Title

Efficient expression of bioactive murine IL12 as a self-processing P2A polypeptide driven by inflammation-regulated promoters in tumor cell lines.

Runing title (50 characters): Inflammation-regulated expression of murine IL12

Authors

Cristina Lorenzo[1,3,*], Gema Pérez-Chacón[2,*], Guillermo Garaulet[1,*], Zulma Mallorquín[1] ,Juan M. Zapata[2] and Antonio Rodríguez[1,#].

1. Department of Molecular Biology, Universidad Autónoma de Madrid, Spain

2. Instituto de Investigaciones Biomédicas "Alberto Sols", CSIC-UAM, Spain

3. Current address: Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares, Spain

*These authors contributed equally to this work.

Correspondence:

Antonio Rodríguez

Facultad de Ciencias, módulo 05, lab 303

Ciudad universitaria de Cantoblanco

28049 Madrid, Spain.

Phone: +34-91-4974869

Fax: +34-91-4974870

E-mail: a.rodriguez@uam.es

Abstract

Interleukin 12 (IL12) is a heterodimeric proinflammatory cytokine that has shown promise as an anti-cancer agent. However, despite encouraging results in animal models, clinical trials involving IL12 have been unsuccessful due to toxic side-effects associated with its systemic administration, prompting investigation into new delivery methods to confine IL12 expression to the tumor environment. In this study we used the self-cleaving property of the 2A peptide to express both codon-optimized murine IL12 subunits (muIL12opt) as a self-processing polypeptide (muIL12opt-P2A). We cloned muIL12opt-P2A driven by different inflammation-induced lentiviral expression systems to transduce murine tumor cell lines commonly employed in syngeneic tumor models. We confirmed the inducibility of these systems in vitro and in vivo and demonstrated the successful expression of both IL12 subunits and the release of bioactive IL12 upon proinflammatory stimulation in vitro. Therefore, IL12 release driven by these inflammation-regulated expression systems might be useful not only to address the impact of IL12 expression in the tumor environment but also to achieve a local IL12 release controlled by the inflammation state of the tumor, thus avoiding toxic side-effects associated with systemic administration.

Introduction

Precancerous and malignant cells can induce an immune response that results in the destruction of transformed cells, a process known as immune surveillance. However, immune surveillance is not always successful, resulting in “edited” tumors. Immunoediting is mediated by an immune selection pressure favoring the development of less immunogenic tumors, which escape recognition by the immune system. Therefore, the immune system has the capacity either to block tumor development and impair established tumors, or to promote carcinogenesis, tumor progression and metastasis. Which of these conditions prevails depends on the balance between the protumor and antitumor mediators of both innate and adaptive immunity(1). This balance is strongly regulated by the tumor microenvironment, which is characterized, among other traits, by inflammation. Nowadays, the role of inflammation in cancer is controversial. On one hand, several immune protumor effector mechanisms are upregulated by chronic inflammation, therefore endorsing the hypothesis of a protumorigenic chronic inflammation, which seems to produce an immune suppressive and tumor-friendly environment, promoting carcinogenesis and tumor growth (2). On the other hand, inflammation is also necessary to create an environment that promotes the induction of antitumor immunity, by the recruitment and activation of several immune effector cells (3).

IL12 is a heterodimeric cytokine composed of two subunits (p35 and p40) covalently linked by a disulfide bridge primarily produced by macrophages, dendritic cells, neutrophils and B cells. When IL12 is secreted, it engages the IL12 receptor (IL12R), also formed by two subunits (β1 and β2) and mostly expressed on activated T and NK cells, dendritic cells and macrophages. IL12-IL12R interaction induces tyrosine phosphorylation of Jak2 and Tyk2, triggering the phosphorylation and activation of STAT4, ultimately leading to the production of IFN-γ, principal mediator of IL12 function. IL12 is one of the main proinflammatory cytokines, being crucial for Th1 differentiation and cytotoxic responses. It induces the production of IFN-γ by T and NK cells, in addition to the proliferation and activation of these immune cells (4). For many years the antitumor effect of IL12 (5) has been known, and its

enhancement of antitumor immunity by acting as a bridge between innate and adaptive immune responses. IL12 induces tumor infiltration, proliferation and activation of effector immune cells (macrophages, NK and T cells), and also inhibits tumor angiogenesis mainly through IFN-γ-dependent production of antiangiogenic factors such as IP10 (6).

Several studies have demonstrated the antitumor activity of IL12 in preclinical models, suggesting its therapeutic use as an anti-cancer agent. However, clinical trials involving IL12 have been unsuccessful due to the toxic side-effects associated with its systemic administration, prompting investigation into new methods of IL12 delivery designed to avoid unacceptable toxicity (7). In this context, gene therapy seems to be a good alternative strategy, as gene transfer methods can be designed to confine IL12 production to the tumor environment, preventing systemic toxicity (8). Several vectors derived from viruses, such as adenovirus (9), adeno-associated virus (10), retrovirus (11) and herpes simplex virus (12), as well as non-viral vectors (13) have been developed to transfer IL12 genes locally to the tumor site, offering encouraging results in preclinical experiences, not only by itself but also when used in combination with other antitumor strategies. In addition, the virus-mimicking effect of these viral vectors should trigger an interferon (IFN)-mediated response, which has proven to be absolutely required for the efficient anti-tumoral effect of IL12, as recently demonstrated by Melero and coworkers using a Semliki forest virus derived vector (14).

An appropriate system for gene therapy in chronic inflammatory processes such as cancer could be a lentivector-based expression system, since lentivectors can infect both dividing and quiescent cells, provide long-term expression and display low immunogenicity. Moreover, an ideal vector system should be disease-regulated, expressing high levels of the transgene only when and where the therapeutic effect of the transgene is required, preventing the toxicity that may be associated with constitutive and systemic expression of the transgene. We have generated a novel inflammation-regulated lentiviral expression system based on the E-selectin promoter (ESELp) that is induced upon acute inflammation. E-selectin is rapidly and transiently expressed in response to early proinflammatory cytokines (TNF-α,

IL1), and is not expressed under basal conditions, making its promoter a good candidate for the design of inflammation-regulated gene therapy vectors (15). We have also generated lentivectors incorporating other previously described inflammation-inducible promoters, such as the human IL6 promoter fused to the enhancer region of the human IL1 promoter (IL1-IL6p) (16) and a chimeric promoter based on NFkB-binding sites (NFkBp) (17). Indeed, we have recently demonstrated that expressing the anti-inflammatory cytokine IL10 with these inflammation-regulated lentiviral expression systems efficiently attenuates zymosan-induced inflammation (15). Since bioactive IL12 is a heterodimer composed of two subunits, p40 and p35, and it has been shown that p40 homodimers are potent IL12 antagonists (18), it is important to develop a system that ensures equimolecular expression of both IL12 subunits to maximize the therapeutic effects of IL12. In this work we have cloned and expressed both murine IL12 subunits as a single coding sequence which is processed into separate subunits during translation by the self-cleaving property of the 2A peptide P2A, thereby guaranteeing their stoichiometric expression. In addition, these IL12 genes are expressed in lentiviral vectors under the control of different inflammation-inducible promoters to confine their expression to inflammation foci. We have studied the inducibility of the inflammation-regulated lentiviral systems and bioactive IL12 production using HEK-293 cells, tumorigenic Lewis lung carcinoma (LLC) cells and melanoma B16-F10 cells.

