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Tumor growth and metastasis suppression by *Glipr1* gene-modified macrophages in a metastatic prostate cancer model

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Abstract

We previously identified the mouse and human *Glipr1* and *GLIPR1/RTVP-1* (respectively) genes as direct p53 targets with proapoptotic activities in various cancer cell lines, including prostate cancer. Intratumoral injection of an adenoviral vector capable of efficient transduction and expression of Glipr1 (AdGlipr1) yielded promising therapeutic results in an orthotopic, metastatic mouse model of prostate cancer. AdGlipr1-transduced macrophages (Mφ/Glipr1) generated greater surface expression of CD40, CD80, and MHC class II molecules and greater production of interleukin (IL)-12 and IL-6 *in vitro* than control macrophages did. Mechanistic analysis indicated that increased production of IL-12 in Mφ/Glipr1 depends on activation of the p38 signaling cascade. Mφ/Glipr1 injected into orthotopic 178-2BMA tumors *in vivo* resulted in significantly suppressed prostate tumor growth and spontaneous lung metastases and longer survival relative to those observed in control-treated mice. Furthermore, these preclinical data indicate the generation of systemic natural killer-cell activity and tumor-specific cytotoxic T-lymphocyte responses. Trafficking studies confirmed that intratumorally injected Mφ/Glipr1 could migrate to draining lymph nodes. Overall, our data suggest that this novel gene-modified cell approach is an effective treatment avenue that induces antitumor immune responses in preclinical studies.

Keywords

prostate cancer; gene-modified cell therapy; Glipr1; macrophages

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Conflict of Interest

Some of the data included in this paper are relevant to intellectual property, co-invented by one of the authors (T.C.T.), held by Baylor College of Medicine, and licensed to Progression Therapeutics Inc., a private biotechnology start-up.

Introduction

Estimates are that there will be 317,080 newly diagnosed cancers of the genitourinary (GU) tract in American men and 51,820 deaths caused by these cancers in 2010; prostate cancer (PCa) alone would account for 69% of those GU cancers and 64% of GU cancer deaths.[1] PCa is androgen sensitive, and androgen-deprivation therapy (ADT) is one of the main treatment modalities in the clinical management of PCa patients with advanced, metastatic disease. Although patients with metastatic PCa can benefit from ADT at the initial stage, this approach is palliative rather than curative. Salvage cytotoxic therapy is associated with considerable morbidity, with little if any survival benefit.[2] Therefore, there is a critical need to develop new and effective therapeutic approaches, including gene- and cell-based therapies.

The tumor-suppressor gene *p53* is the most commonly mutated gene in human cancer.[3] In PCa, *p53* mutations are found in relatively low frequencies in early, localized tumors, but occur in much higher frequencies in advanced, hormone-refractory metastatic tumors, and have prognostic value in locally advanced disease following androgen deprivation and radiotherapy.[4,5]

We previously identified a novel mouse gene, Glioma pathogenesis-related protein 1 (HGNC-approved gene symbol, *Glipr1*), as a p53 target gene and homolog to the human gene Glioma pathogenesis-related protein 1 (HGNC-approved gene symbol, *GLIPR1*).[6]*GLIPR1,* or *RTVP-1* (*RTVP1*[7]), has also been identified in human glioblastoma cells and is a marker of myelomonocytic differentiation in macrophages.[8,9] Moreover, we have confirmed that *GLIPR1* expression in human PCa, especially in metastatic tumors, is significantly lower than it is in normal prostate, owing to methylation in the regulatory region of this gene in PCa cells.[10] Functional analysis demonstrated that overexpression of both *Glipr1* and *GLIPR1 in vitro* in various mouse and human cancer cell lines leads to apoptosis independently of *p53* status.[6,10] Adenoviral vector-mediated *Glipr1* (AdGlipr1) transduction in an orthotopic metastatic murine prostate model resulted in extension of animal survival through multiple effects, such as reduced metastasis to lung, suppression of tumor-associated angiogenesis, and increased infiltration of macrophages and $CD8^+$ T cells into the tumor.[11]*Glipr1*-related antitumor immunostimulatory activities were confirmed and extended in subsequent studies. For example, administration of a novel *Glipr1* gene-modified tumor cell vaccine to mice had substantial antitumor activity in a preclinical model of recurrent PCa.[12] This work led to the conduct of a phase I/II neoadjuvant clinical trial that involves adenoviral vector-mediated *GLIPR1* therapy prior to radical prostatectomy in men with PCa (IND13033).

However, systemic administration of viral vectors is currently not an effective method for targeting metastatic disease, owing to low initial viral titers, immune inactivation, nonspecific adhesion, and loss of particles.[13,14] Alternative approaches have been tested, including gene-modified cell-therapy approaches that focus on the delivery of therapeutic genes to the PCa, such as interleukin 12 (IL-12) gene-modified macrophage therapy.[15] The results of that study demonstrated suppression of tumor growth and spontaneous lung

metastasis, induction of systemic antitumor immune response, and prolongation of survival in mouse orthotopic models of metastatic PCa.

We also previously showed that the number of tumor stroma-associated macrophages is inversely correlated with tumor progression in human PCa.[16] We thus speculate that gene-modified macrophages would not only provide a stable cellular vehicle but also promote intrinsic macrophage-specific tumor cytotoxicity and stimulate antitumor lymphocytes.

Thus, we undertook this study, in which we showed that AdGlipr1-transduced macrophages (Mφ/Glipr1) have enhanced surface expression of CD40, CD80, and major histocompatibility complex (MHC) class II molecules and production of IL-12 via a p38-dependent mechanism. Intratumoral injection of *Glipr1* gene-modified macrophages *in vivo* resulted in statistically significant suppression of prostate tumor growth, suppression of spontaneous lung metastases, and increased overall survival rate in a preclinical mouse model of metastatic PCa. Furthermore, we demonstrated AdGlipr1-stimulated systemic natural-killer (NK) cell activity and tumor-specific cytotoxic T lymphocyte (CTL) responses.

Results

Transduction with AdGlipr1 induces Glipr1 *expression*

Peritoneal-exudate macrophages were transduced with increasing multiplicities of infection (MOI) of AdGlipr1 and incubated for 24 or 48 h, and then an MTS assay was performed to determine the viability of the transduced macrophages (Figure 1a). Twenty-four and 48 h after infection, there was no significant reduction in cell viability with up to 200 MOI, but a significant reduction in cell viability was found at 400 MOI (24 h, $P = 0.0148$; 48 h, $P = 0.0010$) compared with the control group (MOI 0) at the same intervals. Glipr1 protein levels in macrophages were also determined and compared by Western blotting 48 h after AdGlipr1 and Adβgal transduction at the indicated MOI (Figure 1b). AdGlipr1 transduction of macrophages led to a dose-dependent increase in Glipr1 expression, whereas the expression did not change with increasing Adβgal MOI. On the basis of these results, we selected the AdGlipr1 MOI of 100 for use in the studies reported here.

