventually manifest as population gain and loss, respectively, and neutral interactions have no phenotypic manifestation on the participants [14]. In the tumoral ecosystem, negative interaction can inhibit the growth of cells involved in the interaction [14], which is useful for the control of tumor progression. This phenomenon explains why tumor containment tends to leverage negative interactions between tumor cells. Many studies have been conducted on tumor containment leverage competition between tumor cells, especially that between sensitive and resistant tumor cells [40,42]. However, competition is only one type of negative interaction, and tumor containment is designed to utilize all interactions that have negative effects, amensalism is another kind of negative interaction that could be involved [14,38]. Collectively, tumor containment relies on the effective interaction between resistant and sensitive tumor cell populations [46] and is based on the assumption that resistant cells are less fit than sensitive cells and their interaction must be intensive [38,44,47].

For an arbitrary tumor, effective interaction between resistant and sensitive tumor cell populations is not guaranteed. To ensure the effect of tumor containment, the tumor must comprise comparable number of resistant and sensitive cell populations in the first place, otherwise, containment is not effective when most tumor cells are resistant [48]. In addition, the assumption that resistant cells are less fit than sensitive cells can fail [46]. Insensitivity to treatment can be caused by many factors: some mechanisms have shown significant cost in fitness, the fitness cost of some mechanism can be overcome, some mechanisms have no significant cost in fitness, and some reversible mechanisms do not affect the fitness [14,48,49]. If resistant tumor cells are severely deficient in their fitness, theoretically they would not persist; their presence suggests that they are not that weak as imagined [42]. Resistant cells have been shown to exceed sensitive cells in fitness in certain tumors [46,49]. Another difficulty lies in the evaluation of the relationship of fitness between the resistant and sensitive cell populations within a given tumor [46]. Also, the sensitive tumor cells can develop drug resistance [50]. Given that resistance causes a reduction in fitness and places resistant cells in a disadvantage during their interaction with sensitive cells, effective negative interaction is still not guaranteed [49]. Cost of fitness commonly depicts the reduction in the capability of proliferation but does not guarantee the interaction between resistant and sensitive tumor cells [49]. As most of the interactions tend to be neutral [14], the advantage of sensitive cells in fitness is not easy to translate into effective interaction [38]. Absolute neutral interaction seems extreme, but weak interactions that do not satisfy the need of containment treatment are common. Some studies have proposed methods to enhance interactions within a tumor [47], implying the insufficient intensity of intrinsic interactions within tumors. In addition, the interaction between tumor cells occurs at the population level; the result of the interaction between resistant and sensitive tumor cells is not solely determined by the advantage of individual cells and can also be affected by the number and abundance of different tumor cell populations [37].

Under the assumption of the existence of effective interaction, another key process of tumor containment is to achieve the desired effect via the long-term control of the sensitive tumor cell population [48]. The sensitive cell population within a tumor ought to persist without alteration in drug reactivity [50], but this goal is challenging. Many mechanisms can render tumor cells insensitive to the drugs being administered, such as evolution, persistence and tolerance. In addition, sensitive and resistant tumor cells have no sharp boundary. Therefore, ensuring the constant control of the sensitive cell population within a tumor is difficult [51], which greatly increases the uncertainty of tumor containment. In our opinion, the nonguaranteed effective interaction and the controllability of the cells utilized are two obstacles for the implementation of tumor containment strategy.

For the guaranteed impact and controllability of the effector cell, a straightforward strategy is to create cells *in vitro* that can be extensively manipulated *in vitro*. Therefore, we proposed to introduce cells that have strong impact on tumor cells from the outside to delay the progression of the original tumor (Fig. 1). As an alternative to utilizing the interaction between cells within the original tumor, this strategy allows the introduced cells to be potentially engineered *in vitro* with reinforced impact and good control. Two key components are needed for this strategy: a population of cell with strong impact on other tumor cells and a system to control these cells. The introduced cells coexist with the original tumor cells *in vivo*, resembling a scenario of the composition of the original tumor being interfered. Therefore, we call this strategy “compositional intervention.”

Results

Interaction impacts the behavior of leukemic cells

"Compositional intervention" has two key elements: a population of impactive cells and a system to control these cells. To realize this strategy, we first set out to explore whether the interaction between tumor cells has a strong impact on the behavior of involved cells. We chose leukemia as the model system because it is relatively easy to culture and is convenient for model setting and evaluation.

If the interaction between tumor cells has an impact on the behavior of involved cells, then we could expect a change in the behavior when different leukemic cells coexist; otherwise, the cells will stay unaffected. Basing on this assumption, we explored whether the infiltration of some leukemic cells is affected when xenografted with mixtures of different leukemic cells. Therefore, we first established mCherry or GFP stable transformants of the six leukemic cell lines (Fig. S1A, B). For reference, we evaluated the capacity of infiltration of the six leukemic cell lines (Fig. 2A). The result indicated that the number of infiltrated cells of the six cell lines varied greatly when the number of xenografted cells was comparable (Fig. 2B, C, Fig. S2A). In particular, the infiltration of NALM-6 and REH was close to each other and much higher than that of other cell lines. Jurkat, OCI-AML-3, and HL-60 showed comparable and weak bone marrow infiltration, with THP-1 being the weakest (Fig. 2B, C). Although the infiltration of GFP^+ and $mCherry^+$ cells varied for NALM-6 and REH, no significant difference in overall infiltration was detected (Fig. 2C). After setting the referential infiltration of the six cell lines, we then explored the effect of the interaction between leukemic cells on the infiltration of involved cells *in vivo*. Equal amounts of the six cell lines were mixed and injected into the mice through the tail vein (Fig. 2D). With the initial ratio of GFP^+ and $mCherry^+$ cells in each context all close to 1 : 5 (Fig. S2B), the infiltration of GFP^+ cells at D20 was lower than that of $mCherry^+$ cells in most cases (Fig. 2E, F). The infiltration of NALM-6-GFP cell remained high in this scenario and was even higher than that of $mCherry^+$ cells (Fig. 2E, F). In sharp contrast to NALM-6-GFP cells, REH-GFP cells showed dramatic reduction in absolute infiltration (Fig. 2E, F), although both had similar infiltration capacity when xenografted alone.