Materials and Methods

Plasmid constructs: The 19 amino acid 2A region of the picornavirus PTV1 (porcine teschovirus-1), P2A, was generated using the complementary oligonucleotides 2A-1 (5´-GATCCGGAGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGT GGAAGAAAACCCCGGTCCT-3´) and 2A-2 (5´-CTAGAGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGA GAAGTTCGTGGCTCCG-3´) (Sigma-Aldrich). These were annealed to form a duplex with 5' overhang ends and directly cloned into *BamHI-XbaI*-digested pBlueScript to generate the pBS-P2A construct. The murine codon-optimized p35 subunit cDNA (mup35opt) was PCR-amplified from the AG250-DPmuIL12opt plasmid (19) using the oligonucleotides 5´-CGCTCTTCTAGACA**ATG**TGCCAGTCGCGCTACCTCCTCTTC-3´, which added a *XbaI* site (underlined) upstream to the start codon (bold), and 5´-GCGAGAGCGGCCGC**TTA**TCAGGCGGAACTCAGGTAGCCCATC-3´, which retained the stop codon (bold) and added a *NotI* site (underlined) immediately downstream to the coding region. The mup35opt PCR product was further cloned into the *XbaI* and *NotI* pBS-P2A to create pBS-P2A-mup35opt. The mup40opt subunit was also amplified from the AG250-DPmuIL12opt plasmid using the oligonucleotides 5´-CGCTCTGGATCCGCCACC**ATG**TGCCCGCAGAAGCTG ACCATCTCC- 3´, which added a *BamHI* site (underlined) upstream to the start codon (bold), and 5´-CGCAGAAGATCTAATGGA CCGGACCCTGCAGGGGACGC-3´, which removed the stop codon and generated a *BglII* site (underlined) next to the last p40 codon. The mup40opt PCR product was directly cloned into a pGEMT vector (Promega), to generate the pGEMT-mup40opt construct. Next, the P2A-mup35opt sequence was cloned into the pGEMT-mup40opt, using *BglII* and *NotI* restriction sites, obtaining the single ORF mup40opt-P2A-mup35opt encoding for the muIL12opt-P2A. Finally, the mup40opt-P2A-mup35opt cassette was cloned into the *BamHI-NotI* digested pHRSIN HIV 1-derived transfer vectors, including the

ones containing an inflammation-induced promoter (ESELp, IL1-IL6p or NFkBp) and the one containing a constitutive viral promoter (SFFVp) (15). All plasmid sequences were confirmed by sequencing.

Cell culture: Human Embryonic Kidney (HEK-293; ATCC #CRL-1573), murine Lewis Lung Carcinoma (LLC; ATCC #CRL-1642) murine melanoma (B16-F10; ATC #CRL-6475) and RAW 264.7 (ATCC#TIB-71) cell lines were grown in Dulbecco´s modified Eagle´s medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and L-glutamine (2 mM) plus antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). After serum starvation (2%FBS O/N), RAW cells were incubated with lipopolysaccharide (LPS) (2 µg/ml) for 24 hours.

Transient transfection, Brefeldin A treatment and Western Blot: HEK-293 cells were seeded in p100 plates and were transiently transfected by the calcium phosphate method (20). Each plate was transfected with 30 µg total DNA, containing 19 µg of a carrier DNA (pBlueScript), 1 µg of a reporter plasmid (pEGFP-N3; Clontech) and 10 µg of either pHRSIN-SFFVp-muIL12opt-P2A plasmid, AG250-DPmuIL12opt (positive control) or a pHRSIN-SFFVp construct encoding a non-IL12 related transgene (negative control). After 48 hours, the culture media was collected and stored at -70ºC. Fresh culture medium containing the protein transport inhibitor Brefeldin A (BD GolgiPlug^{TM}, Biosciences; cat. #555029) was added to the transfected cells. After 6 hours of treatment, transfected cells were harvested and lysed with Laemmli Buffer for Western Blot analysis. In brief, protein samples were separated on 12% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Whatman). After blocking the membranes with 5% w/v skimmed milk in TBS-T (0.05% Tween-Tris Buffered Saline) for 1 hour at room temperature, the blots were incubated overnight at 4ºC with the corresponding first antibody solution: anti-mouse IL12 antibody (0.1%, v/v; R&D Systems; cat. #AF-419-NA), rabbit polyclonal anti-2A peptide (0.05%, v/v; Millipore; cat. #ABS31); mouse monoclonal anti-β-tubulin (0.1%, v/v; Sigma-Aldrich; cat. #T6074), in TBS-T with 5% skimmed milk. After several washes with TBS-T, blots were incubated with peroxidase-labeled goat anti-rabbit or anti-mouse IgG (0.02%, v/v; Pierce; cat. #4160 and #31430, respectively) in TBS-T with 5% skimmed milk for 1 hour at

room temperature. The blots were then washed again with TBS-T and membrane-bound antibody was detected with ECL (enhanced chemiluminescence) detection reagent (GE Healthcare).

Second generation lentivector production and titration: HEK293 cells were transiently transfected by the calcium phosphate method (20). For viral particle production, the indicated pHRSIN HIV 1-derived transfer vector was cotransfected with two helper plasmids, the 8.91 packaging vector (21) and pMD2-G (VSV-G containing plasmid; Addgene). Supernatants were collected 48 hours after transient transfection and cell debris was removed by centrifugation (10 minutes, 740 x g, 4ºC). Viral particles were concentrated by ultracentrifugation in a swing bucket rotor for 2 hours at 121,986 x g at 4ºC (Ultraclear Tubes, SW28 rotor and L8-70 Ultracentrifuge; Beckman Coulter). After supernatant removal, viral particles were resuspended in phosphate-buffered (PBS) and stored at -70ºC. Total viral content was determined by quantitative PCR (22).

Transduction and induction assay: LLC, B16-F10 and HEK293 cells were seeded in 6-well plates ($5x10^5$ cells per well) and transduced for 5 hours with different lentiviral particles at the indicated multiplicity of infection (MOI). They were then passaged into 24-well plates and incubated for 12 hours under serum deprivation (0.5% FBS for LLC and 2% FBS for B16-F10 and HEK293) before proinflammatory stimulation with tumor necrosis factor (TNF-α) (100 ng/ml) and IL-1β (10 ng/ml) (Peprotech). After 6 hours of stimulation, supernatants were collected and cells were lysed for further analysis. Each experiment was performed at least 3 times.

Luciferase assay: To determine luciferase activity, cells transduced with the luciferase-containing viral particles (pHRSIN-LUC) were collected after stimulation, washed with PBS and lysed with Reporter Lysis Buffer (RLB Promega). Supernatants were saved and employed to measure luciferase activity in a $20/20^N$ luminometer (Turner BioSystems) and for protein quantification (Bradford; Bio-Rad). Reporter gene expression is shown as relative light units (RLU) per microgram of protein.

Lymphoblast proliferation assay: Spleens from C57/BL6 mice (n=4) were mechanically processed and mononuclear murine cells were isolated by density gradient centrifugation (Lympholyte-M; Cedarlane Laboratories, Burlington, NC). Splenocytes were resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 µM 2-mercaptoethanol (2-ME), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and incubated in 96-well plates (5 x 10^4 cells per well) with concanavalin A (ConA, 4 µg/ml) for 30 hours at 37ºC. After this incubation, different amounts of codon-optimized murine IL12 were added and cell survival (proliferation) was determined 48h later using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Recombinant muIL12 (Peprotech) was used as a positive control.

Interferon gamma release: Splenocytes from C57/BL6 mice (n=3) were isolated as described above andsubsequently incubated for 30 hours in the presence of ConA (4 µg/ml), washed with PBS and cultured in 96-well plates (5 x 10^4 cells per well) in triplicates with different amounts of codon-optimized murine IL12. After 48 h supernatants were harvested and stored at -20ºC until use. In parallel, cell viability in these conditions was measured using the CellTiter-Glo Luminescent Cell Viability Assay. Recombinant muIL12 (Peprotech; cat. #210-12) was used as a positive control. Supernatant from cells cultured in the absence of ConA was used as a negative control of interferon production.