In vitro characterization of AdGlipr1-transduced macrophages

To investigate the characteristics of Mφ/Glipr1, we analyzed the cell surface expression and production of cytokines. Cytometric analysis showed that Mφ/Glipr1, compared with the control nontransduced macrophages (Mφ) and those transduced with β galactosidase (Mφ/βgal), had up to a threefold increase in cell surface expression of MHC II and CD40 molecules, a moderate increase of CD80 antigen expression, and a slight change in CD86 expression (Figure 2).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis revealed significant changes in cytokine RNA expression levels, specifically in Mφ/Glipr1, compared with Mφ and Mφ/βgal (Table 1). Among these changes, the most interesting were those involving type 1 (M1) and type 2 (M2) cytokines.[17] Significantly increased expression levels of specific proinflammatory and M1 cytokines IL-1α, IL-12 p40, IL-12 p35, and IL-6

were documented. In contrast, the mRNA levels of the M2 cytokines IL-2, IL-4, and IL-10 were minimally affected by both Adβgal and AdGlipr1 transduction, and the TNF-α mRNA levels were moderately increased by AdGlipr1 transduction. Additionally, iNOS (NOS2) and Arg1 expression increased by 33 and 3 times, respectively, in Mφ/Glipr1.

Protein levels of the cytokines in the culture medium of Mφ/Glipr1, as analyzed by ELISA, were similar to those found on qRT-PCR: secretion of IL-12 and IL-6 proteins increased significantly, whereas TNF-α and IL-10 levels did not change statistically significantly (Figure 3).

Analysis of MAPK-signaling pathways in AdGlipr1-transduced macrophages

To investigate the role of mitogen-activated protein (MAP) kinases in the production of cytokines by the transduced macrophages, we analyzed the phosphorylated forms of JNK (P-JNK), p38 (P-p38), and ERK (P-ERK) by using Western blotting with specific anti-phosphokinase antibodies (Abs). Figure 4a shows that transduction of macrophages by AdGlipr1, compared with the nontransduced and β–galactosidase-transduced controls, increased the induction of phosphorylation of JNK, p38, and ERK. The total levels of these proteins were unaffected (Figure 4a), indicating that Glipr1 stimulated the activity of but not the levels of the MAP kinases.

To further understand the role of the individual MAPK pathways on cytokine production by Mφ/Glipr1, we used SP600125, SB203580, and U0126, which are specific inhibitors of JNK, p38, and ERK MAPK activity, respectively (Figure 4b). As expected, SP600125, SB203580, and U0126 suppressed AdGlipr1-mediated phosphorylation in a dose-dependent manner. We also tested the effect of these MAPK inhibitors on the secretion of IL-12 in Mφ/Glipr1 by using ELISA (Figure 4c). SB203580 dose-dependently suppressed the secretion of IL-12 protein, with a statistically significant difference between the 0-μM and 25-μM concentrations ($P = 0.0005$). However, SP600125 and U0126 did not affect the secretion of IL-12 protein with the same doses. These results suggest that the p38 pathway regulates Glipr1 induction of IL-12 in peritoneal exudate cells.

AdGlipr1-transduced macrophages suppress tumor growth and metastasis and enhance survival in a preclinical mouse model of PCa

To determine the possible therapeutic effect of Mφ/Glipr1 *in vivo*, we conducted experiments in an orthotopic mouse model of a metastatic PCa cell line, 178-2 BMA. Seven days after orthotopically injecting 178-2 BMA cells, we injected the tumors with Mφ, Mφ/βgal, Mφ/Glipr1, Hanks balanced salt solution (HBSS), Adβgal, or AdGlipr1 in a volume of 25 μl. Fourteen days after the treatment we harvested and extensively analyzed both primary tumors and their metastases. It was interesting that, as depicted in Figure 5a, Mφ/Glipr1 treatment significantly suppressed the growth of primary tumors (1029 mg) compared with treatment with HBSS (3064 mg), Mφ (2691 mg), Adβgal (2799 mg), and Mφ/βgal (2414 mg) ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, and $P = 0.0002$, respectively). The effect of tumor growth suppression by Mφ/Glipr1, however, was not statistically significantly different from that of AdGlipr1 alone (1029 mg vs 1570 mg; $P = 0.1086$). These results suggest that Mφ/AdGlipr1 is as effective as AdGlipr1 inhibiting tumor growth and reducing a number of lung

metastases. However, there are advantages of using the Mφ/AdGlipr1 system compare to a viral vector delivery as further discussed.

To evaluate potential antimetastatic effects of Mφ/Glipr1 in this PCa model, we analyzed the extent of lung metastasis 14 days after the treatment. As indicated in Figure 5b, the Mφ/Glipr1-treated group had significantly fewer macroscopic spontaneous lung metastases (mean, 2.0) than the HBSS- (7.1), Mφ- (5.9), Adβgal- (6.7), and Mφ/βgal- (5.9) treated groups had ($P = 0.0036$, $P = 0.0204$, $P = 0.0071$, and $P = 0.0204$, respectively). However, the number of lung metastases in the Mφ/Glipr1-treated group was not statistically significantly different than that in the AdGlipr1-treated group (2.0 vs 2.8; $P = 0.6205$).

Figure 5c is a cumulative Kaplan-Meier survival plot for the treated mice; the mean survival times for the control groups treated with Mφ and Mφ/βgal were 22.7 days and 23.0 days, respectively, whereas that for the Mφ/Glipr1-treated group was 27.8 days. These differences were statistically significant according to Mantel-Cox log-rank analysis (vs Mφ, $P < 0.0001$; vs Mφ/Adβgal, $P = 0.0011$). On necropsy, no obvious cause of death was found other than extensive tumor load, splenic trauma, or abundant ascites.