To further reveal the pattern of the infiltrated cells, we performed whole mount immunofluorescence staining and imaging on the femurs of the mice inoculated with cell mixture including NALM-6-GFP or REH-GFP cells, which shown remarkable changes in infiltration. The result revealed high and comparable infiltration for NALM-6 and REH cells when xenografted alone (Fig. 2G), and this finding was consistent with the flow cytometry detection. The NALM-6 and REH cells with different labels filled the bone marrow indifferently (Fig. 2G). Also consistent with the flow cytometry detection, the area occupied by stained GFP^+ cells in the bone marrow varied greatly among the mice xenografted with the mixture of six cell lines including NALM-6-GFP and REH-GFP cells (Fig. 2G). In the bone marrow of mice xenografted with mixtures of six cell lines including NALM-6-GFP cells, the stained GFP^+ cells occupied most of the infiltrated area, and the other five mCherry+ cell lines together took a small area (Fig. 2G). Therefore, the NALM-6 cells largely maintained their pattern as xenografted alone. In the bone marrow of mice xenografted with the mixture of six cell lines including REH-GFP cells, the infiltration pattern of REH-GFP cells largely differed from that when xenografted alone. Most of the REH-GFP cells scattered at the edge of bone marrow or epiphysis and appeared to be squeezed by $mCherry^+$ cells (Fig. 2G). On the basis of the difference in the number and the distribution of infiltrated cells, the interaction of different leukemic cells influences the infiltration of involved cells. These

results also suggested that NALM-6 cells possess advantages in the interaction with the other leukemic cell lines involved in this study.

NALM-6 cells strongly impact other cells *in vivo*

To confirm the impact of NALM-6 cells, we set up xenograft experiment with mixtures of NALM-6 cells and other cell lines (Fig. 3A). With the initial ratio of GFP^+ and $mCherry^+$ cells in each mixed contexts close to that of designed (Fig. S3), the result would reflect the impact of NALM-6 cells on other cells. As indicated by the results, the infiltration of NALM-6-mCherry cells far exceeded that of other cell lines in most cases, except for the mice injected with mixture of NALM-6-GFP and NALM-6-mCherry cells (Fig. 3B, C). Similar to the result of xenograft experimental with the mixture of six cell lines, REH-GFP cells shown dramatic reduction in infiltration when xenografted with NALM-6-mCherry cells (Fig. 3B, C). This result was in sharp contrast to its high infiltration when xenografted alone. For other cell lines (HL-60-GFP, Jurkat-GFP, and OCI-AML-3-GFP), infiltration was also greatly affected compared with that in xenografts alone (Fig. 3B, C), but the absolute reduction was far less that of REH-GFP cells. The results of whole mount immunofluorescence staining and imaging of the femurs of these mice were consistent with corresponding flow cytometric detections. In the bone marrow of mice xenografted with NALM-6-mCherry and NALM-6-GFP cells, NALM-6 cells with different labels filled the bone marrow (Fig. 3D). By contrast, NALM-6-mCherry cells occupied the most space, and REH-GFP cells occupied a limited space in the bone marrow of mice xenografted with mixture of REH-GFP and NALM-6-mCherry cells (Fig. 3D). The pattern of infiltration in mice xenografted with mixture of NALM-6-mCherry cells and REH-GFP cells was distinctive, with NALM-6-mCherry cells showing unlimited distribution and scattered REH cells largely confined to the edge and epiphysis bone marrow (Fig. 3D). On the basis of the above evidence, NALM-6 cells have an impact on the infiltration of other leukemic cells and thus could serve as the first key element of "compositional intervention" strategy.

Suicide system effectively controls NALM-6 cells *in vivo*

The second key element of "compositional intervention" is a system to control the first key element. Therefore, we plan to introduce a control system into NALM-6 cells. In theory, the most commonly used herpes simplex virus thymidine kinase/ganciclovir (HSVTK/GCV) suicide system in tumor gene therapy would match the requirement [52]. Many HSVTK variants with enhanced efficiency had been created, with SR39 being one of the best [53]. Therefore, we cloned SR39 into a lentiviral vector and under the control of EF-1α promoter (Fig. S4A), and the cell killing effect of SR39/GCV system was confirmed *in vitro* (Fig. S4B). We then established NALM-6 cell stable transformants of SR39 (Fig. S4C, D). To test the effectiveness of SR39/GCV system on NALM-6 cells *in vivo*, we xenografted NALM-6-SR39-mCherry cells into immunodeficient mice (Fig. 4A). At D20, symptoms were observed in the PBS-treated mice but not in the GCV-treated mice (data not shown), and the infiltration of NALM-6-SR39-mCherry cells in the GCV-treated mice was significantly lower than that in the control mice (Fig. 4B, C). These results indicated the capability of SR39/GCV system in controlling NALM-6 cells *in vivo*.