ELISA: Cell culture supernatants were collected for IL12 and IFNγ detection by employing mouse IL12 ELISA Kit (Thermo Scientific; cat. #EMIL 122) and mouse IFNγ Quantikine ELISA Kit (R&D systems; cat. #MIF00), respectively. Samples were measured in a microplate spectrophotometer (xMark; Bio-Rad) following manufacturer's recommendations.

Animals: Six-week-old female C57/BL6 mice (Charles River, Burlington, MA) were fed lab chow and kept on a 12 hours light/dark cycle. The animals were cared for according to the UAM Animal Facility guidelines for the care and use of laboratory animals.

Statistical analysis: Prism 5 for Windows (GraphPad Software Inc.; version 5.03) was employed for statistical analysis. Data shown in figures 1-4 were analyzed by *t*-test (unpaired, two-tailed test) compared to control or untreated samples. Data shown in figure 5 were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test. EC_{50} was calculated by Gaddum/Schild EC50 shift. Statistical significance was assigned at $P<0.05$. SD, standard deviation.

Results

Generation of muIL12opt-P2A self-processing construct

As human IL12 is inactive in mice and mouse IL12 is active in both humans and mice (23), we decided to clone codon-optimized murine IL12 subunits (muIL12opt) (19) as a single coding sequence and employ the self-cleaving property of the P2A peptide to express this heterodimeric cytokine as a self-processing polypeptide. A similar strategy has been successfully employed with bovine (24) and ovine (25) IL12. The 2A peptide was first discovered in the foot-and-mouth disease virus (FMDV) (26). It allows the coordinated co-expression of multiple proteins from a single transcript and, even more importantly, enables the co-expression of the two IL12 subunits in stoichiometric amounts (27). In brief, we first designed the 2A coding oligonucleotides for the porcine teschovirus-1 2A peptide (P2A), annealed them and cloned them into a plasmid. Furthermore, we performed PCR amplification of the sequences encoding the murine IL12 subunits, p35 and p40, from a codon-optimized muIL12-containing construct (muIL12opt) (19) (Figure 1a; top). Next, both PCR products were subcloned into different intermediary plasmids to finally generate the single ORF p40-P2A-p35 coding for the codon-optimized self-cleaving murine IL12 (muIL12opt-P2A). This cDNA cassette was further cloned into a HIV 1-derived transfer vector (pHRSIN) under the transcriptional control of the constitutive viral promoter from Spleen Focus Forming Virus (SFFVp) (Figure 1a; bottom).

Efficient murine IL12 expression

To assess whether we had successfully cloned murine IL12 and if the interleukin was correctly expressed, we first performed a transient transfection experiment in HEK293 cells. For this experiment, we employed the pHRSIN transfer vector that contains the muIL12opt-P2A cassette under the control of SFFVp. As a positive control, we used the parental codon-optimized muIL12-containing construct (muIL12opt). In addition, we employed a pHRSIN-SFFVp construct encoding a non-IL12 related transgene as a negative control. Cells were transfected by the calcium phosphate method with one of

these pHRSIN-based constructs. To test whether IL12 was successfully released, the culture medium collected from transfected cells was analyzed by ELISA (Fig. 1b). To confirm the correct expression of both IL12 subunits, we analyzed the transfected cells by western-blotting. Since IL12 is a secreted cytokine, to detect it in cell extracts, intracellular protein transport must be blocked to accumulate IL12 within the Golgi complex (28). Thus, after collecting the culture medium 48 hours post-transfection, cells were treated with Brefeldin A, a protein transport inhibitor that blocks protein release. After treatment, cells were harvested and total cell extracts were analyzed by western blotting using an anti-IL12 antibody that recognizes both IL12 subunits, p40 and p35 (Figure 1c). As a protein loading control we used an antibody specific for β-tubulin. These cell extracts were also tested for P2A expression by employing an anti-2A peptide antibody. As expected, we were able to detect the P2A peptide fused to p40 subunit only in the total cell extracts from cells transfected with the muIL12opt-P2A-containing construct (last lane). As CMV promoters are more transcriptionally active than SFFVp, lower IL12 levels were expressed when the lentivector was employed (Fig. 1b-c). These results show that muIL12opt-P2A is successfully expressed and efficiently released. They also suggest that the P2A sequence does not interfere with the heterodimeric conformation of IL12.

Inducibility of the inflammation-regulated promoters in human and murine tumor cell lines

Since we want to express the muIL12opt-P2A transgene under the control of inflammation-regulated promoters we first tested the inducibility of the different expression systems in a variety of tumor cell lines, including HEK293 human cells, murine Lewis lung carcinoma (LLC) and murine B16-F10 melanoma cells (29). To address this, we employed pHRSIN transfer vectors encoding the luciferase reporter gene under the control of different inflammation-induced promoters: the E-Selectin promoter (ESELp), the IL1-IL6 hybrid promoter (IL1-IL6p) and the synthetic 6x NFkB promoter (NFkBp) (15). As a control, a pHRSIN vector expressing luciferase under the control of the constitutive SFFVp was used. The different cell lines were transduced with these lentivectors and then stimulated for 6 hours, harvested and cell extracts were made to measure luciferase activity and protein content. We first

employed TNF plus IL-1β as we have previously shown that these proinflammatory cytokines are able to induce these inflammation-regulated promoters in other cell lines (15). As shown in figure 2a, luciferase activity from the constitutively active SFFVp was not affected by the treatment, while proinflammatory treatment increased the luciferase activity in the three transduced cell lines when the inflammation-regulated promoters were employed. Notably not all inflammation-inducible promoters responded similarly in the different cell lines. In this regard, we observed that all promoters efficiently responded to the inflammatory stimulus in LLC cells. By contrast, transduced B16-F10 cells showed low luciferase activity and low fold induction with the ESELp, while the IL1-IL6p failed to induce any luciferase activity in response to the inflammation stimulus. In these cells, the NFkBp was also efficiently stimulated by TNF and IL1 (Figure 2a middle). HEK293 cells showed similar results to those obtained with LLC cells, with the exception of the IL1-IL6p, which failed to respond to the stimulus (Figure 2a right). When we compared the transcriptional inducibility of the different inflammation-inducible promoters, we found that the NFkBp displays the highest basal luciferase activity in all cell lines tested; in all cases, the NFkB-controlled luciferase activity was further increased upon proinflammatory stimulation. Importantly, the ESELp displays very low basal luciferase activity in all cell lines assayed, which was consistently increased after proinflammatory stimulation, showing the highest fold induction in both LLC and HEK293 cell lines. The IL1-IL6p showed the lowest luciferase activity with no, or modest fold induction in all cell lines tested. To further confirm these results, we incubated LLC and B16F10 transduced cells with a cytokine cocktail-containing media collected from LPS-treated RAW cells. We found that treatment with these murine cytokine cocktails mimicked the results obtained with TNF plus IL-1β (Figure 2b). As LLC express TLR4 (30), LPS treatment was able to increase the luciferase activity in LLC transduced cells (Figure 2b, top). Altogether these results demonstrate that ESELp and NFkBp are transcriptionally upregulated upon proinflammatory treatment of transduced LLC and B16F10 cells.

Induction of Interferon gamma (IFNγ) is one of the antitumoral activities of IL12. As this cytokine will be present within the tumor, we tested whether this cytokine was able to upregulate the inflammation-regulated promoters. An IFNγ-dependent induction would generate a problematic activation loop in terms of therapeutic applications. As shown in figure 2c, IFNγ did not induce the luciferase activity under the control of these inflammation-regulated promoters. Therefore, the transcriptional activity of these promoters will not be affected by the presence of IFNγ within the tumor microenvironment.