Kinetics analysis of treatment activities

The kinetics of the treatments' activities were analyzed in tumors from 3 or 4 mice harvested at sequential points before the end of the time course. As shown in Figure 6a, the tumors grew rapidly between 7 and 14 days after injection of the 178-2 BMA cells. However, significant suppression of tumor weight was observed on both days 7 and 14 after treatment in the Mφ/Glipr1-treated group compared with the Mφ- (day 7, 313 mg vs. 825 mg, $P = 0.0054$; day 14, 1200 mg vs. 2910 mg, $P < 0.0001$) and Mφ/βgal-treated groups (day 7, 313 mg vs. 690 mg, $P = 0.0372$; day 14, 1200 mg vs. 2400 mg, $P < 0.0001$). Each animal's spleen was also weighed at the time of euthanasia (Figure 6b); the average weight on day 0 was 62.7 mg. In the Mφ/Glipr1-treated group, the spleen weight significantly increased to a maximum of 185.3 mg on day 7, compared with that in the Mφ- (142.5mg) and Mφ/βgal (134.8mg) -treated groups ($P = 0.0227$ and $P = 0.0077$, respectively), after which it decreased to 118.25 mg (similar to the control groups) on day 14.

The serum levels of IL-12 in the Mφ/Glipr1-treated animals gradually increased, with a peak on day 5, and then decreased until day 14 (Figure 6c), whereas the levels in the Mφ- and Mφ/βgal-treated animals did not change significantly during the time course.

AdGlipr1-transduced macrophages induce a systemic immune response

To detect whether systemic immune responses possibly resulted from the treatment of the mouse prostate tumors with Mφ/Glipr1, we isolated splenocytes from mice bearing Mφ-, Mφ/βgal-, and Mφ/Glipr1-treated tumors and analyzed the NK and CTL activities. Figure 7a shows that NK activities in splenocytes isolated from the Mφ/Glipr1-treated mice were statistically significantly greater than they were in the Mφ- and Mφ/βgal-treated mice 2 days after treatment at effector-to-target (E:T) ratios of 100:1 ($P = 0.0012$ and $P = 0.0067$, respectively) and 50:1 ($P = 0.0003$ and $P = 0.0027$, respectively). Additionally, splenocytes isolated from mice 14 days after treatment were evaluated for their specific ability to lyse

178-2 BMA cells *in vitro* (Figures 7b and c). A statistically significant increase in CTL activity in Mφ/Glipr1-treated mice was observed relative to that in Mφ- and Mφ/βgal-treated mice 7 days after treatment at the E:T ratios of 100:1 ($P = 0.0081$ and $P = 0.0381$, respectively) and 25:1 ($P = 0.0055$ and $P = 0.0007$, respectively; Figure 7b). The difference in CTL activity between treatment groups was less pronounced at day 14, although on average it was still greater in Mφ/Glipr1-treated mice (Figure 7c). To confirm that $CD8^+$ T cells were responsible for this activity, we selectively depleted $CD4^+$ cells and $CD8^+$ cells from the splenocytes by antibody-mediated complement lysis before performing the CTL assay (Figure 7d). A significant reduction of lytic activity was observed when $CD8^+$ effector cells were depleted but not when $CD4^+$ effector cells were depleted ($P = 0.0071$, Mann-Whitney test), thus confirming our hypothesis that $CD8^+$ cells play a role in tumor cell lysis.

AdGlipr1-mediated macrophages migrate to draining lymph nodes

We prepared fluorescently labeled macrophages using the cell-linker compound PKH26-PCL, as described in Materials and Methods. We then infected these fluorescent macrophages with AdGlipr1 and Adβgal and injected them directly into 178-2BMA orthotopic tumors. The prostate, draining lymph nodes, livers, and lungs were harvested 24 and 72 h after the injection and were evaluated for macrophage migration by studying tissue sections under a fluorescence microscope. At 24 h, fluorescent macrophages were restricted to the prostate (not shown). At 72 h, the Mφ/Glipr1-treated cells had migrated to the cortices of the lymph nodes draining from the prostate with greater efficiency than had the Mφ/βgal-treated cells (Figure 8). The migration seemed to be confined to lymph nodes at this interval because fluorescent macrophages were not detected in the livers and lungs (data not shown).

Discussion

We previously demonstrated that orthotopic delivery of adenoviral vector-mediated *Glipr1/mRTVP-1*, a direct p53 target gene, into mouse PCa tumors significantly suppressed their growth and metastasis to the lung.[11] In addition, we documented that intratumoral injection of AdGlipr1 generates widespread immunostimulatory activities *in vivo*, including the induction of macrophages within the primary tumor and the increased generation of IL-12. Additional studies showed that a *Glipr1* gene-modified tumor cell vaccine had significant antitumor activity—accompanied by systemic antitumor immunity—in a preclinical model of recurrent PCa in mice.[12] We further demonstrated the successful genetic modification of murine peritoneal exudate macrophages with adenoviral vectors and presented evidence that intratumoral injection of murine IL-12 recombinant adenoviral vector (AdmIL-12)-transduced macrophages is safe and effective in a preclinical model of metastatic PCa.[15] On the basis of these results, we decided to conduct this study to evaluate the potential efficacy of *Glipr1* gene-modified macrophage therapy in our established preclinical mouse models of metastatic PCa.

Macrophages are prominent in the stromal compartment of virtually all types of malignancy. Tumor-associated macrophages (TAMs) arise from monocyte precursors that migrate from the bloodstream into both primary and secondary tumors at an early stage in their development.[17] Some TAMs, recognized as M1 macrophages, are activated by

proinflammatory cytokines such as interferon (IFN)-γ and interleukin (IL)-1β, and tumor necrosis factor (TNF)-α and are capable of lysing tumor cells, presenting tumor-associated antigens to T cells, and expressing immunostimulatory (type 1) cytokines such as IL-12 to stimulate the proliferation and antitumor functions of T cells and NK cells.[17-19] However, these activities can be suppressed by other tumor-derived cytokines (type 2), including IL-4, IL-10, transforming growth factor (TGF)-β1, prostaglandin E_2, and macrophage colony-stimulating factor,[20-23]. In contrast to M1 TAMs, M2 TAMs are activated through an "alternative" program by IL-4 and IL13 and have poor antigen-presenting capability and produce factors that suppress T-cell proliferation and activity, and promote angiogenesis.[17,24]

We previously showed that the numbers of TAMs in direct contact with PCa cells correlate positively with the Gleason score, whereas the numbers of TAMs within the cancer stroma were inversely associated with multiple clinical and pathologic markers of disease progression and with its recurrence after surgery.[16] The presence of large numbers of TAMs correlate with a favorable prognosis in gastric and colorectal cancers and melanoma.[25-27] In stark contrast, increased numbers of TAMs are also associated with shorter survival in patients with breast and esophageal squamous cell carcinomas.[28,29] Overall, it is apparent that the status of tumor progression, tumor-cell compartmentalization and more importantly, the activation status of macrophages are critical determinants of macrophage activities and their effects.