After confirming the effectiveness of the HSVTK-SR39/GCV system, we determined whether the introduction of SR39 could attenuate the impact of NALM-6 cells. Given that the REH-GFP cells showed the greatest reduction in infiltration when xenografted together with NALM-6-mCherry cells, we mixed and xenografted equal numbers of NALM-6-SR39-mCherry cells and REH-GFP cells (Fig. 4D). With comparable initial number of NALM-6-SR39-mCherry cells and REH-GFP cells, the infiltration of the former was extremely higher than that of the later at D20 (Fig. 4E, F). The results of

whole mount immunofluorescence staining and imaging of the femurs were consistent with the flow cytometric detection. NALM-6-SR39-mCherry cells filled most of the bone marrow, and scattered REH-GFP cells largely confined to epiphysis and the edge of the bone marrow (Fig. 4G). These results were consistent with those in the xenograft experiment of REH-GFP cells and NALM-6-mCherry cells, indicating that the introduction of SR39 does not attenuate the impact of NALM-6 cells, at least for REH cells. On the basis of this evidence, the SR39/GCV system could serve as the second key element of the "compositional intervention" strategy.

Effectiveness of "compositional intervention"

With NALM-6 cells as the first key element and HSVTK-SR39/GCV suicide system serve as the second key element, we preliminarily established the "compositional intervention" strategy. Prior to the evaluation of the effectiveness of this strategy, we evaluated the survival time of mice xenografted with NALM-6-SR39-mCherry cells and REH-GFP cells alone and in the mice xenografted with their mixture (Fig. 5A). The survival time of the mice xenografted with REH-GFP cells was significantly longer than that of the mice xenografted with NALM-6-SR39-mCherry cells alone and the mixture of NALM-6-SR39-mCherry cells and REH-GFP cells; however, the difference was small at only about 2 days at the median level (Fig. 5B). The survival time of the mice xenografted with mixture of NALM-6-SR39-mCherry cells and REH-GFP cells did not significantly differ from that of the mice xenografted with NALM-6-SR39-mCherry cells alone (Fig. 5B). Therefore, the NALM-6 cells mainly directed the survival of mice xenografted with mixture of NALM-6-SR39-mCherry and REH-GFP cells. This finding implied that the survival time of the mice could be prolonged by controlling the NALM-6 cells. We then evaluated the effectiveness of "compositional intervention" upon the administration of GCV (Fig. 5C). As indicated, GCV administration significantly ($P<0.0001$) prolong the survival time of the mice xenografted with the mixture of NALM-6-SR39-mCherry and REH-GFP cells by about 7 days at the median level (Fig. 5D). Upon the administration of GCV, the survival time of the mice xenografted with the mixture of NALM-6-SR39-mCherry and REH-GFP cells significantly exceeded that of mice xenografted with REH-GFP cells alone ($P < 0.0001$) (Fig. 5D). Although the above evidence preliminarily indicated the effectiveness, we further explored whether improved results could be achieved via the schematic change of GCV administration (Fig. 5E). When all mice were xenografted with the mixture of NALM-6-SR39-mCherry and REH-GFP cells, the mice treated with inconsecutive GCV showed significantly longer ($P = 0.0004$) survival time than the mice treated with consecutive GCV (Fig. 5F). In particular, the maximum survival time was extended from 30 days to 34 days (Fig. 5D, F). With these findings, we are confident with the effectiveness of the strategy. However, it still does not conform to the clinical context, in which therapeutics always fall behind the disease. Therefore, we tested whether the therapeutic effect could still be achieved even when the introduction of NALM-6-SR39-mCherry cells lags behind the injection of REH-GFP cells (Fig. 5G). Although the introduction of NALM-6-SR39-mCherry cells was 2 days behind the injection of REH-GFP cells, the survival time of these mice was significantly ($P = 0.0055$) longer than that of the mice injected with REH-GFP cells alone upon GCV administration (Fig. 5H). Moreover, the maximum survival was 33 days, which was not hugely different from the maximum survival of 34 days achieved in the previous experiments (Fig. 5F, H). These results indicated that "compositional intervention" is still effective in context that resembling actual clinical application.

Discussion

Here, we proposed a novel strategy that delays tumor progression via the introduction of engineered impactive cells. The strategy comprises two key elements: a population of impactive cells and a control system. We applied the proposed method in leukemic mouse model. In this study, the impactive cells and the cells being intervened were selected from different origins due to several considerations. First, the most straight and effective way to screen for impactive cells is to compare the impact of different clones in vivo; the use of different cell lines can greatly accelerate the screening. If the screening is conducted in single cell line, then clones ought to be effectively distinguished at first. As a result, a large number of clones must be isolated to compare their impact in vivo and find a clone that influences most other clones. This process is extremely challenging. Alternatively, we mixed six cell lines together to create a "reconstructed tumor" and take the individual cell line as "clone" of the "reconstructed tumor" to easily distinguish different "clones". Moreover, each "clone" is composed of a large number of "sub-clones", therefore, the impactive "clone" obtained could involve multiple "sub-clones", which greatly increases the possibility and enhances the operability of finding a cell population that is consistently impactive. By using this strategy, we found that cell population from the NALM-6 cell line consistently affected the infiltration of other cell lines. Still, given that the cell lines comprising the "reconstructed tumor" were all of human origin, the differences between them were mainly the manifestation of differences in gene expression. Therefore, the interaction between different "clones" of the "reconstructed tumor" did not essentially differ from that between intra-tumoral clones of human origin. Although the "reconstructed tumor" was artificially created in our study, the results derived from it successfully revealed the intensity and impact of tumor cell interaction, which is needed by the tumor containment strategy.