The ESELp and NFkBp inflammation-regulated promoters are upregulated within the tumor microenvironment.

We decided to test whether these promoters were also induced in the tumor microenvironment. For this we employed LVs encoding the luciferase transgene under the control of the inflammation-induced promoters (ESELp, NFkBp) or the constitutive viral promoter (SFFVp) to transduce LLC cells (Figure 3). As in vivo experiments last for several weeks and silencing of transgene expression may occur, we first tested if the promoter inducibility was maintained. For this, we transduced LLC cells, kept them in cell culture and treated them with either LPS or TNFα plus IL1β at different times (Figure 3a). We found that ESELp and NFkBp were efficiently induced even at 38 days post-transduction, a period of time longer than that needed for the in vivo experiments. Therefore, we decided to test whether these inflammation-regulated promoters were upregulated in vivo. For this, LLC cells were transduced with the indicated luciferase-encoding LVs and implanted subcutaneously into mice. Tumor burden and bioluminescence (Figure 3b and 3c, respectively) were monitored during 28 days. We found that the transduced cells successfully engrafted after implantation. We observed that at late time points the tumor volumes were smaller than those observed when control cells were employed (i.e., non-transduced LLC). Regarding the in vivo luciferase activity, the results show that luciferase expression under the control of ESELp and NFkBp was induced 21 days after implantation (Figure 3c). At the end of this in vivo experiment

(day 28th), tumors were isolated and the luciferase activity was measured in tumor homogenates. As shown in figure 3d, in the case of NFkBp none of the tumors displayed luciferase activity. In the case of ESELp, only two out of four tumors still showed very low luciferase activity. These results indicate that these inflammation-regulated promoters are transiently upregulated within the tumor environment. As transcriptional activity of these promoters is upregulated by pro-inflammatory cytokines, these results suggest that in LLC-based tumors inflammation is transiently upregulated within tumor microenvironment.

Efficient murine IL12 release upon proinflammatory stimulation

Once we had determined the inducibility of the inflammation-regulated promoters, we proceeded to clone the muIL12opt-P2A coding sequence in the inflammation-regulated lentivectors (Fig 4a), as inflammation is one of the main characteristics of tumor environment and IL12 has shown potential as an anti-cancer agent. We generated the lentiviral particles encoding the muIL12opt-P2A under the control of the different inflammation-regulated promoters (ESELp, IL1-IL6p and NFkBp) or a constitutive promoter (SFFVp) and used them to transduce HEK293, LLC and B16-F10 tumor cells. As in the previous experiments, transduced cells were treated for 6 hours with TNF plus IL-1β. After treatment, cell culture medium from transduced cells was collected and the IL12 concentration was measured. As expected, cells transduced with the constitutive strong viral promoter SFFVp released high levels of IL12 (Figure 4b-d). Regarding the inducible promoters, the IL12 levels detected mostly correlated with the inducibility observed in the luciferase assays (Figure 2). Thus, the HEK293 and LLC cell lines showed inflammation-dependent IL12 induction when ESELp and NFkBp were employed (Figure 4b and 4d). In the case of B16-F10 cells, we only detected significant amounts of IL12 when NFkBp was employed (Figure 4c). Similar to the results shown in Figure 2, NFkBp supported the highest fold induction of IL12 expression in the three cell lines assayed. However, ESELp showed the lowest basal

activity while its activity was consistently increased upon proinflammatory stimulation. These features (low basal activity and high fold induction) are particularly important for biomedical application to reduce toxicity associated with constitutive and systemic IL12 release.

muIL12-P2A induces murine lymphoblast proliferation and IFNγ release.

Although the fusion of P2A to muIL12 did not appear to adversely affect expression of the IL12 subunits and the heterodimeric interleukin is secreted correctly from the cells, thus suggesting its proper folding, we performed a functional assay to test its bioactivity. We collected supernatants from HEK293 cells containing either the parental muIL12opt or the P2A-based IL12 (muIL12opt-P2A) and tested them in a proliferation assay using ConA-stimulated murine lymphoblasts (Figure 5). As a positive control, recombinant and purified murine IL12 (muIL12R) was employed in these proliferation assays. The results show that all murine IL12 samples employed were able to induce proliferation of ConA-stimulated murine lymphoblasts in a dose-dependent manner (Figure 5a). Similar results were obtained with α-CD3-stimulated murine lymphoblasts (data not shown). Notably, although similar amounts of IL12 were used, muIL12opt and the P2A-based IL12 were more efficient in inducing proliferation (2.1 fold) than recombinant purified IL12 (1.6 fold); the difference could be due to loss of specific activity during purification. Finally, to further characterize these cytokines we determined the half maximal effective concentration (EC_{50}) of each murineIL12. As shown in figure 5b, muIL12opt-P2A showed the lowest EC_{50} (0.16 ng/ml), closely followed by the muIL12opt (0.27 ng/ml). As expected, the purified muIL12R showed a significantly higher EC_{50} (8.07 ng/ml).

Treatment with IL12 induces IFNγ release, which is essential for the anti-tumor activity of IL12. Therefore, in addition to lymphoblast proliferation, we also measured the amount of IFNγ released by ConA stimulated murine lymphoblasts and further treated with IL12. Freshly isolated splenocytes were first stimulated with ConA and then incubated for 48h with increasing concentrations of IL12. As shown

in figure 5c, we found that incubation with IL12 induced IFNγ in a dose-dependent manner. As in the case of cell proliferation, recombinant purified IL12 was less efficient and the amount of IFNγ was lower than that induced by muIL12opt and muIL12opt-P2A. Overall, these results confirmed that 2A self-processing murine IL12 is bioactive and efficiently induces lymphoblast proliferation and IFNγ release.

Discussion

The production of IL12 requires the coordinated expression of p35 and p40 subunits to form the functional heterodimeric interleukin. The biosynthesis of IL12 depends on the interaction of its p35 and p40 subunits, not only to form the functional heterodimer but also because these two subunits regulate each other. For example, it has been reported that the p40 subunit stabilizes p35 and promotes its secretion (19). In the present work, we have used an expression-optimized IL12 plasmid (muIL12opt) described by Dr. Felber and coworkers (19), which encodes significantly higher levels of bioactive IL12 compared to the wild-type IL12 sequences. We have generated a P2A-based IL12 construct encoding both IL12 subunits in a single coding sequence in order to achieve concomitant, stoichiometric production of both IL12 subunits and high levels of bioactive interleukin. The 2A peptide was first discovered in the foot-and-mouth disease virus (FMDV), which encodes a single ORF in which two of the gene products are separated by the short 2A sequence, F2A (19 aa) (26); it is now known that other picornaviruses also have a 2A peptide, such as the porcine teschovirus-1 (PTV1), whose 2A peptide (P2A) has been used in this work. During its translation, P2A interacts with the exit tunnel of the ribosome to induce “skipping” of the last peptide bond at the C-terminus of 2A, so that the ribosome is able to continue translating the downstream gene product, after releasing the first protein fused at its C-terminus to 2A. This approach provides a tool to allow the coordinated co-expression of multiple proteins from a single coding sequence and enables the achievement of stoichiometric production (27) of the subunits. This is especially important in the case of IL12, whose bioactive form requires equimolar expression of the two separate genes encoding p40 and p35 and the subsequent formation of the heterodimeric complex, since p40 homodimers are potent IL12 antagonists (18). This makes the P2A strategy more suitable for the expression of heterocomplexes such as IL12 than other multicistronic strategies such as use of internal ribosome entry sites (IRES), which allow translation to be initiated from downstream translational start codons but often with reduced efficiency, leading to unequal expression of the gene products. In addition, this strategy reduces the size of the vector since

it eliminates the need of alternative promoters and regulatory sequences. Additional advantages of the P2A approach are that 2A-based exon skipping has been observed to occur in all tested eukaryotic systems, and that the fusion of the small size P2A sequence neither interferes with the functionality of the chimeric protein nor displays immunogenicity in immunocompetent individuals (31). Our results show that 2A cleavage occurred at its C-terminus, similarly to its role in the processing of PTV1 polypeptide, as we were able to detect the mup40 subunit fused to P2A in the transient transfection experiment in HEK 293 cells by western blotting (Figure 1c). The cellular processing of muIL12opt was apparently successful in all the tumor cell lines tested: HEK293, LLC and B16-F10, as transduced cells produced and released functional heterodimeric IL12 (p70), measured by ELISA (Figure 4). IL12 bioactivity was further demonstrated in lymphocyte proliferation experiments (Figure 5). Thus, our results confirm that the IL12 polypeptides are processed normally and secreted from the cell and that the P2A sequence employed does not interfere with heterodimeric IL12 folding, secretion and activity.