Our *in vitro* data demonstrated that the AdGlipr1-transduced macrophages (Mφ/Glipr1) expressed significantly higher levels of type 1 cytokines (IL-1α, IL-6, and IL-12) and secreted more IL-12 and IL-6 than the Mφ control and Mφ/βgal cells did, although no statistically significant changes in the expression levels of type 2 cytokines (IL-2, IL-4, and IL-10) were found between the Mφ/Glipr1, Mφ/βgal, and Mφ control groups. It was notable that *Glipr1* transduction also increased expression of cell surface MHC II molecules, which may indicate polarization toward the M1 phenotype.[30] The Mφ/Glipr1 cells also demonstrated a remarkably strong increase in inducible nitric oxide synthase (NOS2 expression, an M1 marker) and a moderate increase in Arg1 expression (an M2 marker) relative to the expression levels in Mφ and Mφ/βgal cells. In accordance with *in vitro* results, the serum levels of IL-12 gradually increased in the Mφ/Glipr1-treated group, with a peak on day 5, but did not increase in the control groups. Overall, these results indicate that AdGlipr1 might stimulate peritoneal exudate macrophages to develop into polarized M1 macrophages.

In the current study, we found increased NK activity on day 2 and CTL activity on day 7 after treatment with Mφ/AdGlipr1. These increases in activity may have resulted from the generation of a Th1 response and the increased surface expression of MHC class II, CD80, and CD40 that was induced by AdGlipr1 in peritoneal exudate macrophages. However, this CTL activity in the Mφ/AdGlipr1-treated group decreased by day 14, perhaps because of the decreased serum levels of IL-12 levels. The increase in CTL activity observed in Mφ/AdGlipr1-treated mice was clearly CD8 dependent because it was almost completely ablated by depletion of $CD8^+$ cells but not by depletion of $CD4^+$ cells. These immunostimulatory

activities are very similar to those demonstrated previously after AdGlipr1 injection into orthotopic tumors using a similar model system.[11]

AdGlipr1 also induced phosphorylation of JNK, p38, and ERK in murine peritoneal exudate macrophages; inhibition of P-JNK and P-ERK had no effect on IL-12 production, whereas inhibition of P-p38 by SB203580 suppressed IL-12 production. These results indicate that the p38 pathway mediates Glipr1-induced IL-12 production in peritoneal exudate macrophages. The expression of p38 family members is regulated differently in various tissues and cell lineages. The p38 MAPK pathway has been proposed to function in the regulation of cytokine production, B- and T-cell proliferation and differentiation, the innate immune response, cell cycle control, and apoptosis.[31] The results of several studies have demonstrated that activation of the p38 MAPK pathway is involved in IL-12 regulation.[32,33] Mice deficient in MKK3, an upstream kinase of p38, were defective in the production of IL-12 by antigen-presenting cells.[34] Lipopolysaccharide-stimulated macrophages isolated from MKK3-deficient mice produced normal levels of IL-6, IL-1β, TNF-α, and IL-1β but, in contrast, IL-12 production was significantly reduced in MKK3-deficient macrophages.[34]

We previously showed that GLIPR1 up-regulation activates the JNK signaling cascade and is required for GLIPR1-mediated apoptosis in cancer cells.[35] Consistent with those previously reported findings, overexpression of GLIPR-1 increased the phosphorylation of JNK in macrophages in this study. However, activated JNK signaling does not lead to apoptosis in peritoneal exudate macrophages. This, together with our previous results, suggests that the apoptotic responses to JNK inhibition are cell-line dependent and that the involvement of JNK in apoptosis is complex and still not well understood.[36]

Another interesting finding in this study was the increased degree of migration of macrophages to the draining lymph node in mice injected with *Glipr1* gene-modified macrophages. In addition to their role in innate immunity, M1 macrophages have been shown to phagocytose apoptotic tumor cells. This function, together with the production of higher levels of IL-12, and increased surface expression of CD40 and MHC class II molecules, suggests that some Mφ/Glipr1 cells could function as tumor antigen-presenting cells. Their increased migration to the lymph nodes may be anatomic evidence of this possibility. In the cortex of a lymph node, the Mφ/Glipr1 subgroup could come into contact with naive lymphocytes, thereby initiating the process of antigen presentation. This process could be a bridge between the phagocytotic function of M1 macrophages and the induction of systemic immunity, such as the increased CTL activity seen in the Mφ/Glipr1-treated mice. Further studies are warranted to determine the mechanisms underlying the effect of Glipr1 in promoting the migration of macrophages to the lymph nodes.

Overall, the results of this study demonstrate that *Glipr1* gene-modified macrophages generated antitumor effects in a preclinical mouse model of metastatic PCa. Intratumoral injection of AdGlipr1 and Mφ/Glipr1 produced similar antitumor effects, such as reduced tumor weights and fewer lung metastases. However, treatment with a viral vector alone has limitations owing to systemic vector toxicity and production of antivirus antibody (Ab) that preclude repeated injections. Gene-modified cell therapy, such as we investigated,

overcomes both of these limitations and could be considered for further studies, including clinical trials.

Materials and methods

Mϕ collection and cultivation

Peritoneal exudate macrophages were collected by lavage 5 days after intraperitoneal (IP) injection of 2 ml of thioglycollate medium (Becton, Dickinson and Company Diagnostic Systems, Sparks, MD) into 129/Sv mice. The collected cells were seeded in petri dishes with Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation, Carlsbad, CA) enriched with 10% heat-inactivated fetal bovine serum (FBS). The cells were allowed to attach for 2 h before being washed with phosphate-buffered saline (PBS) to eliminate nonadherent cells. Macrophage purity was assessed by flow cytometry, and the cells were replated before being infected with the adenoviral vector.

Glipr1/mRTVP-1 cDNA was cloned and sequenced as previously described and inserted into pCA3.[6] Replication-defective adenoviral vector was generated by cotransfection with pJM17 into the 293 human embryonic kidney cell line.[37] As a control adenoviral vector, Adβgal was prepared as described previously.[38] Each adenoviral vector was isolated from a single plaque, expanded in 293 cells, and purified by double cesium-gradient ultracentrifugation; the titer was assessed by performing a plaque assay on the infected 293 cells and expressed as plaque-forming units (PFUs). The adenoviral vectors were added to the prepared macrophages at the indicated MOI in serum-free medium for 2 h, and then fresh complete medium was added for overnight incubation. Cells were collected and analyzed at indicated time points as described below.