"Compositional intervention" utilizes interaction between tumor cell populations; however, the exact identity of the interaction that is effective remains unknown. Given that a tumor is a complex ecosystem, the interaction between tumor cell populations is complicated and similar to that between species in a natural ecosystem; this similarity may be one of the reasons why many tumor containment strategies are implemented according to mathematical models [38,49]. The interactions in ecosystem exists in three broad categories: positive, negative, and neutral. The interaction most frequently reported in tumor containment studies is competition, which is a negative interaction. The goal of tumor containment is to achieve maximum delay of progression rather than eradicate, interactions beside competition could also play a role in tumor containment. Therefore, the introduced cell of "compositional intervention" should be impactive instead of being only competitive, and any type of interaction that has an effect on delaying tumor progression could be utilized.

In our opinion, "compositional intervention" has some peculiar advantages over conventional therapeutic strategies. As the infinite uncertainty of tumor heterogeneity, cancer therapy ought to be as dynamic as the tumor being treated, which is almost impossible for conventional therapies [44]. The therapeutic effect of "compositional intervention" is provided by impactive tumor cells, which can be as inherently dynamic as the tumor being treated. Interestingly, regardless of the how dynamic are the impactive tumor cells, they are supposed to be tightly constrained by the control system, thus making "compositional intervention" a combination of uncertainty and certainty. In addition, the effector cell is introduced from outside the body, which creates room for extensive engineering before being introduced into the body, thus making "compositional intervention" highly optimizable. To our knowledge, this strategy could

be enhanced in at least two directions: the first is to enhance the impact of the introduced cells, and the other is to optimize the control system. With ever evolving bio-technology, this task will not be challenging, even the use of nontumor cells with great impact and good control that match the requirements of is imaginable. Our ongoing work preliminarily indicated that the impact of NALM-6 cells can be enhanced by the over expression of certain gene (data not shown). In addition, "compositional intervention" achieves therapeutic effect by the impact of the introduced cells, this feature is apparently independent of the exact target of the tumor being treated. Therefore, the therapeutic effect of "compositional intervention" is potentially not seriously attenuated by the heterogeneity of the tumor being treated and supposed to not suffer from resistance encountered by conventional therapeutics. Finally, the effect of "compositional intervention" is achieved by the impactive cells introduced from outside the body in a non-killing way. This pathway is logically different from conventional therapeutics and other containment strategies, making "compositional intervention" highly compatible with strategies of other modal. In our opinion, combining with tumor therapeutics of other modal to achieve good outcomes is more reasonable for the implementation of "compositional intervention" than conducted alone.

With the blood-borne nature of leukemia and the convenience in model setting and model evaluation, leukemia plays disproportional role in pioneering tumor research with its relatively low incidence[3]. These advantages also prompted us to use leukemia as the model system to realize our strategy. However, extending this strategy to other types of tumors is challenging because other tumors, especially solid tumors, do not disperse through blood as effective as leukemic cells. Another problem is the slow responsiveness of the HSVTK/GCV suicide system, which is only applicable as a demonstration of the principle of the control system. Several reasons can explain this finding. The NALM-6 cells are resistant to apoptosis because they need an extremely high dose of chemotherapy drugs to achieve visible effect [54]. Gap junction can augment the effectiveness of the HSVTK/GCV suicide system; however, leukemic cells lack this function [52]. Tumor containment leverages endogenous tumor cell interactions, and the drug administered is the same as that in conventional therapies. Although tumor containment has been proved to be superior to conventional treatments in certain contexts, patient acceptance remains difficult, the main reason is that tumor containment is inherently noncurative [50]. Instead of exploiting endogenous tumor cell interactions, our strategy utilizes the engineered impactive tumor cells introduced from outside the body. This condition intensifies the willingness to reject this approach as an option because it concerns raised about the safety of the introduced tumor cells, although it could guarantee the impact and controllability of the effector cell. Extensive efforts are further warranted to improve the effectiveness and the acceptability of this strategy in the future.

Using leukemia as the model system, we established a novel strategy that delays tumor progression via the introduction of engineered tumor cells, this extends the tumor containment strategy with guaranteed impact and controllability. This strategy is inherently dynamic, highly optimizable, and highly compatible to other therapeutics. Potentially, it won't be seriously attenuated by the heterogeneity of the tumor. With these features, this approach could be instructive to the development of novel tumor therapies.

Materials and Methods

Cell culture

In brief, 293T cells were cultured in DMEM (high glucose) basal medium supplemented with 10% FBS and 1% 100× penicillin-streptomycin at 37 °C with 5% CO_2 and passaged every 2 days. Leukemic cells (HL-60, Jurkat, NALM-6, OCI-AML-3, REH, and THP-1) were cultured in RPMI 1640 basal medium supplemented with 10% FBS and 1% 100× penicillin–streptomycin at 37°C with 5% CO_2 and passaged every 2 days.

Generation of stable transformants of cell lines

The lentiviral vector that expressing gene of interest was constructed using the ClonExpress II One Step Cloning Kit (Vazyme) following the manual, and the expression of gene of interest was driven by EF-1alpha promoter and coupled with reporter gene via the 2A sequence. Sequences were confirmed by sanger sequencing. After the mass preparation of endotoxin-free plasmids, lentivirus was produced and concentrated as previously described [55]. In brief, 3 mL of leukemic cells were inoculated into each well of a six-well plate at a density of 2×10^5 cells/mL to generate stable transformants. Afterward, 2–8 μL of concentrated virus was added to each well and expanded for two passages. The cells were then harvested, and positive cells were sorted on a flow cytometer (Beckman Coulter, MoFlo Astrios EQ) under the purity mode.

