**Title: Enhanced anti-cancer activity of microglia by AAV2-mediated IL-12 in the therapy of glioblastoma multifrome**

Running title: GBM therapy through AAV2/IL-12 activated microglia

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**Abstract:**

Microglia has been found to diversify its function by cancerous cells or in a cancerous environment, thereby contributing to cancer growth and metastasis. Its immuno-activity, however, can be modulated by interleukin-12. So a strategy was designed using AAV2 carrying IL-12 to activate microglia then to eliminate cancerous cells. The transduction efficacy of AAV was evaluated with AAV2 encoding GFP and IL-12 on cancerous and CNS cells. The bioactivity of microglia modulated by IL-12 was examined and death receptors 4 and 5 were detected on cancerous cells. The effects of IL-12 and AAV2/IL-12 on microglial cytoxoxicity were evaluated too. The results demonstrated human cell line DBTRG, surgical specimen of GBM, and rat astrocyte expressed GFP quite well. Tremendous IL-12 secretion was detected in DBTRG, RG2, and astrocyte after transfection of AAV2/IL-12. TRAIL releasing and phagocytotic activity of microglia were significant, increasing ($p<0.05$) after the stimulation of IL-12. DR4 and DR5 were expressed in all of the examined GBM cells. MTT assay of microglial cytotoxicity elicited significant increase ($p<0.05$) when the IL-12 protein or RG2-secreting IL-12 could have contact with microglial cells. Conclusively, AAV2 is an effective vector in transferring therapeutic genes such as IL-12 to induce or enhance microglial anti-cancer activity.

**Introduction:**

**Interleukin-12**

Among the results of recent anti-cancer research, immuno-modulation with cytokines is one of the modalities with the most potential.[1-4] Many pro-inflammatory cytokines have been investigated and clarified for their anti-cancer effects and mechanisms. Among these, interleukin-12 (IL-12) exhibits the most power in enhancing either innate or adaptive responses for anti-cancer activity. In the cascade of IL-12 activity, the antigen presenting cells, such as dendritic cells, macrophage, and neutrophile, produce and secrete IL-12 after encountering the invading pathogen or transformed cells. Subsequently, IL-12 induces proliferation of hematopoietic progenitor cells and generation of IL-3 and stem-cell factors, along with stimulating a pre-activated natural killer cell, natural killer T cell, and cytotoxic T cell to increase in number and cytotoxicity and to secrete interferon-γ, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor and IL-8. In addition, IL-12 may facilitate the $T_H1$ pathway to promote the naïve T cells to effectiveness and becoming memory $T_H1$ cells.[5] In anti-cancer activity, IL-12 has been demonstrated to stimulate NK cells or T cells to enhance anti-angiogenesis through secreting interferon-γ, to induce tumor cytotoxicity by secreting the family of tumor necrosis factor (TNF), and to promote tumor antigen-specific adaptive immunity through the $T_H1$ pathway.[6]

Nevertheless, the adverse effect of systemic administration of pro-inflammatory cytokines shown in the human clinical trail of interleukin-12 protein was found to be extremely harmful.[7] Furthermore, from the experience of clinical practice, malignant brain tumors always recur or relapse within a short period after the complete initial treatment, even in the imaging curative cases.[8-10] Therefore, a superior method should be designed as a long term expression of cytokines and local tissue affection for the purpose of maximal therapeutic effectiveness and restricted systemic toxicity.

**Adeno-associated virus**

For the purpose of long term expression and local delivery of cytokine, an effective vehicle to transfer the cytokine into the focal tissue is superior to direct protein injection or naked DNA implantation. In the choice of a mediating vector, acuminating evidence has illuminated the feasibility of adeno-associated virus (AAV) in genomic transportation.[11,12] AAV is of the parvovirus family and is characterized by being deficient in replication, non-pathogenic to humans, and able to transfect most cell types.[13] The genes mediated by AAV could be expressed up to 15 months and sustained in high levels during the $2^{nd}$ to $4^{th}$ months when inoculated in the mouse brain.[14] Its competence and safety have been elucidated in several clinical human trials from early 1996, where cystic fibrosis was converted with AAV2 transferring the cystic fibrosis transmembrane conductance gene into lung tissue, to 2007, where

advanced Parkinson's disease was treated with AAV2 transferring the glutamic acid decarboxylase gene into the subthalamic nucleus.[15,16]