Cell viability assay

Isolated Mφ were infected with increasing MOI of AdGlipr1 as described above and incubated in 96-well dishes at 2.0×10^4 cells/well for 48 h. The viability of Mφ was evaluated using an MTS Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, Mφ were cultured for 48 h in conditions as described above. MTS substrate was added to the medium at a ratio of 20 μl of MTS for every 100 μl of culture medium. Then, Mφ were incubated for 1 h at 37°C. After incubation, 100 μl from each well was analyzed in triplicate on a colorimetric plate reader at 490 nm absorbance.

In vitro *characterization of transduced macrophages*

Peritoneal exudate macrophages were seeded in 24-well plates in triplicate. The next day, the medium was aspirated, the adherent cells were washed gently with PBS, and the macrophages were infected with 100 MOI of AdGlipr1 or Adβgal. Fresh medium was added after 2 h. $0.5–1.0 \times 10^6$ cells from each group of Mφ, Mφ/βgal, and Mφ/Glipr1 were then harvested and stained with FITC-conjugated monoclonal Abs against murine cell surface molecules (F4/80 [AbD Serotec, MorphoSys UK Ltd., Oxford, United Kingdom], I-A^b, CD80, CD86, CD40, and appropriate isotype controls [Pharmingen, San Diego, CA]). Cytometric analysis was performed by using an EPICS XL-MCL (Coulter Electronics,

Westbrook, ME) flow cytometer. The mean fluorescence intensity and percentage of positive cells were used to make the between-group comparisons.

Analysis of cytokine expression in transduced macrophages

For RT-PCR analysis of cytokine expression, total RNA was isolated from cultured macrophages 48 h after transduction by using a RiboPure RNA isolation kit (Applied Biosystems/Ambion, Austin, TX). Total RNA (2 μg) was reverse transcribed using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA). The generated cDNA (1 μl per reaction) was analyzed by performing qRT-PCR with validated primer sets from the Harvard Medical School Primer Bank[39] (http://pga.mgh.harvard.edu/primerbank/index.html and Table 1) and using a Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) according to the manufacturer's protocol. Reactions were monitored by using Invitrogen's One-Step real-time PCR system with the SYBR Green protocol settings. The relative changes in gene expression were calculated by using the $\Delta\Delta C_t$ method (gene expression level = $2^{-\Delta\Delta Ct}$) according to the following formula: $\Delta C_t = C_t$ (treated) – C_t (control), in which C_t represents the cycle threshold value normalized to the expression levels of *GAPDH*, a house keeping gene. Melting curve analysis was done for each pair of primers to confirm the specificity of the RT-PCR reaction.

We also used the Taqman qRT-PCR assay (Applied Biosystems) to assess the levels of mouse IL-12 p35 and IL-12 p40.

Quantitation of cytokines by ELISA

Blood was drawn from the inferior vena cava of the mice at the time of euthanasia and allowed to clot, and the serum was collected after centrifugation. The serum and tissue culture supernatants were stored at –80°C. The levels of IL-12 and IL-6 were quantitatively determined by commercially available ELISA kits per the manufacturer's protocol (Invitrogen/BioSource, Carlsbad, CA).

Western blotting

Peritoneal exudate macrophages transduced with Adβgal or AdGlipr1 at 100 MOI were incubated for 48 h as described before. The cell lysates were then electrotransferred and immunoblotted against primary Abs. Next, the primary Abs were detected by using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and visualized by using enhanced chemiluminescence. The primary Abs that selectively recognize the phosphorylated forms of JNK, p38 MAP kinase, and ERK were all purchased from Cell Signaling Technology, Inc., Beverly, MA. Generation of Glipr-1(mRTVP-1) was previously described.[6] To determine the amounts of precipitated JNK, p38 MAP kinase, and ERK, blots were stripped and reprobed using phosphorylation state-independent anti-JNK, anti-p38 MAP kinase, and anti-ERK Abs.

Orthotopic mouse prostate cancer model

The mouse PCa cell line 178-2 BMA was originally derived from a bone metastasis obtained from Zipras/myc9-infected urogenital sinus tissue from p53-heterozygous 129/Sv mice in the metastatic mouse prostate reconstitution (MPR) model system, as previously

described.[40,41] Cell preparation and the orthotopic injections were carried out as described previously.[15] Uninfected macrophages (Mφ), Adβgal-transduced macrophages (Mφ/βgal), and AdGlipr1-transduced macrophages (Mφ/Glipr1) were each resuspended in HBSS at a concentration of 4×10^7 cells/ml, and 25 μl of the resulting suspension was injected into established prostate tumors 7 days after the tumor cell injection. For the selected groups, *in situ* delivery of Adβgal or AdGlipr1 at an optimal dose of 5×10^8 PFUs was made by injection into established tumors. A 30-gauge needle was placed through an intracapsular, transprostatic tract before tumor penetration to minimize leakage at the time of needle withdrawal. Animals were euthanized on the 14th day after the treatment or at selected times for kinetic analyses. However, for the survival analysis, animals were monitored daily and euthanized when they became moribund.

The sample size was 10 mice per treatment group, except for the kinetics analyses, in which 3 or 4 mice per treatment group were euthanized on each specified day. At necropsy, all animals were carefully evaluated for gross metastases. The primary tumors and spleens were removed and weighed. The spleens were placed in sterile medium and splenocytes were purified and used for NK and CTL assays as described below. Tumor tissues were fixed in formalin, embedded in paraffin cut into 5-μm sections, and stained with H&E for histologic examination. The lungs were removed and placed in Bouin's solution for fixation. The next day, two independent observers counted the total number of spontaneous lung metastases with the aid of a dissecting microscope, and the average of their two counts was reported.[38]

All mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and all experiments were conducted in accordance with the principles and procedures outlined in the NIH's *Guide for the Care and Use of Laboratory Animals*.

In vitro *cytolytic assays*

Splenocytes were collected at selected times and used for NK and CTL assays. NK activity was determined by lysis of ^{51}Cr-labeled YAC-**1** tumor cells (the prototypic NK-cell target cells) with splenocytes derived from tumor-bearing animals, essentially as described previously.[38] The YAC-**1** cell line was obtained from the American Type Culture Collection (Manassas, VA). CTL activity was determined by lysing target ^{51}Cr-labeled, interferon γ (IFN-γ)-stimulated 178-2 BMA cells with splenocyte-derived T lymphocytes.