Mouse and xenograft

All mice involved were **NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju** and maintained under sterile conditions at the animal facility in accordance with local regulations. All mice involved were maintained under sterile conditions at the animal facility in accordance with local regulations. All animal experiments were approved by the Experimental Animal Ethical Committee at Shanghai Jiao Tong University School of Medicine, China and performed in accordance with the "Animal Research: Reporting of In Vivo Experiments" guidelines of the National Center for the Replacement, Refinement, and Reduction of Animals in Research. Leukemic cells were harvested, counted, and resuspended to desired concentration with PBS in accordance with the experimental design. Finally, 250 μL of cell suspension was injected into each mouse using an insulin syringe through the tail vein. For experiments that require cell mixing, the cells were mixed according to the experimental design after counting, then centrifuged for a second time, and resuspended to the desired concentration with PBS. A 250 μL volume of cell suspension was injected into each mouse using an insulin syringe through the tail vein. The mice were sacrificed following the experimental designs, and the tibia, femur, and ilium of each mouse were dissected and cleaned of excess tissues. For the detection of infiltration, the tibia and ilium were first fractured, and the cells in the bone marrow were flushed out with PBS. The suspension was then filtered with a 70 μm filter, erythrocytes were eliminated, and a cell suspension was finally obtained and subjected to flow cytometry (Beckman Coulter, Cytoflex-S).

Drug administration

Ganciclovir sodium (MedChemExpress) powder was first dissolved in PBS to prepare the storage solution (50 mg/mL), and stored at −80 °C. The storage solution (50 mg/mL) was diluted into working solution (5 mg/mL) with PBS before the administration, and a dose of 10 mL drug per kilo gram body weight were intraperitoneally injected into each mouse using an insulin syringe according to the experimental design. For the mice that only receive PBS, 10 mL of PBS per kilo gram body weight was administered via intraperitoneal injection using an insulin syringe.

Whole mount immunofluorescence staining, imaging, and image processing

Stain reagent was prepared using the following: blocking buffer (PBS added with 1% BSA, 2% FBS, 0.2% Triton X-100), Chicken anti-GFP (Abcam), Mouse anti-mCherry (Biolegend), Goat Anti-Mouse 633 (Life Technology Corporation), Goat Anti-Chicken 488 (ThermoFisher Scientific), and DAPI (Selleck). After the mice were sacrificed, the femurs were dissected and removed of excess tissues. The femurs were then fixed with 4% paraformaldehyde at 4 °C for 7 h, then treated with 20% sucrose at 4 °C for 1 day, and embedded with OCT (SAKULA). After complete curing at −20 °C, the embedded tissue was cut with a frozen slicer (LEICA, CM1950) to expose the bone marrow and then equilibrated at room temperature for 30 min. Residual OCT was gently washed with PBS on a horizontal shaker. Staining was performed at 4 °C in a 600 μL tube. For each femur, 500 μL of block solution containing primary antibody (dilution rate equals 1∶500) was added, and excess antibody was washed with PBS after immersion for 3 days. Afterward, 500 μL of block solution containing DAPI (final concentration 1μg/mL) and fluorescent conjugating secondary antibody (dilution rate equals 1∶500) was added to each femur and immersed for another 2 days. excess antibody was then washed with PBS. The stained femurs were either immediately subjected to imaging or temporarily stored in PBS at 4 °C. Imaging was conducted on a laser confocal microscope (Nikon, A1R-SI) using 405, 488, and 633 nm lasers, and panoramic fluorescence images of each femur were captured under a 20× objective lens with confocal mode. FIJI [56], an open-source software for scientific image processing, was used to process all the fluorescent images on a DELL Precision M6800 mobile workstation equipped with Core-i7-4910M, 32 GB RAM, 2 TB ROM, and NVIDIA Quadro K5100M graphic card.

Statistical analysis and plotting

For all data presented by bar plot, statistical inference was conducted with t-test under the R_3.53 environment when needed [57]. All bar plots were generated using ggplot2 [58]. For survival analysis, statistical inference was conducted with Log-rank (Mantel-Cox) test in GraphPad Prism 7.04, and the survival curve was also generated using GraphPad Prism 7.04. In all statistical inferences, "#" represents a p-value larger than 0.05, "*" represents a p-value range from 0.01 to 0.05, "**" represents a p-value range from 0.001 to 0.01, "***" represents a p-value range from 0.0001 to 0.001, and "****" represents a p-value less than 0.0001.

Data and materials availability

All data are available in the main text or the supplementary materials.

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Competing interests:

Authors declare that they have no competing interests.

Figures and Tables

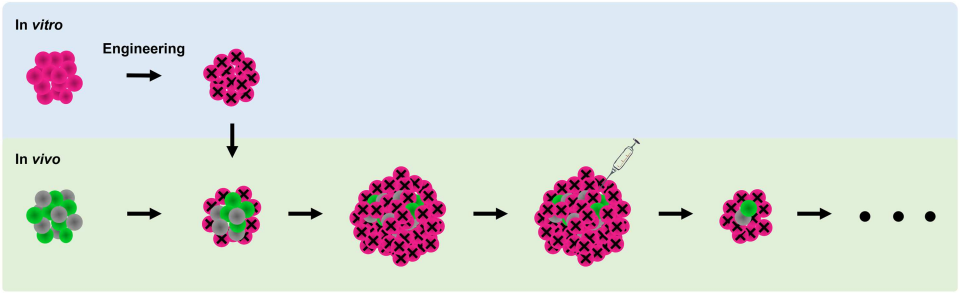

Fig. 1. Delay tumor progress with controllable impactive tumor cells. Schematic diagram showing the compositional intervention strategy, the globules represent tumor cell populations, tumor cells are engineered to be controllable *in vitro* before being introduced. The introduced cell has strong impaction on tumor cells *in vivo*, and therapeutic benefit is achieved via the control of the introduced cells.