**Microglia**

Microglia - the central nervous system (CNS) immune cells - comprise 2-20% of the population of CNS cells, which execute the defense function against invading microbes and execute the clearance of transformed cells.[17,18] Prospectively, in a tumor environment, microglial cells infiltrate the tumor considerably more than other peripheral immune cells, which have been estimated to be up to 10-34% of the composition of tumor mass.[19] Microglial cells represent an antigen presenting function in CNS through major histocompatibility complex type 2 and present phagocytotic ability. Furthermore, they generate cytokines, including IL-1α, IL-β, IL-6, IL-12, IL-16, IL-23, TNF-α, and transforming growth factor and chemokines, including MCP-1, MIP-1, IL-8, MIG, and IP-10.[20,21] Nevertheless, the immune performance of microglia in the tumor environment was diversely and continuously affected by the tumor-released factors, such as immunosuppressive cytokines, that potentiated to loss or reduction in their antigen presenting activity and innate immune response.[22,23] Furthermore, the extracellular matrix protease released by microglia which infiltrated the tumor and was changed in behavior by the tumor-released factor was postulated to promote the invasion and migration of tumor cells.[24] Therefore,

readjusting or reinforcing the native immune activity of tumor-infiltrating microglial cells will achieve better anti-cancer activity than the peripheral immune cells by disrupting the phenomenon of tumor-promoted effects of microglial cells and restoring the anti-tumor ability of the abundant tumor-infiltrating microglial cells. In this study, we constructed AAV type 2 (AAV2) encoding IL-12 to survey the expressive efficacy of astrocyte, microglial cell and glioblastoma multiforme (GBM) cell and to judge the feasibility of AAV2 in malignant brain tumor therapy. Further, owing to the high composition of microglia in tumor mass, we evaluated the anti-tumor effect of microglia pre- and post-stimulation with IL-12 on account on its secretion of interferon-gamma (IFN-γ), nitric oxide, tumor necrosis factor-alpha (TNF-α), and TNF related apoptosis inducing ligands (TRAIL) and also its phagocytotic activity.

**Materials and methods:**

**Construction of AAV2 encoding IL-12**

The full length cDNA of IL-12 (InvivoGen) was amplified using polymerase chain reaction and subcloned into pAAV-MCS (Stratagene), then verified by DNA sequencing (Protec). The production of AAV2 encoding IL-12 included 5 main steps - transformation of E-coli, plasmid extraction, culture of HEK 293 cells, package of AAV2 in 293 cells and AAV2 purification. In brief, E-coli (ECOS 101) cells were used as competent cells and transformed by pAAV-RC (Stratagene), pHelper (Stratagene), or pAAV-GFP, which were cultured with 2YT –Broth A50 (Invitrogen) medium containing ampicillin (50μg/ml) at 37℃. The transformed E-coli were resuspended in 2YT-Broth medium A50 and prepared for extraction of the three plasmids. The plasmid DNAs were purified with a plasmid Mega preparation kit (Qiagen). Each of the extracted plasmids was quantified with OD260/280. The frozen HEK 293 cells were thawed under 37℃ and cultured with DMEM (Cellgro), 10% FBS (Hyclone), and 1% penicillin/streptomycin (Gibco). The extracted plasmids, pAAV-RC, pAAV-IL-12, and double the pHelper, were mixed and shaken with $CaCl_2$. The mixture was spread into HEK 293 cell culture dish and incubated with DMEM, 10% FBS, and 1% P/S at 37℃ for AAV-GFP package. The packaged AAVs were purified through heparin column (Heparin Actigel Sterogen).