Effector cells were generated *in vitro* by incubating spleen cells from tumor-bearing mice (1.8×10^7 cells per well) with mitomycin C-treated 178-2 BMA cells (1.2×10^7 cells per well) in 12-well plates for 5 days in the presence of anti-transforming growth factor β_1 (TGF-β_1) Ab (30 mg/ml) and IL-2 (20 U/ml). Target 178-2 BMA cells were incubated with IFN-γ (100 U/ml) for 2 days and then radiolabeled with 100 μCi of ^{51}Cr for 45 min at 37°C. Cells with different effector-to-target (E:T) ratios were incubated for 4 h at 37°C. Supernatants were collected and counted in a gamma counter, and the percentage of specific lysis was calculated as described previously.[42]

In vitro depletion of $CD8^+$ and $CD4^+$ T cells was accomplished treating splenocytes immediately after their isolation with supernatants from the hybridoma HO-2.2 (anti-Lyt

2.2) or GK1.5 (anti-L3T4) (American Type Culture Collection) in a cytotoxicity medium (RPMI-1640 with 25 mM HEPES and 0.3% bovine serum albumin) purchased from Cedarlane Laboratories, Ltd., Burlington, ON, Canada. Antibody was allowed to bind at 4°C for 1 h, and then rabbit complement (Accurate Chemical & Scientific Corp., Westbury, NY) was added, and the cells were incubated at 37°C for 1 h. The cells were then incubated as described above for preparing effector cells. The specificity and effectiveness of the procedure were confirmed by flow cytometric analysis of splenocytes 24 h after complement-mediated lysis.

Trafficking study of transduced macrophages in vivo

The fluorescent cell-linker compound PKH26-PCL (Sigma-Aldrich Corp., St. Louis, MO) for phagocytic cell labeling was injected IP into 129/Sv donor mice. Labeled macrophages were collected from the donor mice 2 days later by peritoneal lavage with culture medium and plated as described above. Macrophages were infected with AdGlipr1 or Adβgal at an MOI of 100. Then 1×10^6 infected macrophages were injected into orthotopic 178-2 BMA tumors. The mice were euthanized 24 and 72 h after treatment with macrophages, and the prostates, draining lymph nodes, lungs, and livers were collected and fixed in Zamboni's fixative (2% paraformaldehyde, 10% picric acid, and 0.1 M phosphate buffer; pH 7.2) for 12 h at 4°C, embedded in O.C.T. compound, and frozen. Serial 6-μm sections were made from these samples using a cryostat mounted in antifading medium and examined with the use of a fluorescence microscope.

Statistical analysis

ANOVA analysis of variances software (unpaired t-test) was used to compare the tumor weights, number of spontaneous metastases, the results of ELISA, and cytolytic assay. Kaplan-Meier survival analysis results were evaluated by using the Mantel-Cox log-rank test. All analyses were performed by using Statview 5.0 software (SAS Institute, Cary, NC).

Acknowledgements

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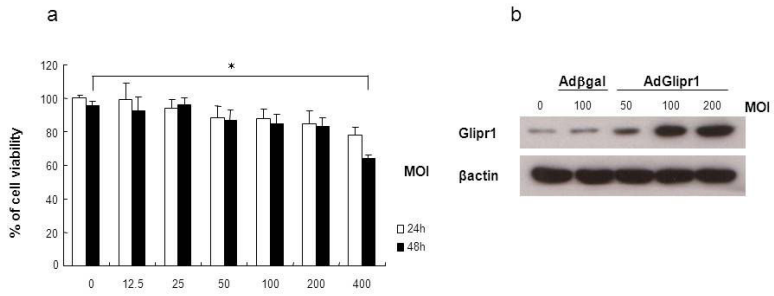

Figure 1.

AdGlipr1-mediated gene transfer into macrophages *in vitro*. Isolated macrophages were transduced with Adβgal or AdGlipr1 at the indicated MOI and incubated for 24 and 48 h. (a) Viability of the macrophages infected with increasing MOI of AdGlipr1 was evaluated by using an MTS assay. The viability at an MOI of 0 was set at 100% as the control. Experiments were performed 3 times. *Bars*, SE. **P* < 0.05. (b) Protein levels of Glipr1 in macrophages were determined by Western blotting 48 h after transduction with either AdGlipr1 or Adβgal at the indicated MOI.

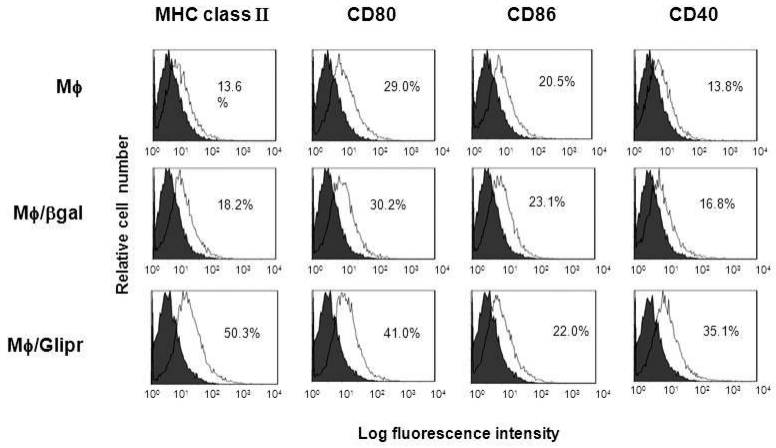

Figure 2.

Flow-cytometric detection of specific cell-surface markers expressed by AdGlipr1-transduced macrophages (Mφ). Isolated Mφ were transduced with Adβgal (Mφ/βgal) or AdGlipr1 (Mφ/Glipr) at 100 MOI and incubated for 48 h. Macrophages were analyzed flow cytometrically to determine the percentages of MHC class II-, CD80-, CD86-, and CD40-positive cells.

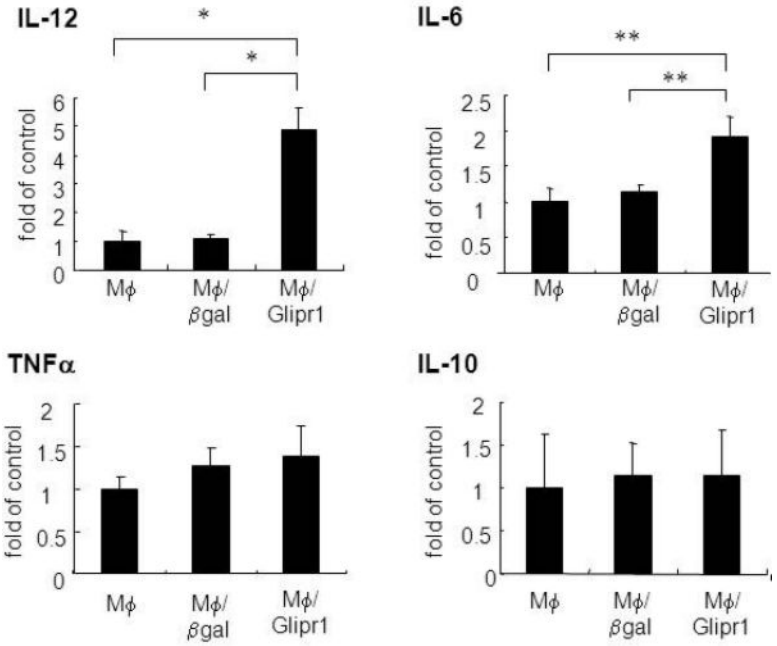

Figure 3.