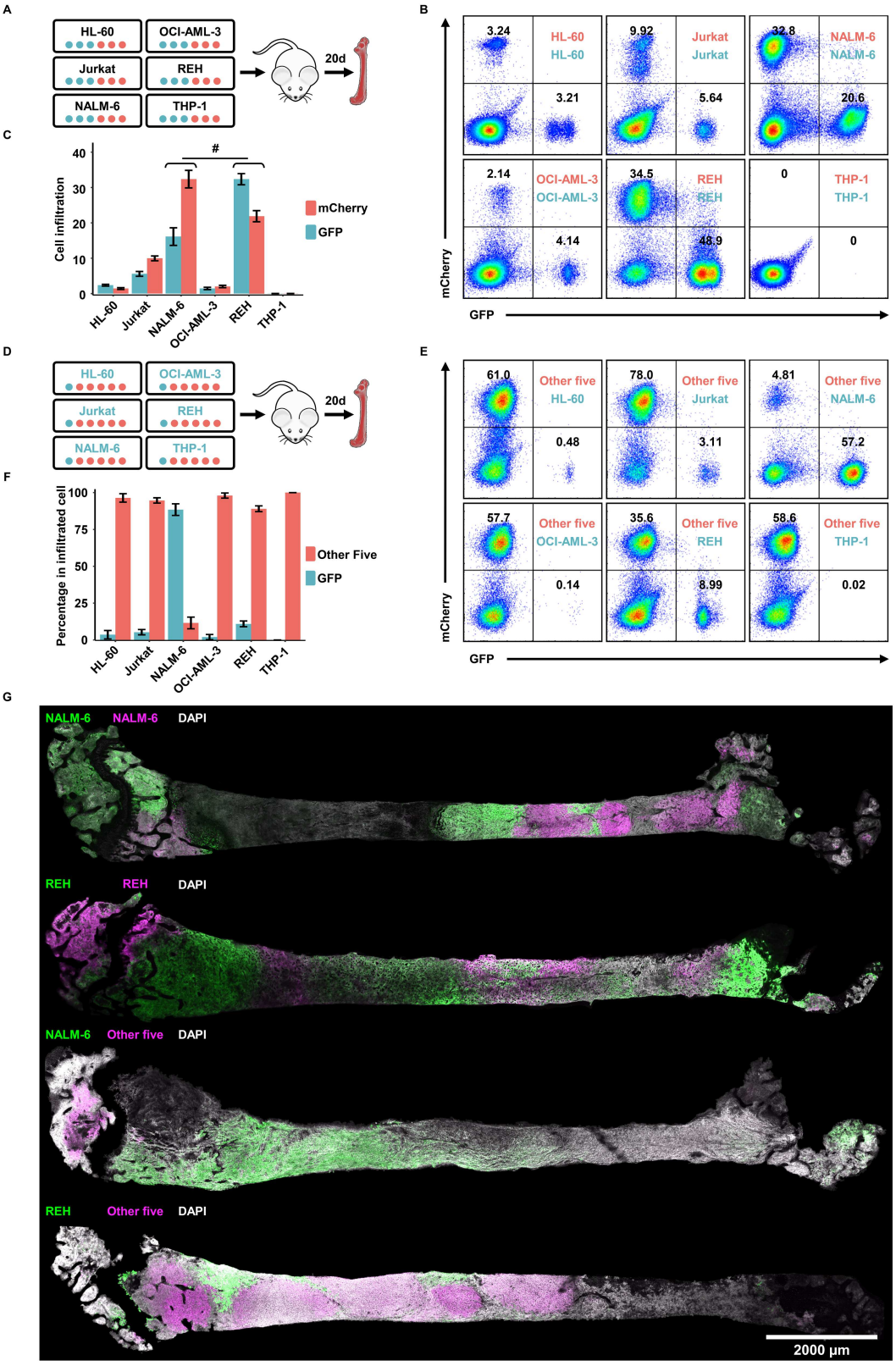

Fig. 2. Interaction of leukemic cells impaction cell infiltration *in vivo*. (**A**), Schematic diagram showing the design to evaluate the capability of infiltration of 6 leukemic cell lines, red and turquoise represents mCherry+ and GFP+ cells respectively. (**B**), Representative flow cytometric graphs showing the composition of infiltrated cells in mice of **A** at D20. (**C**), Bar plot showing the summary of the composition of infiltrated cells in mice of **A** at D20, n=3. (**D**), Schematic diagram showing the design

to evaluate the impaction of interaction on the infiltration of 6 leukemic cell lines, red and turquoise represents mCherry+ and GFP+ cells respectively, the number of all cell lines is equal in all contexts. (**E**), Representative flow cytometric graphs showing the composition of infiltrated cells in mice of **D** at D20. (**F**), Bar plot showing the summary of the composition of infiltrated cells in mice of **D** at D20, n=3. (**G**), Whole mount immunofluorescence imaging of femur of mice in **A** and **D** showing the distribution of infiltrated leukemic cells, magenta and green represents mCherry+ and GFP+ cells respectively, scale bar is 2000 μm.