Generally, IL-12 based therapies can be divided into three categories: active non-specific immunotherapy [aimed at activation of predominantly innate mechanisms], active specific (vaccine) approach [directed mainly to the stimulation of adaptive antitumor response] and gene therapy. Up to 58 clinical trials based on IL-12 therapy have been started or completed to date (32). Recombinant IL-12 has shown relevant antitumor activity both in experimental models and in humans. However, its clinical use has been hampered by dose-limiting side effects after systemic delivery of the recombinant protein. Hence, the rationale for the new IL12-based gene therapy is that local expression of this cytokine may result in enhanced antitumor activity and reduced toxicity. In this regard, a number of local gene therapy approaches have been undertaken in veterinary clinical oncology (33). By employing viral and non-viral gene delivery methods local antitumor effects have been achieved in cats, horses and dogs. They demonstrate that IL-12 based gene therapy is an effective approach. Altogether these results support and upgrade the antitumor IL-12 features observed in preclinical experiments performed in rodents. Most recently initiated IL-12-based clinical trials are focused on local tumor treatment by

gene therapy (32). These strategies are trying not only to minimize the IL-12-dependent toxic effects but also to induce specific antitumor mechanisms by overcoming the strong immunosuppressive tumor microenvironment (34). In this regard it is important to mention that the inflammation-regulated promoters ESELp and NFkBp are still induced in the presence of IL10 [(15) and data not shown], a potent immunosuppressive cytokine commonly expressed in tumors. Therefore, their transcriptional regulation will not be hampered by the presence of this immunosuppressive factor. Clinical trials based on intratumoral IL-12 expression have proven that local production of IL-12 inside a tumor can stimulate tumor infiltration by effector immune cells and that in some cases this is followed by tumor regression. Therefore IL-12 is still considered as an anticancer cytokine and its importance in cancer immunotherapy keeps growing.

Systemic expression of IL12 has proved to cause significant toxicity and negative side-effects (7). Thus, a major challenge in the treatment of cancer using IL12 is the development of expression systems restricted to and tightly regulated by the tumor environment, in order to confine transgene expression only to the tumor site. We have tested three different lentiviral expression systems based on different inflammation-induced promoters (ESELp, IL1-IL6p and NFkBp) in different cell lines, including two commonly employed in syngeneic mouse tumor models (LLC and B16-F10). Our results show significant differences in terms of promoter activity, fold induction and IL12 production among the different cell lines and promoters (Figure 4). Among them, the IL12 concentration obtained with the ESELp-based system upon stimulation (480 pg/ml in LLC; 160 pg/ml in HEK-293) might be enough to induce IL12-dependent antitumor immunity. This expression system would fulfill the requirements of an inflammation-induced system as it produces very low IL12 in basal conditions (90 pg/ml in LLC, 25 pg/ml in HEK-293), thus preventing toxic side-effects in the absence of inflammation. In B16-F10 cells, the basal IL12 production of the NFkBp-based system was very low (39 pg/ml) while it was significantly increased after proinflammatory stimulation (666 pg/ml); therefore, the NFkB-based system would be the best option for this cell line. These long-term lentiviral expression systems might be useful not only

to address the impact of IL12 expression in the tumor environment but also to achieve local IL12 release controlled by the inflammation state of the tumor, hopefully avoiding toxic side-effects associated with systemic IL12 administration.

Recent preclinical studies in solid tumor models have shown that the level of IL12 expression is essential for tumor clearance and protection (11). For this, the authors isolated and characterized IL12-producing tumor cell clones. Once the bioactivity of muIL12opt-P2A has been confirmed in cell culture-based assays and the inducibility of the promoters has been tested in vivo, we will test the bioactivity of the muIL12opt-P2A in mice by employing IL12-producing clones from transduced LLC and B16-F10 cells. We will employ them to perform syngenic tumor experiments, in which LLC or B16-F10 clones transduced with the selected inflammation-inducible lentivector encoding muIL12opt-P2A will be subcutaneously implanted in mice to examine the protective role of the induced IL12 expression in tumor growth. It would be important to address the mechanisms implicated in this effect and determine which immune cell populations are required to trigger it. It will also be important to study whether long-term protection can be establish by immune memory, to find out how many IL-12-secreting cells are able to protect mice from tumor development and to determine how much IL12 is needed. These experiments will constitute a proof-of-concept to study the antitumoral efficacy of our inducible LV systems after local intratumoral administration.

Although gene therapy approaches exploiting the antitumoral activity of IL-12 have not yet achieved forecasted clinical success, they shed some light to design future strategies. The most relevant concerns to explain the disappointing results in those trials were the duration and intensity of transgene expression, as they employed short term expression vectors expressing IL-12 under the control of non-inducible promoters (35-37). Therefore, new vectors with regulated, long-term production of IL-12 might have better results and deserve clinical testing. The LV-based expression systems here described fulfill these two features, long term and inducible transgene expression; therefore we think it is worth employing them in preclinical experiments to test their in vivo efficacy after intratumoral administration.

We will address the therapeutic role of inducible IL12 expression in syngenic tumor growth by employing the wild type tumor cell lines, LLC and B16F10, followed by in vivo intratumoral injection of the inflammation-regulated lentiviral systems encoding muIL12opt-P2A. For future clinical applications, our expression systems can be transferred to vector platforms safe enough for human applications.

An important characteristic of IL-12 is that it synergizes with several other cytokines (38). The fact that IL-12 as a monotherapy displayed limited clinical efficacy raised the investigation of combined treatments. A number of combined approaches have been tested, but only a few showed encouraging results (32). Nevertheless, despite these results, IL-12 remains as a recognized anticancer agent with a great potential for synergistic combinations with other immunotherapies and/or conventional cancer treatments (39,40).

Finally, there is increasing evidence showing the importance of host responses to viral vectors for successful experimental cancer therapies. There is evidence of IL12 and IFN interplay in the control of tumor growth (6,38). In this regard, it has recently been shown by Melero and coworkers (14) that the antitumor efficacy of a virally expressed murine IL12 was strongly dependent on the induction of the IFN response. These results suggest the need of viral vectors mimicking a viral infection to trigger an IFN response needed to sustain efficient IL12 anti-tumor activity.

In summary we have produced viral vectors expressing p2A-dependent stoichiometric amounts of p40 and p35 IL12 subunits under the control of inflammation-dependent promoters. These vectors meet the criteria of 1) producing equimolar amounts of IL12 subunits that are secreted as bioactive IL12, 2) restricting their expression to inflammation sites. In addition, the virus mimicking effect of these viral vectors would likely trigger the IFN-response needed for efficient IL12 dependent immunotherapeutic effects, improving its anti-tumor properties.