**Transduction of rAAV2 on targeted cells**

Five cell types – Human GBM cell line DBTRG, rat GBM cell line RG2, primary culture of rat microglia and rat astrocyte, and primary culture of human GMB cells – were surveyed for rAAV2 transduction efficacy. The RG2 and BV2 were purchased from FIRDI Taiwan, astrocytes were extracted from the embryonic brain of rat, and human GBM cells were cultured from a surgical specimen proven by a pathologist. $1\times10^5$ cells of DBTRG were spread into each well of 6-well plate and cultured with 10% FBS, DMEM (Gibco 11995), and Penicillin-Streptomycin sulfate (Gibco 15140-122) for 24 hours. 100µl AAV2-GFP ($4.28\times10^{10}$ particle/ml) was added in and inspected daily under florescent microscope to the fourth day. The expression of GFP was evaluated and pictured at 24 hours and at four days after the addition of rAAV2. The same procedure was practiced on the other cell types.

**ELISA assay of IL-12 expression**

Five cell types — rat GBM cell line RG2, primary culture rat astrocyte, microglia cell line BV2, human GBM cell line DBTRG and HEK 293 — were transfected by AAV2/IL-12 and were evaluated for IL-12 expression. $1\times10^5$ cells of each cell type were transferred to 6 wells of a 96-well plate and cultured with 10% FBS, DMEM, and Penicillin-Streptomycin sulfate for 24 hours. AAV2-mIL-12 in three concentrations — $1\times10^{10}$, $1\times10^{11}$, and $1\times10^{12}$ particle/ml — were added into

cultured cells with one concentration to two wells and were incubated for 4 days. The supernatant of each well was aspirated and span, then the upper clear fluid was obtained for estimating the mIL-12 concentration by ELISA Kit (M1270 Quantikine). The same procedures were performed with AAV2-GFP instead of AAV2-mIL-12 for negative control.

**ELISA assay of microglia-secreted cytokines**

Mouse IL-12 protein (mIL-12) was purchased from eBioscience and stored at 4 ℃. $5x10^4$ BV2 cells were transferred to each well of a 96-well microplate then cultured with 2% FBS for 24 hours. The experiment was divided into three groups – non-stimulated, IL-12-stimulated, and LPS-stimulated. Therefore, 100ng mIL-12 and LPS were added into 18 wells individually to active the BV2 cells. After 24, 48, and 72 hours of stimulation, the supernatant was aspirated and collected for detection of IFN-γ. The same procedures were repeated for detection of TNF-αand TNF-related apoptosis inducing ligand (TRAIL). The amount of IFN-γ and TNF-α were estimated with ELISA kit (Quantikine, R&D) following the product introduction. The TRAIL was detected with rat anti-mouse TRAIL/TNFSF10 monoclonal antibody as the primary antibody and biotinylated anti-rat IgG antibody as the secondary antibody (R&D systems, Inc.).

**Western blot assay of TRAIL receptor**

A total of 6 human cancer cell types — GBM cell lines DBTRG, G5T-VGH, GBM 8401, GBM 8901, along with pathologically verified GBM specimen No. 3 and No. 4 — were evaluated by SDS-PAGE for death receptors 4 (DR4) and 5 (DR5). The protein was extracted with lysis buffer PRO-PREP (Asia life Science). Before that, the tissue was ground with ceramic zirconium oxide beads. The protein concentration was determined by BCA assay read by ELISA reader at OD 590 nm. The protein was analyzed on 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes at 400 mA for 2.5 hr. Goat anti-human TRAIL R1 and R2 antibodies were used as primary antibody. Rat anti-goat IgG couple with horseradish peroxidase was used as secondary antibody and the activity of peroxide was detected with enhanced chemiluminescence.

**Phagocytosis of microglia**

BV2 cells were cultured in a 10cm culture dish with 10% FBS + DMEM. The phagocytotic analysis was done using a 96-well microplate with cover and fluorescence microplate reader (Vybrant, Phagocytosis Assay Kit). Briefly, $4x10^5$ BV2 cells were transferred into a well for a total of 18 wells and incubated with 10% FBS+DMEM. Six wells without BV2 cells were added in 150 µL of DMEM as negative control. 18 wells were equally divided into IL-12-treated, LPS-treated and

non-treated three groups. The stimulating reagent 100ng IL-12 or 100ng LPS was added and the culture medium was changed to 2% FBS + DMEM. After 18 hrs of incubation, 100 µL of prepared E-coli fluorescent BioParticle suspension (Vybrant) was added into each well then incubated 2 hrs. 100 µL trypan blue was added in and incubated for one minute after removing the BioParticle loading suspension by vacuum aspiration. The fluorescence was read at ~480 nm excitation and ~520 nm emission. The phagocytotic efficacy was estimated with the formula of "% Effect = Net Experimental Reading/Net Positive Reading x 100%".