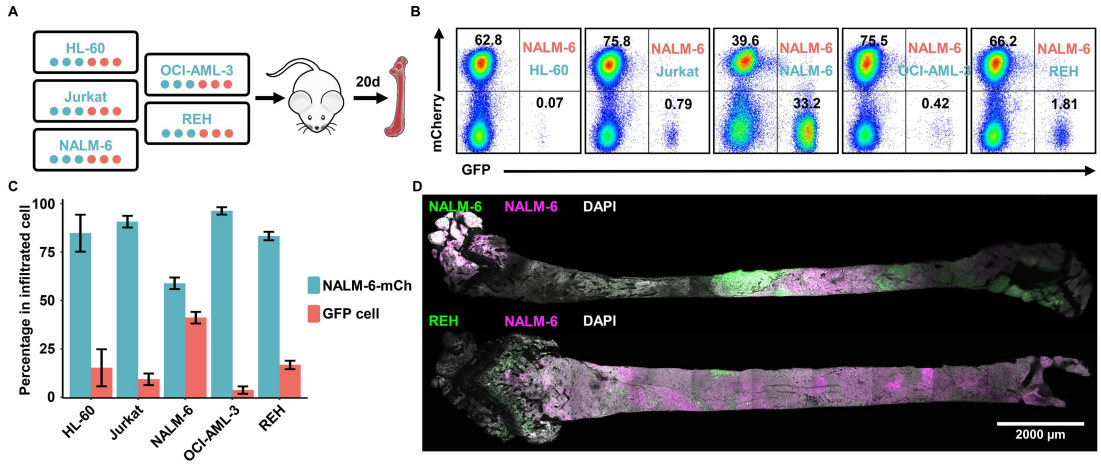

Fig. 3. NALM-6 cells impact the infiltration of other leukemic cells *in vivo*. (**A**), Schematic diagram showing the design to evaluate the impaction of NALM-6 cells on other leukemic cell lines, red and turquoise represents mCherry+ NALM-6 cells and other GFP+ cells respectively, the number of all cell lines is equal in all contexts. (**B**), Representative flow cytometric graphs showing the composition of infiltrated cells in mice of A at D20. (**C**), Bar plot showing the summary of the composition of infiltrated cells in mice of **A** at D20, n=3. (**D**), Whole mount immunofluorescence imaging of femur of mice in **A** showing the distribution of infiltrated leukemic cells, magenta and green represents mCherry+ and GFP+ cells respectively, scale bar is 2000 μm.

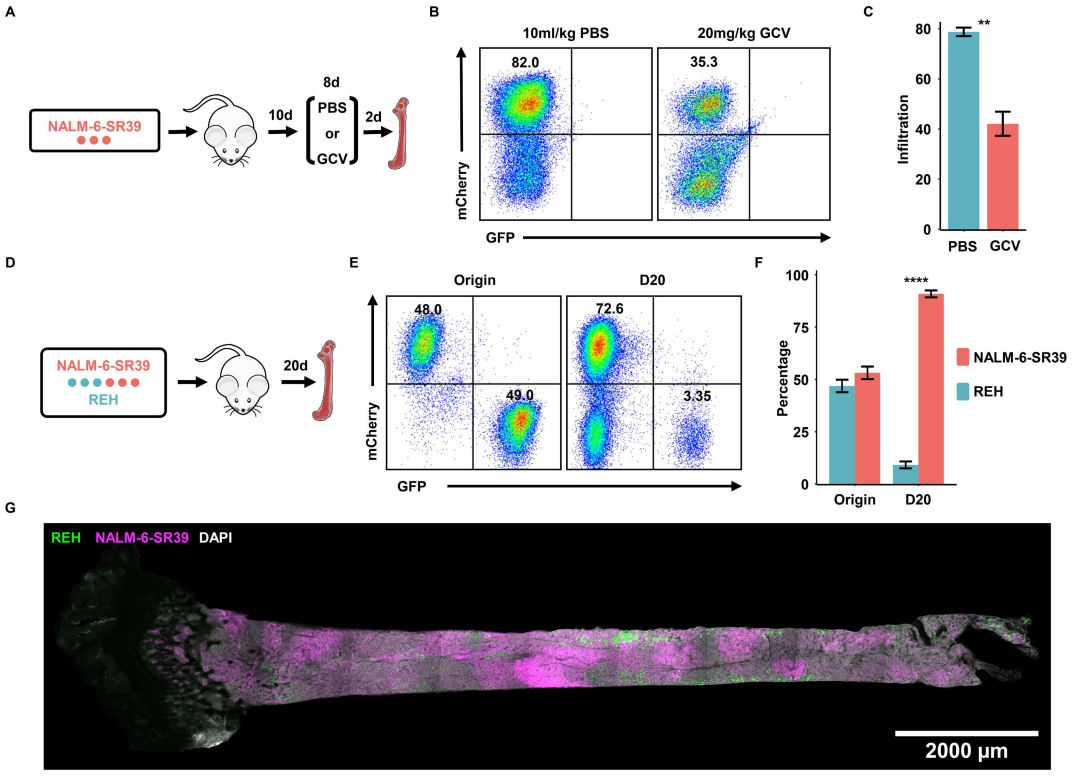

Fig. 4. Suicide system effectively control NALM-6 cells *in vivo*. (**A**), Schematic diagram showing the design to evaluate the cell killing effect of HSV-TK-SR39/GCV system *in vivo*. (**B**), Representative flow cytometric graphs showing the infiltration of NALM-6-SR39-mCherry cells of mice in **A** at D20. (**C**), Bar plot showing the summary of the infiltration of NALM-6-SR39-mCherry cells of mice in **A** at D20, n=3. (**D**), Schematic diagram showing the design to evaluate the impaction of NALM-6-SR39-mCherry on REH-GFP cells *in vivo*, red and turquoise represents NALM-6-SR39-mCherry and REH-GFP cells respectively. (**E**), Representative flow cytometric graphs showing the original composition of cell mixtures in **D** and the composition of infiltrated cells in mice of **D** at D20. (**F**), Bar plot showing the summary of the original composition of cell mixtures in **D** and the composition of infiltrated cells in mice of **D** at D20, n=3. (**G**), Whole mount immunofluorescence imaging of the femur of mice in **D** at D20 showing the distribution of infiltrated leukemic cells, magenta and green represents NALM-6-SR39-mCherry and REH-GFP cells respectively, scale bar is 2000 μm.