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Conflict of interest

The authors declare no competing financial interests.

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Figure legends

Figure 1. Murine IL12 is successfully expressed as a P2A self-processing polypeptide. (**a**) Schematic representation of the murine IL12-encoding plasmids. The murine codon-optimized sequences encoding the IL12 subunits (p35 and p40) were amplified by PCR from the muIL12opt plasmid and employed to generate the single ORF mup40opt-P2A-mup35opt sequence (muIL12opt-P2A). The sequence of the P2A peptide (bold and underlined) flanked by the linker (gray) and murine IL12 specific (bold) sequences are detailed. Arrowhead indicates the cleavage site. (**b**,**c**) HEK 293 cells were cotransfected with a GFP-encoding reporter plasmid plus either the parental muIL12opt plasmid, a control plasmid (Control) or the P2A-based muIL12 construct, muIL12opt-P2A. (**b**) At 48h post-transfection, culture medium was collected and assayed for IL12 by ELISA. The IL12 concentration was calculated based on a recombinant murine IL12 (p70) standard curve. (**c**) After collecting the culture medium, transfected cells were treated with Brefeldin A (protein transport inhibitor) for 6 hours. Cell extracts were analyzed by western blotting using either anti-IL12, anti-2A peptide, anti-GFP or anti-β-tubulin antibodies. huCMV=human Cytomegalovirus promoter; siCMV=simian CMV; SFFVp=Spleen Focus Forming Virus promoter. ***P*< 0.01, and ****P*< 0.001 versus control; n=3.

Figure 2. Inflammation-regulated promoters are induced upon pro-inflammatory stimulation of transduced LLC and B16-F10 tumor cells. The indicated lentiviral vectors encoding the luciferase reporter transgene under the control of the inflammation-induced promoters (ESELp, IL1-IL6p, NFkBp) or the constitutive viral promoter (SFFVp) were employed to transduce LLC (**a-c**), B16-F10 (**a-c**) and HEK-293 (**a**) cell lines (MOI=1.0). After serum deprivation, cells were incubated with tumor necrosis factor (TNF) (100ng/ml) plus IL1β (10ng/ml) (**a,c**), LPS (**b**,**c**), supernatant from LPS-treated RAW cells (RAW SN) (**b**) or Interferon γ (**c**) for 6 hours, and the luciferase activity was determined after cell harvesting. Reporter gene expression is shown as relative light units (RLU) per microgram of protein (a,c) or fold induction (b). Numbers in panel a indicate the fold induction. **P* < 0.05, ***P*< 0.01, and

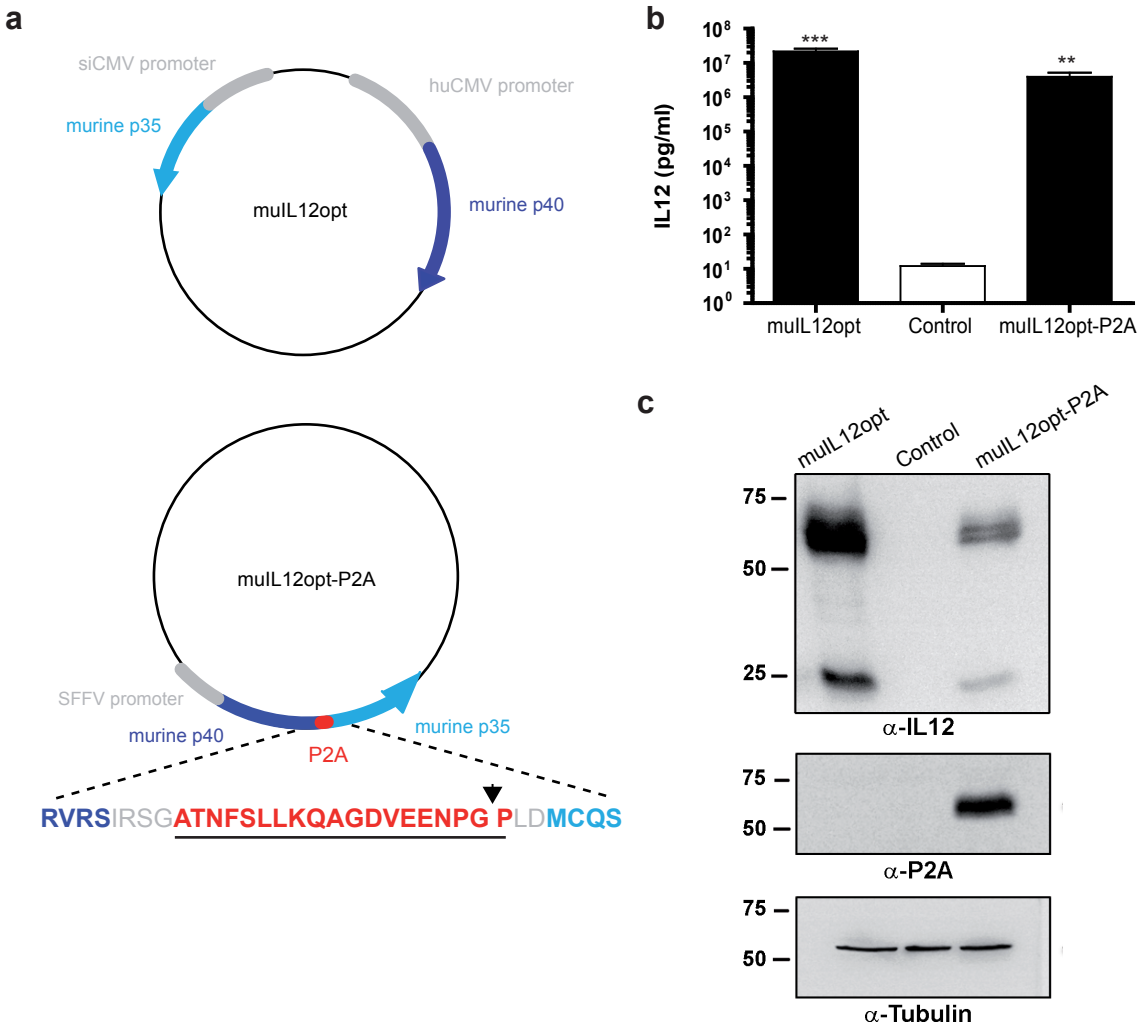
***P< 0.001 versus untreated cells; n=3. ESELp=E-selectin promoter; IL1-IL6p= IL1 enhancer-IL6 promoter; NFkBp=6xNFkB sites; SFFVp=Spleen Focus Forming Virus promoter.

Figure 3. Intratumoral upregulation of inflammation-regulated promoters in vivo. The indicated lentiviral vectors encoding the luciferase reporter transgene under the control of the inflammation-induced promoters (ESELp, NFkBp) or the constitutive viral promoter (SFFVp) were employed to transduce LLC cells (MOI=1.0). Transduced cells were either grown in cell culture (**a**) or implanted ectopically in mice (**b-d**). (**a**) At the indicated day post-transduction (17, 25, 38), transduced cells were deprived of serum and incubated with either LPS or tumor necrosis factor (TNF) (100ng/ml) plus IL1β (10ng/ml) for 6 hours, and the luciferase activity was determined after cell harvesting. Reporter gene expression is shown as relative light units (RLU) per microgram of protein. *P < 0.05, **P< 0.01, and ***P< 0.001 versus untreated cells; n=3. (**b-d**) Transduced LLC cells (7.5 x 10^5) were implanted subcutaneously in C57BL6 mice (n=4 per group) and tumor growth (**b**) and luciferase activity (**c-d**) were followed up for 28 days. Non-transduced LLC cells were employed as a control (n=4). (**b**) Tumor diameters were measured and total tumor volumes were calculated. Plots show tumor volume calculated by the measured tumor diameter. Each line represents the individual growth of tumor volume for each animal. (**c**) In vivo luciferase activity was monitored at the indicated days post-implantation by employing an IVIS. Scatter plots show in vivo luciferase activity (flux) of each animal. (**d**) Twenty-eight days after LLC implantation, animals were euthanizedand luciferase activity was measured in tumor homogenates. Scatter plots show RLU (relative light units) per microgram of protein (μg).