**MTT assay of microglial cytotoxicity on RG2 cells**

MTT solution (5mg/ml) was diluted with PBS in a ratio of 1:9 and stored at 2-6 ℃ for further utility. Two different culture modes were performed:  the co-culture mode using a 96-well plate and the trans-well culture mode using a trans-well 96-well plate. Four conditions were set in the co-culture mode, which included 2ml MTT medium only, 2ml MTT medium + $5x10^4$ RG2 cells, 2ml MTT medium + $5x10^4$ RG2 cells + $5x10^4$ BV2 cells, and 2ml MTT medium + $5x10^4$ RG2 + $5x10^4$ BV2 cells + 100ng IL-12. The same conditions were applied in the trans-well mode, however, RG2 cells were incubated on the bottom plate with 2 ml MTT medium and BV2 cells were incubated on the upper plate with 2ml MTT medium. The plates were incubated in a 37℃ incubator for 4 hours. After centrifugation of the plate at 2000 rpm for 5

minutes, the upper fluid of every well was removed and 1 ml DMSO was added. The plate was incubated for 5 minutes then transferred to the reader for measurement of absorbance at 590 nm.

**MTT assay of microglia on AAV2/IL-12-transfected RG2 cells**

The procedures were the same as above method except the experimental groups were changed to $5x10^4$ RG2 cells, $5x10^4$ AAV2/IL-12-transfected RG2 cells, $5x10^4$ RG2 + $5x10^4$ BV2 cells, $5x10^4$ AAV2/IL-12-transfected RG2 cells + $5x10^4$ BV2 cells in co-culture model and $5x10^4$ RG2 cells, $5x10^4$ AAV2/IL-12-transfected RG2 cells on the bottom plate and $5x10^4$ BV2 cells on the upper plate in the trans-well model.

**Statistical Analysis**

The data is depicted with mean value $\pm$ standard deviation. Student's t-test was applied to examine the difference when only two groups were compared. For comparison of multiple groups, one-way ANOVA with Bonferroni post test was applied. The difference was assumed to be significant when $p<0.05$.

**Results:**

**GFP expression of targeted cells**

All of the 5 targeted cells types could be transfected by AAV2/GFP and expressed GFP after 4 days incubation. In cancer cells, the expression of GFP of human cell line DBTRG and GBM cells from excised specimen were near 100%, however, the RG2 cells displayed a lower expression rate of near 20% (Fig. 1). In CNS cells, the rat astrocytes illustrated good expression of GFP as well as the DBTRG, but the rat microglial cells showed moderate expression between the DBTRG and RG2 (Fig. 1).

**Secretion of IL-12 of targeted cells**

The DBTRG cells exhibited the highest ability, even greater than HEK 293 in the secretion of IL-12 after transfection of AAV2/IL-12. The astrocytes and RG2 cells could express IL-12 in abundant amounts, too. Nevertheless, microglial cell line BV2 didn't express detectable IL-12 after four days of transduction. All of the five cell types that transfected with AAV/GFP did not secrete IL-12 (Tab. 1).