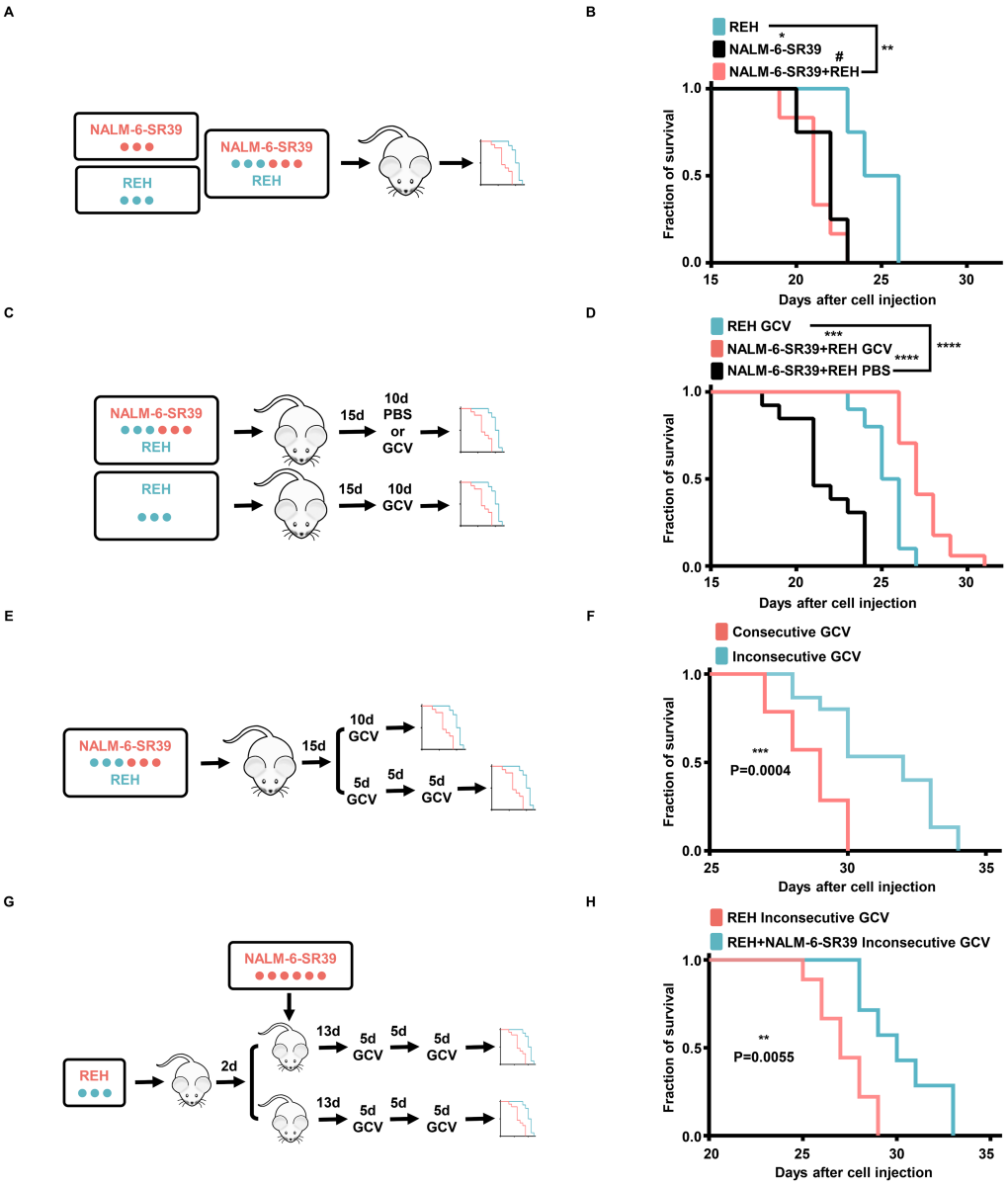

Fig. 5. Compositional intervention effectively elongates the survival of mice. (**A**), Schematic diagram showing the design to evaluate the survival time of mice inoculated with NALM-6-SR39-mCherry and REH-GFP cell alone or mixture of them, red and turquoise represents NALM-6-SR39-mCherry cells and REH-GFP cells respectively. (**B**), Survival curve of mice in **A**. (**C**), Schematic diagram showing the design to evaluate the effectiveness of GCV on the survival of mice inoculated with mixture of NALM-6-SR39-mCherry and REH-GFP cells, red and turquoise represents NALM-6-SR39-mCherry cells and REH-GFP cells respectively. (**D**), Survival curve of the mice in **C** upon different treatment. (**E**), Schematic diagram showing the design to evaluate the impaction of regimen modification of GCV administration on the survival of mice inoculated with mixture of NALM-6-SR39-mCherry and REH-GFP cells, red and turquoise represents NALM-6-SR39-mCherry cells and REH-GFP cells respectively. (**F**), Survival curve of the mice in **E** upon consecutive or inconsecutive GCV administration. (**G**), Schematic diagram showing the design to evaluate the effectiveness of delayed "compositional intervention", red and turquoise represents NALM-6-SR39-mCherry cells and REH-GFP cells respectively. (**H**), Survival curve of the mice in **G** upon inconsecutive GCV administration.

Supplementary Files

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