Production of various cytokines by AdGlipr1-transduced macrophages. Isolated macrophages (Mφ) were transduced with Adβgal (Mφ/βgal) and AdGlipr1 (Mφ/Glipr) at 100 MOI and incubated for 48 h. The growth medium was changed 48 h after transduction and the cells recultured. After 48 h, the supernatant was collected from triplicate wells and the cytokine concentration relative to that of the controls was determined on ELISA. *Bars*, SE. **P* < 0.05, ***P* < 0.01.

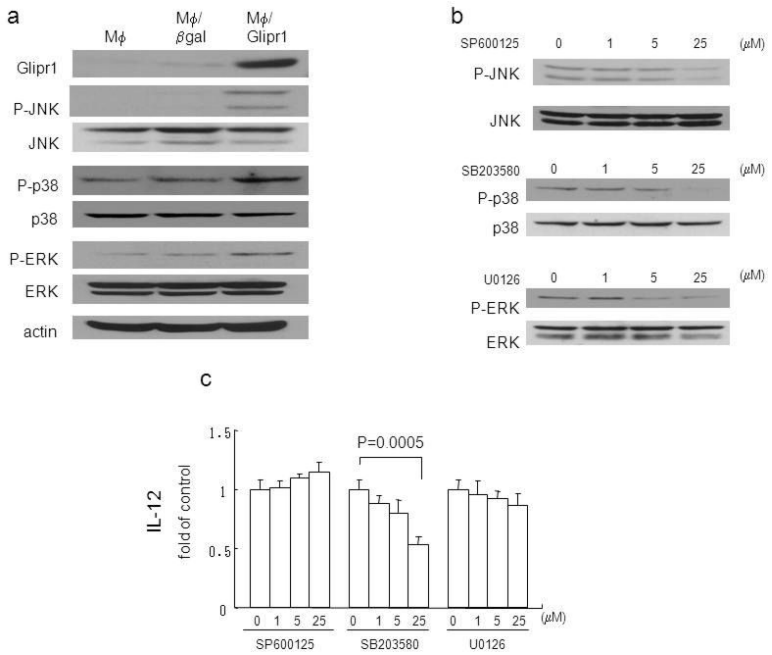

Figure 4.

Analysis of MAPK-signaling pathways in AdGlipr1-transduced macrophages and the effect of specific MAPK inhibitors on the production of cytokines. (a) Isolated macrophages (Mφ) were transduced with Adβgal (Mφ/βgal) and AdGlipr1 (Mφ/Glipr) at 100 MOI and incubated for 48 h. Cell lysates were prepared and analyzed by Western blotting using 10% SDS-polyacrylamide gels and antibodies against the indicated proteins. P-, phospho-specific antibodies. (b, c) Macrophages were pretreated with the indicated concentrations of the inhibitors SP600125, SB203580, and U0126 before being transfected with AdGlipr1 and cultured for 48 h. The culture medium was then changed, and the cells were recultured with these MAPK inhibitors. After 48 h, the supernatant and cell lysates were collected from triplicate wells and analyzed for relative IL-12 levels with Western blotting (b) and ELISA (c). *Bars*, SE.

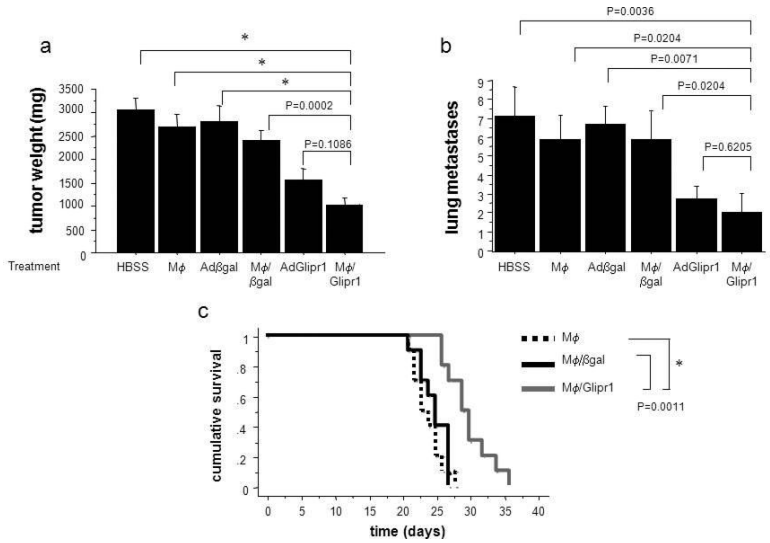

Figure 5.

Beneficial effects of AdGlipr1-transduced macrophage (Mφ/Glipr1) treatment on prostate tumors. When compared with the effects of the other treatments, injection of Mφ/Glipr1 into prostate tumors resulted in (a) tumor suppression (as determined by tumor weight), (b) fewer lung metastases, and (c) prolonged survival. The sample size was 7–10 animals per group: Mφ, Mφ/βgal, Mφ/Glipr1, Hanks balanced salt solution (HBSS), Adβgal, or AdGlipr1. *Bars*, SE. **P* < 0.0001.

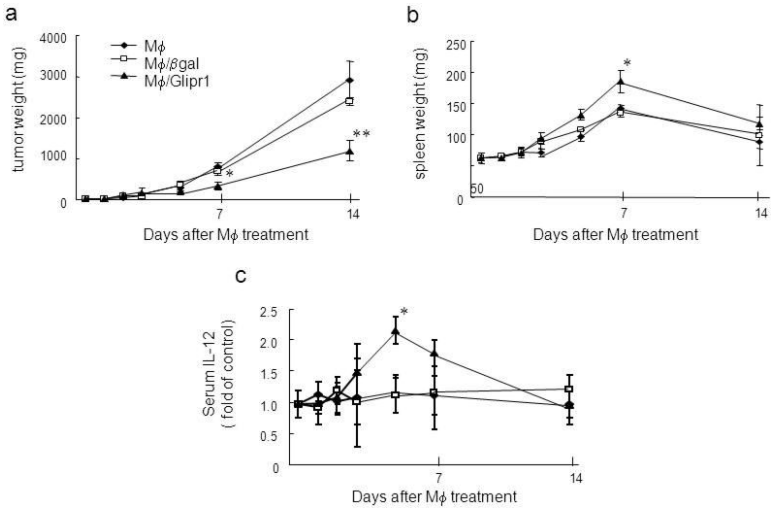

Figure 6.