**Cytokines secreted from BV2 after IL-12 stimulation**

The BV2 didn't secrete detectable IFN-γ in either the non-stimulated (control group), the IL-12-stimulated, or the LPS-stimulated group. In contrast, the BV2 secreted substantial TNF-α, over the estimating scale, after stimulating by LPS. BV2,

however, secreted only minimal TNF-α(27.3±9.2pg/ml) after the stimulation of IL-12. The t-test disclosed no significant difference increasing of TNF-α in IL-12-stimulated group compared with the non-stimulated group, which had a level of 9.7±6.1 pg/ml (Fig. 2). The BV2 stimulated by IL-12 also secreted TRAIL, which was 827.2±162.6 pg/ml after 6 hrs stimulation, 755.2±46.4 pg/ml after 12 hrs stimulation , and 828.8± 111.3 after 24 hrs stimulation. Interestingly, the LPS didn't stimulate BV2 to secrete TRAIL as well as IL-12 as the levels were 640.0±20.3 pg/ml, 632.8±10.1 pg/ml, and 640.0±20.2 pg/ml after 6, 12, and 24 hrs stimulation. The ANOVA analysis elicited significant differences between the three groups, and the IL-12-stimulated group exhibited significant differences with the LPS-stimulated and non-stimulated groups in all three time points (Fig. 3).

**Expression of death receptor of tumor cells**

All of the 6 tested GBM cell types – DBTRG, G5T-VGH, GBM 8401, GBM 8901, GBM specimen No. 3, and GBM specimen No. 4 – expressed DR4 and DR5 by western blot assay (Fig. 4).

**Phagocytotic activity of BV2**

The Phagocytotic activity of BV2 was enhanced obviously by either IL-12 or LPS. The phagocytotic efficacy was 76.7±12.9% in IL-12-stimulated BV2 and 100.0± 7.6% in LPS-stimulated BV2. The ANOVA analysis elicited significant differences

between the three groups. The phagocytotic activity in BV2 of the IL-12-stimulated group exhibited significant differences with the non-stimulated groups (Fig. 5).

**Cytotoxicity of BV2 and IL-12-enhanced BV2 on RG2 cells**

The cytotoxicity of BV2 on the GBM cells RG2 was evaluated by MTT assay. The IL-12-stimulated BV2 cells exhibited significantly increasing cytotoxicity on RG2 cells either in co-culture or trans-well model. In contrast, the non-IL-12-stimulated BV2 cells exhibited no significantly increasing cytotoxicity on RG2 cells in both models (Fig. 6). In the secondary MTT assay of BV2 cytotoxicity, only the group of BV2 cells co-cultured with AAV2/IL-12-transfected RG2 cells displayed significantly increasing cytotoxicity. Differing from the experiment of IL-12 protein stimulation, the trans-well model of BV2 plus AAV2/IL-12 showed no cytotoxic activity of the BV2 cells. This phenomenon was interpreted as showing that BV2 cells could not be stimulated by the RG2-secreted IL-12 since BV2 cells were incubated on the upper plate. Furthermore, the survival of RG2 cells exhibited no difference after being transfected by AAV2/IL-12 as compared with the non-transfected cells (Fig. 7).

**Discussion:**

**AAV2 application on the therapy of malignant brain tumor**

AAV has been verified as a competent vehicle to transfer therapeutic genes into many cell types of animals and humans,[11,25] but its transductive efficiency still exhibits cell type-dependency. From the results of this experiment, the human GBM cells displayed remarkable AAV transduction to express GFP and IL-12, but the rat GBM cells disclosed weak transduction by AAV. In CNS cells, in contrast to rat astrocyte and rat microglia, which showed good transduction by AAV2, the cell line BV2 displayed very weak expression of IL-12 after being transfected by AAV2/IL-12. Therefore, it is important to inspect the transductive efficiency of AAV-targeted cells – cancer cells or tumor-infiltrated cells – if AAV is to be considered as a vector carrying therapeutic genes. The hypothesis for treatment of malignant brain tumor by local AAV2 delivery presumes to follow the sequential events from AAV2/IL-12 transfecting to cancer cells, to cancer cells expressing IL-12 to induce or enhance the cytotoxicity of tumor-infiltrated microglial cells and, further, to eliminate cancer cells. Under this strategy, the undesired side effects from excessive IL-12 will be reduced when IL-12-secreting cancer cells are destroyed by microglial cells – since the microglial cells don't secrete IL-12.