Figure 4. The P2A self-processing IL12 is released upon pro-inflammatory stimulation of transduced murine tumor cells. (**a**) Schematic representation of the different transfer plasmids generated. The muIL12opt-P2A coding sequence was cloned in constructs to place it under the control of the inflammation-regulated promoters, ESELp, IL1-IL6p and NFkB. (**b**-**d**) The indicated transfer vector was employed to generate lentiviral particles to transduce LLC (**a**), B16-F10 (**b**) and HEK 293 (**c**)

cells (MOI=10.0 for LLC and B16-F10; MOI=1.0 for HEK 293). After serum deprivation, cells were incubated with tumor necrosis factor (TNF) (100ng/ml) plus IL-1β (10ng/ml) for 6 hours, and supernatants were collected for IL12 detection by ELISA. The IL12 concentration was calculated based on a recombinant murine IL12 (p70) standard curve and expressed as picograms of IL12 per ml. One representative experiment is shown. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus untreated cells; n=3.

Figure 5. Murine IL12 induces murine lymphoblast proliferation and IFNγ release. (**a-b**) Purified mononuclear cells ($5x10^4$) from spleens of C57 mice (n=4) were stimulated with concanavalin A (ConA, 4 µg/ml) for 30 hours, and then co-stimulated with murine IL12-containing supernatants from HEK 293 transfected cells with either muIL12opt-P2A or muIL12opt. Commercially available recombinant murine IL12 (muIL12R) was used as a positive control. Forty eight hours after muIL12 stimulation, increase in cell number was determined using the CellTiter-Glo Luminiscent Assay from Promega. (**a**) Proliferation ratio between co-stimulated (IL12+ConA) and ConA only treated mononuclear cells (n=4). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus lowest IL12 dose employed. (**b**) EC_{50} value determination. GraphPad Prism (version 5.01) was used for the analysis and for the EC_{50} value calculation. Samples were measured in triplicates. Statistical analysis shows mean ± standard error of the mean (n=4). (**c**) Purified mononuclear cells ($5x10^4$) from spleens of C57 mice (n=3) were stimulated with concanavalin A (ConA, 4 µg/ml) for 30 hours, washed, and then co-stimulated with murine IL12-containing supernatants as above. Forty eight hours later supernatants were collected and the concentration of IFNγ measured by ELISA.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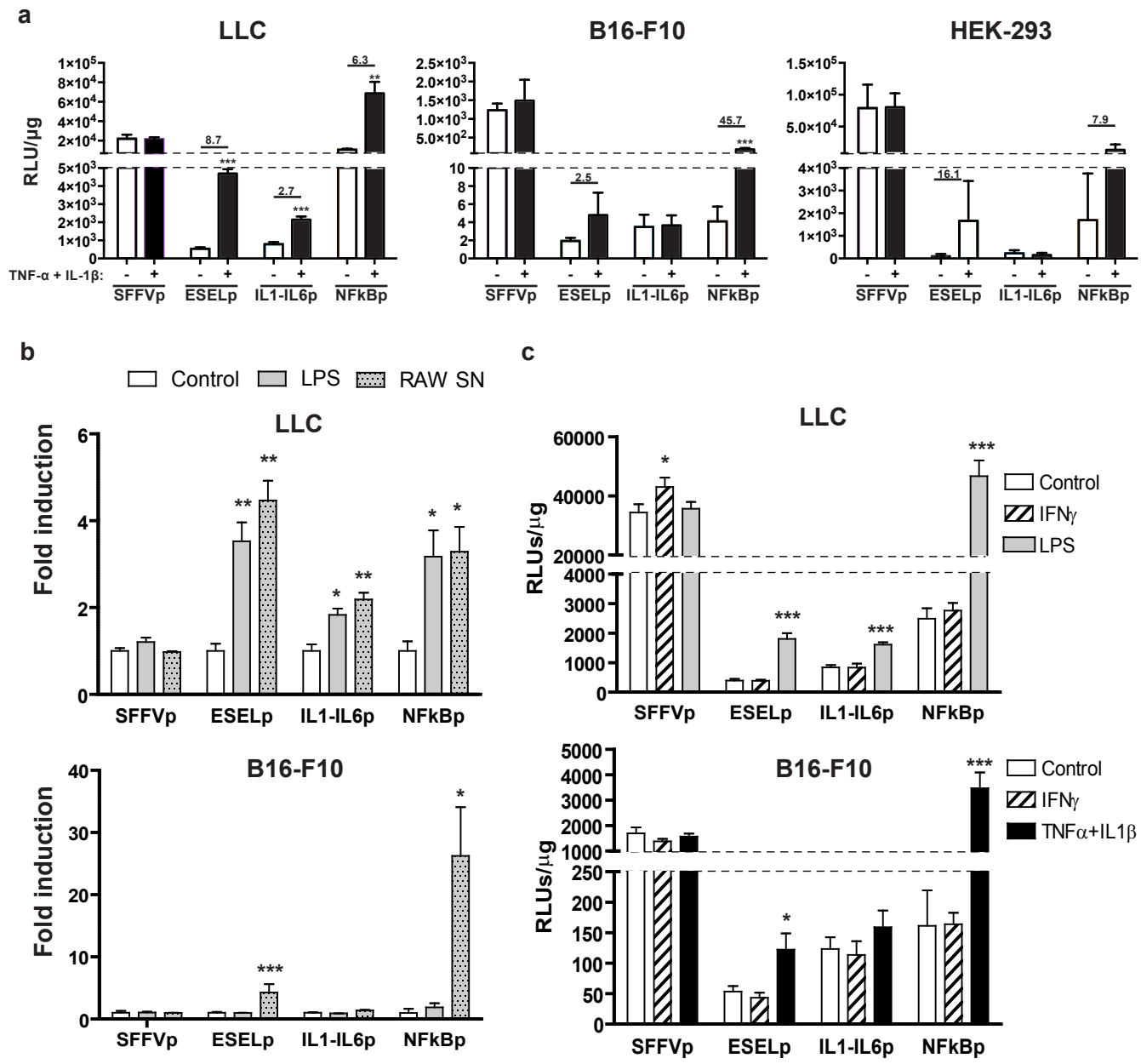
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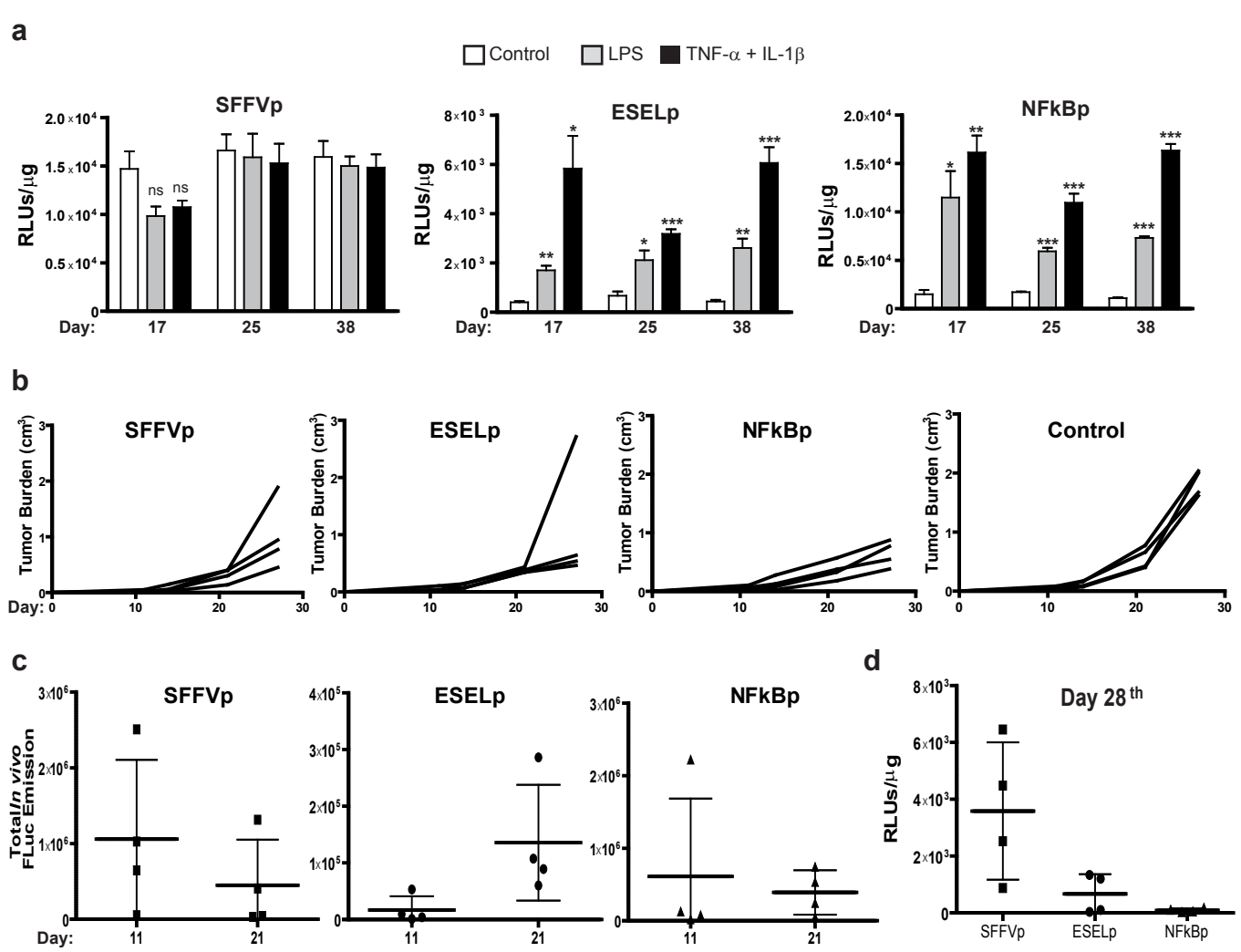
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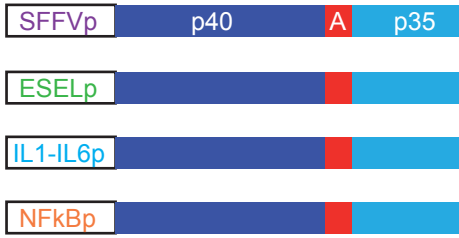
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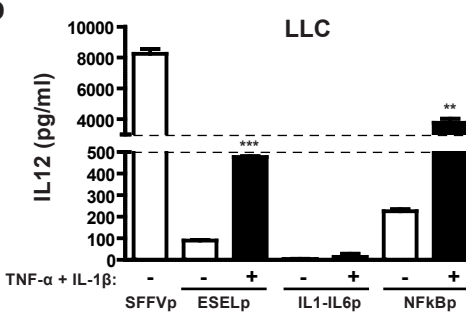
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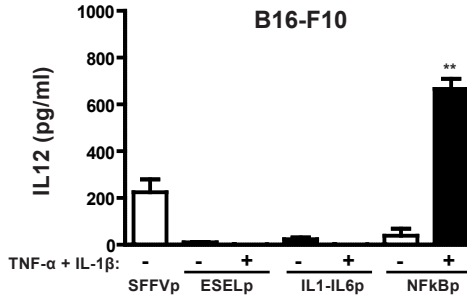
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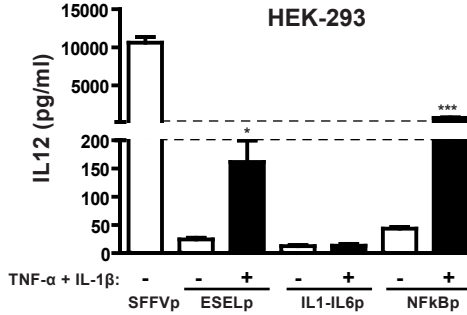
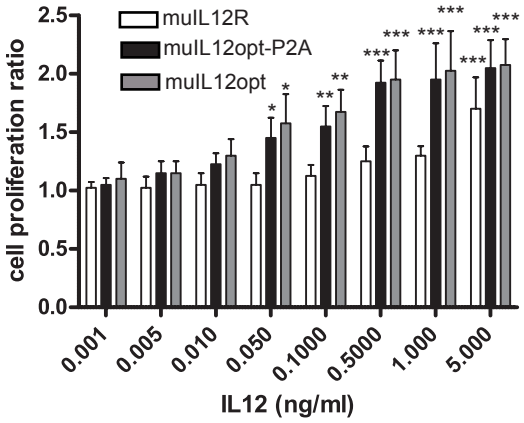
NFkBp