Kinetics analysis. (a) The tumor weight was lower (ie, growth suppression) in the Mφ/Glipr1-treated group than that in the control groups by day 7 and continued throughout the day 14 after the treatments. (b) The spleen weight was significantly higher in the Mφ/Glipr1-treated group than that in the control groups at day 7 after the treatments. (c) Serum IL-12 levels gradually increased in Mφ/Glipr1-treated group, with a peak on day 5, but the level returned to that of the control groups at day 14. *Bars*, SE. **P*<0.05, ***P*<0.001.

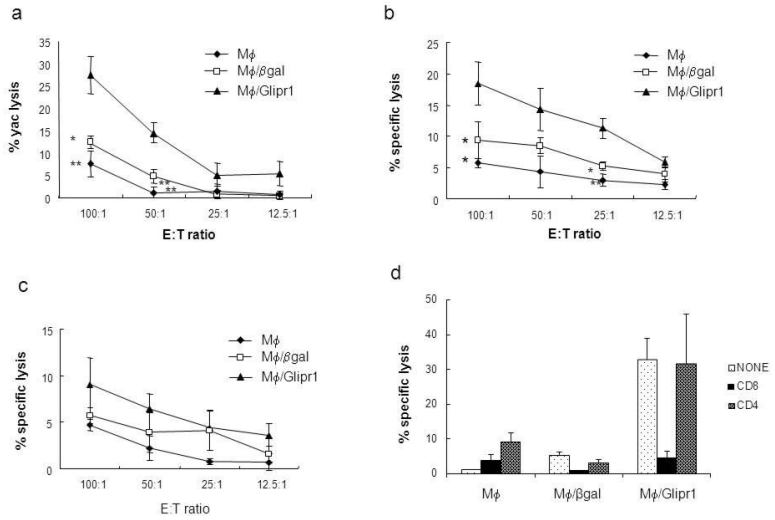

Figure 7.

Treatment with AdGlipr1-transduced macrophages (Mφ/Glipr1) induced systemic immune responses. (a) NK activity in splenocytes 2 days after treatment relative to that of YAC-**1** cells. CTL activity in splenocytes 7 (b) and 14 (c) days after treatment. Increased NK activities on day 2 and CTL activities on day 7 were demonstrated in the Mφ/Glipr1-treated group relative to those in the control groups. The sample size was 4 mice per group. (d) CTL activity in splenocytes from animals euthanized on day 7 after vector injection and then depleted of $CD8^+$ or $CD4^+$ cells *in vitro*. *Bars*, SE. **P*<0.05 ***P*<0.005

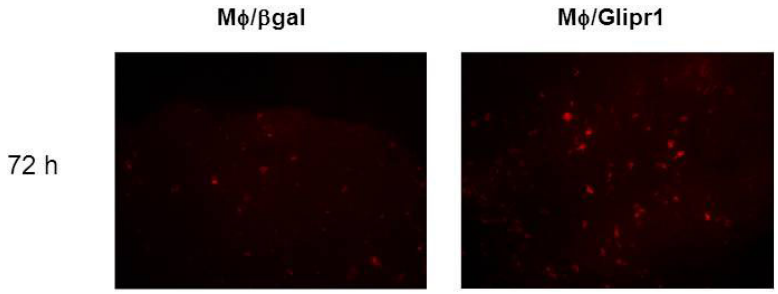

Figure 8.

In vivo macrophage trafficking. On day 7 after injection of 178-2BMA metastatic prostate cancer cells, the resulting orthotopic tumors were injected with PKH-26CL–fluorescently labeled macrophages that had been transduced with Adβgal (Mφ/βgal) or AdGlipr1 (Mφ/Glipr1). The draining lymph nodes were harvested 72 h after macrophage treatment, and tissue sections were examined with fluorescence microscopy. These data imply that the Mφ/Glipr1 migrated into the cortex area of draining lymph nodes with higher efficiency than that observed with Mφ/βgal cells.

Table 1

Change in RNA relative expression levels of selected cytokines and related genes in Mϕ/Glipr1 or Mϕ/αgal-transduced macrophages - compared with those nontransduced macrophages (Mϕ) *in vitro*, as assessed on qRT-PCR

Cytokine	*Mϕ/pgal*	*Mϕ/Glipr1*	*Primer ID*
IL-1α **	1.18±0.24	2.89±0.22	6754328a1
IL-1β	1.36±0.23	5.79±3.95	6680415a1
IL-2	1.39±0.08	1.53±0.01	7110653a1
IL-4	1.03±0.17	1.58±0.24	10946584a1
IL-6*	1.23±0.15	2.14±0.31	13624311a1
IL-10	1.12±0.26	1.06±0.78	6754318a1
IL-12b*	0.86±0.20	5.62±2.08	6680397a1
IL-12 p35*	1.22±0.48	11.22±4.16	Mn00434165_m1†
IL-12 p40*	0.82±0.15	5.63±1.89	Mn01288992_m1†
TNF-α*	0.81±0.17	1.73±0.22	7305585a1
IFN-γ	1.29±0.37	1.41±0.58	33468859a1
NOS2**	1.11±0.41	33.05±10.04	6754872a1
Arg1**	1.16±0.18	3.11±0.09	7106255a1

For each gene, a validated primer set from the Harvard Medical School Primer Bank was used (primer ID http://pga.mgh.harvard.edu/primerbank/index.html). Levels of expression were determined on quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis by using the $2^{-\Delta\Delta Ct}$ method. Each value for Mϕ/βgal and Mϕ/Glipr1 represents the fold changes relative to the value for the nontransduced macrophages (Mϕ, set as 1 for each gene). The data presented as means with standard deviation calculated based on the results of four independent measurements. Analysis of variances between Mϕ/αgal and Mϕ/Glipr1 group were performed using unpaired t-test and P-values were calculated (ANOVA). Significant differences between Mϕ/Glipr1 and Mϕ/βgal expression levels of cytokines are indicated as

* P<0.01 or

** P<0.001.

† Taqman assay was used (Applied Biosystems) for IL-12 p35, IL-12 p40.