**Strategy of AAV2/IL-12 mediated immunotherapy**

Malignant brain tumor is still an incurable and fatal cancer despite the current therapeutic modalities and it usually relapses or recurs within 1-2 years.[8,10] The majority of this failure is highly related to the immune escape of cancer or the immune reduction of the host.[26,27] Therefore, fundamentally, to restore or to augment the innate or adaptive immune activity could overcome this situation of decline. Microglia, the resident immune cell, naturally represents antigen-presenting and innate immunity in CNS and has been found infiltrating malignant brain tumors. In acuminating evidence, however, their functions were converted from cancer-defense to cancer-contribution by cancerous cells or cancerous microenvironment.[24,28] Therefore, a therapeutic strategy for brain cancer can be to enhance or reform the anti-cancer tumor ability of microglia. In this study, a microglial cell was confirmed to secrete TRAIL and increased phagocytosis after stimulation of IL-12. In contrast with this, microglia did not express significant, IFN-γ(data not shown) and TNF-α after stimulation of IL-12.

**Anti-cancer mechanism of microglia modulated by IL-12**

TRAIL is a member of the TNF superfamily, referring to type II trans-membrane proteins, which triggers cell apoptosis through an extrinsic pathway.[29] Different from TNF-alpha, TRAIL is characterized by specific identity of cancerous cells by binding TRAIL death receptors of cancerous cells to initiate apoptosis and by binding decoy

receptors of normal cells to avoid death destiny.[30] Several immune cells, such as dendritic cells, monocyte, macrophage, NK cells, and cytotoxic T cells were demonstrated to secrete TRAIL in many organs after stimulation with IFN-γ and LPS.[31,32] Besides, microglia also showed its ability in secreting TRAIL after LPS stimulation.[33] In this study, we examined the influence of IL-12 on microglia secreting TRAIL using LPS as positive control. The results derived from three repeats, however, illustrated that TRAIL secreted from microglia could be promoted by IL-12 but not by LPS.

The TRAIL-induced apoptosis is highly depended on the TRAIL receptor on cancerous cells, so we interpreted the DR4 and DR5 in four human GBM cell lines and two excised GBM specimens. As expected, all the GBM cells expressed DR4 and DR5, which enhances the conviction of the feasibility of anti-cancer therapy by IL-12 stimulating microglia through the TRAIL pathway. Other than this, phagocytosis, one of the powerful defensive bioactivities of microglia, also was strongly induced by IL-12.

**Conclusion:**

AAV2 is an effective vehicle to transfer therapeutic genes in anti-GBM therapy. Among the defensive bioactivities of microglia, phagocytosis and the TRAIL death pathway were documented to be induced by AAV2-mediated IL-12. The cytotoxocity of microglia on GBM cells were confirmed to be significantly increasing via the stimulation of AAV2-mediated IL-12 through the TRAIL death pathway and phagocytosis. In summary, microglial cells can be modulated by AAV2-mediated IL-12 to increase anti-cancer activity and play an important role in the therapy of malignant brain tumors.

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**Legends:**

Fig. 1A. Photographs of GBM cell lines, surgical specimens of GBM, and CNS cells caught under microscope 4 days after being co-cultured with AAV2-GFP. The left photograph was pictured under a green fluorescent microscope and the right under phase contrast. A: DBTRG cells were inspected under a green fluorescent microscope, left; under phase contrast, right (high power field, x400). B: RG2 cells (low power field, x100) and C: Surgical specimen No.1 (high power field, x400). D: Microglial (low power field x100). E: Astrocytes (high power field x400).

Table 1: The secreting amount of IL-12 from BV2, astrocyte, HEK 293, RG2 and DBTRG were estimated 4 days after being co-cultured with AAV2/IL-12 in three different concentrations - $1x10^{10}$, $1x10^{11}$ and $1x10^{12}$ particle/ml. The data were the mean of three wells in the same concentrations. * IL-12 secretion from DBTRG was over the maximal datum (2000 pg/ml) of estimating scale in all three AAV2 concentrations.

Fig. 2: TNF-alpha secreted from BV2 after 24 hours stimulation of IL-12 protein. The ctrl. represented non-IL-12-stimulating group.