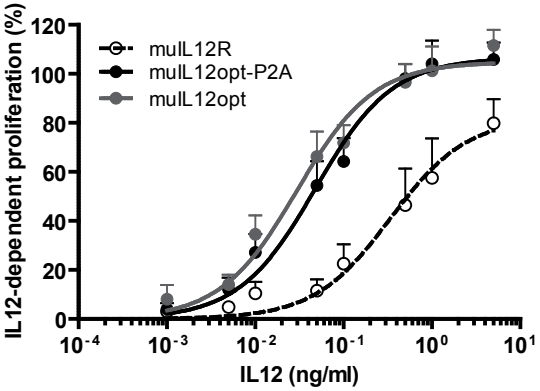
d


HEK-293
IL12 (pg/ml)
15000
10000
5000
200
150
100
50
0
*
***
TNF-α + IL-1β:
-
-
+
-
+
-
+
SFFVp
ESELp
IL1-IL6p
NFkBp

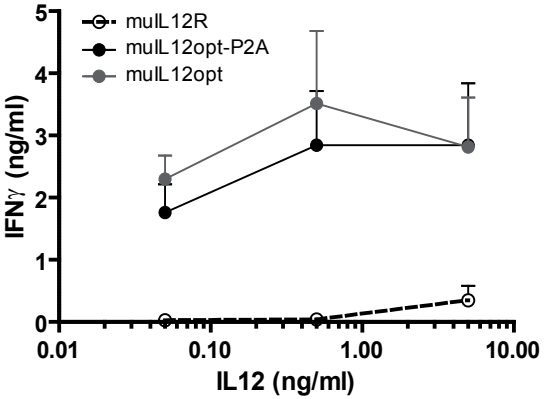
a


mulL12R
mulL12opt-P2A
mulL12opt
cell proliferation ratio
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.005
0.010
0.050
0.1000
0.5000
1.000
5.000
IL12 (ng/ml)

b


mulL12R
mulL12opt-P2A
mulL12opt
IL12-dependent proliferation (%)
120
100
80
60
40
20
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
IL12 (ng/ml)

c


mulL12R
mulL12opt-P2A
mulL12opt
IFNγ (ng/ml)
5
4
3
2
1
0
0.01
0.10
1.00
10.00
IL12 (ng/ml)