Fig. 3: TRAIL secreted from BV2 after stimulation of IL-12 and LPS at the time intervals of 6 hrs, 12 hrs, and 24 hrs. The ctrl represented non-stimulating group. * $p<0.05$, significant difference of IL-12-stimulated group compared with LPS and ctrl
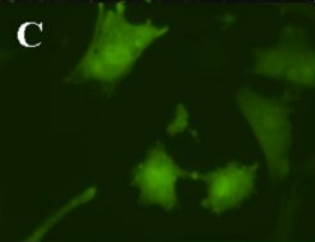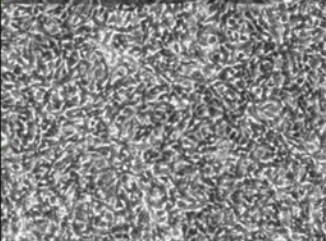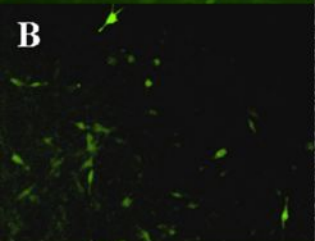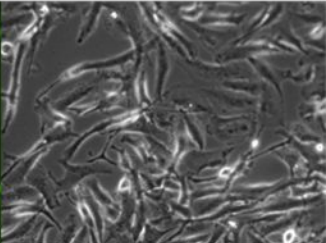
at different time interval by ANOVA analysis.

Fig. 4: The SDS-PAGE of DR4 and DR5 which were extracted from human GBM cell lines DBTRG, G5T-VGH, GBM8401, GBM9801, along with primary culture GBM cells No. 3 and No. 4.
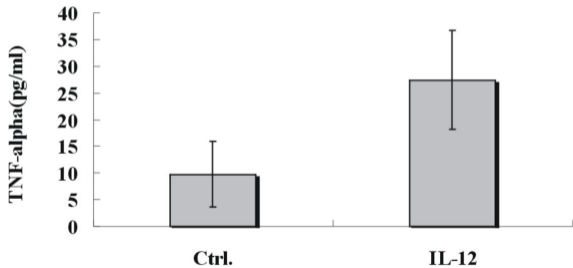
Fig. 5: The Phagocytotic activity of BV2 after stimulation of IL-12 and LPS. The phagocytotic efficiency was estimated with fluorescent particles engulfed by BV2 cells. The ctrl represented the non-stimulated group. * P<0.05 versus control.

Fig. 6: The cytotoxic effects of BV2 on RG2 with and without stimulation of IL-12 protein were evaluated by MTT assay in trans-well and co-culture two different environments. The data of absorbance were collected and presented to 5 groups. * P<0.05, RG2+BV2+IL-12 in trans-well compared with RG2, RG2+BV2 in trans-well, or RG2+BV2 in co-culture; RG2+BV2+IL-12 in co-culture compared with RG2, RG2+BV2 in trans-well, and RG2+BV2 in co-culture.
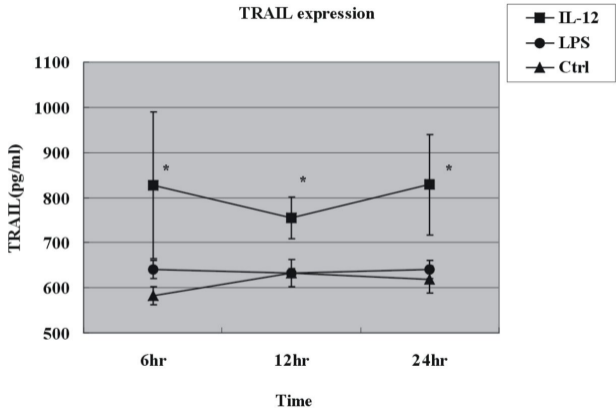
Fig. 7: The cytotoxic effect of BV2 on RG2 and AAV2-IL-12-transfected RG2 was evaluated by MTT assay in two different environments – trans-well and co-culture. The data of absorbance were collected and presented to 6 groups. RG2-AAV denoted RG2 cells transfected with AAV2/IL-12. * p<0.05, RG2-AAV+BV2 compared with the other groups.
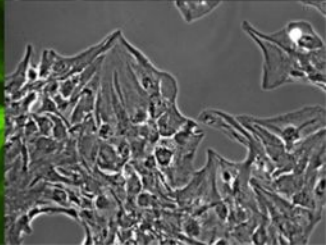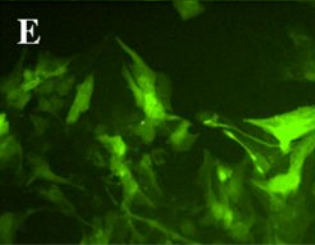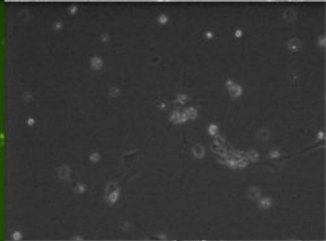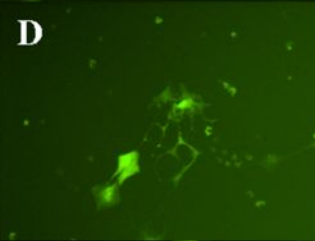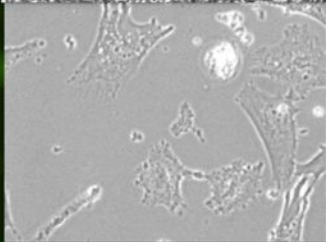
**BV2 secrtes TNF-alpha**

**TRAIL expression**

Legend: IL-12, LPS, Ctrl
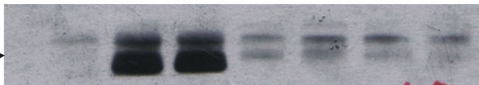
Y-axis: TRAIL(pg/ml), ranging from 500 to 1100

X-axis: Time (6hr, 12hr, 24hr)

DR4

| | ctrl | No.4 | No.3 | DBTRG | G5T-VGH | GBM8401 | GBM8901 |
|---|---|---|---|---|---|---|---|

55kDa →

B-actin

DR5

| | ctrl | No.4 | No.3 | DBTRG | G5T-VGH | GBM8401 | GBM8901 |
|---|---|---|---|---|---|---|---|

45kDa →

B-actin

**BV2 phagocytosis**
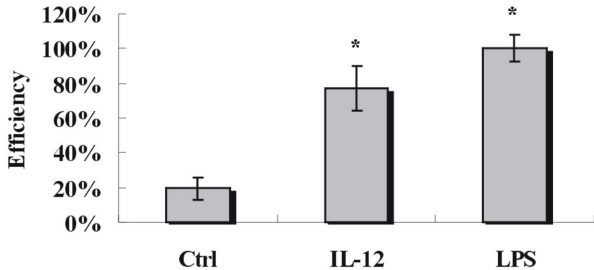
MTT assay of RG2 survival

MTT assay of RG2 survial
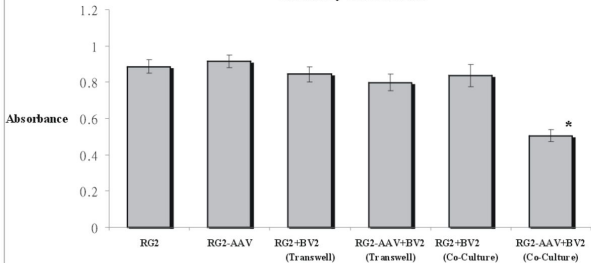
| | AAV-GFP | | | AAV-IL-12 | | |
| | mIL-12(pg/ml) | | | mIL-12(pg/ml) | | |
| | $10^{10}$VP | $10^{11}$VP | $10^{12}$VP | $10^{10}$VP | $10^{11}$VP | $10^{12}$VP |
|---|---|---|---|---|---|---|
| BV2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Astrocyte | 0 | 0 | 0 | 365.5 | >2000 | >2000 |
| HEK 293 | 0 | 0 | 0 | 1624.8 | >2000 | >2000 |
| RG2 | 0 | 0 | 0 | 129.3 | 1000.8 | >2000 |
| DBTRG | 0 | 0 | 0 | *>2000 | >2000 | >2000 |