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Facultad de Ciencias

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Summary

Inflammatory processes are present in different pathologies including chronic inflammatory disorders, neuroinflammatory conditions and cancer. We hypothesized that an ideal lentiviral expression system for gene therapy treatments of such diseases could make use of the pathological inflammation grade, specific to each pathological condition and to the course of the disease, to regulate the transgene expression levels; ideally the expression levels of the therapeutic agent would parallel the local inflammatory status and therefore might avoid undesirable side effects associated with a constant transgene expression. These inflammation-regulated systems might also circumvent drug efficacy attenuation due to a continuous exposure to the therapeutic agent.

Our approach has consisted on the generation of lentiviral vectors (LVs) that contain inflammation-regulated promoters to drive transgene expression. Antonio Rodríguez's research group has developed a novel LV system based on the human E-selectin promoter. In addition we have also generated other two inflammation-regulated LVs based on previous reports: a hybrid promoter based on the human IL-6 promoter plus an enhancer region of the human IL-1 promoter and a chimeric promoter based on six NF-κB transcription factor binding sites in tandem. We have confirmed their *in vitro* inducibilities in different cell types (immune, endothelial, glia and tumor cells) in response to pro-inflammatory stimuli such as TNF-α, IL-1 or LPS.

LVs represent the chosen gene therapy tool to obtain stable and long-lived transgene expression *in vivo*. This study shows that the combination of LVs with inflammation-inducible promoters allows to monitor inflammatory processes such as those taking place in acute inflammation and solid tumor models. Moreover our results indicate that the expression of the proper cytokine (either murine IL-10 or IL-12) under these inflammation-regulated LV systems constitute an efficient strategy to provide disease-regulated cytokine expression with therapeutic effects in the mouse model assayed. These systems also reduce side effects as cytokine expression is confined to inflammatory sites. These inducible promoters might represent excellent systems for the inflammation-regulated expression of different agents for future clinical applications.

Resumen

Los procesos inflamatorios están presentes en diferentes patologías incluyendo enfermedades inflamatorias crónicas, neuroinflamación y cáncer. Nuestra hipótesis de partida consiste en que un vector lentiviral ideal para el tratamiento con terapia génica puede aprovecharse del nivel de inflamación, específico de cada patología y del curso de la enfermedad, para regular los niveles de expresión del transgén, de forma que se produzcan diferentes niveles de la molécula terapéutica en respuesta al estado inflamatorio local y, por lo tanto, se puedan evitar los efectos secundarios asociados.

Nuestro abordaje ha consistido en el desarrollo de vectores virales que contienen promotores inducibles por inflamación para controlar la expresión del transgén. El laboratorio del Dr. Antonio Rodríguez ha desarrollado un nuevo sistema lentiviral inducible por inflamación basado en el promotor de la Selectina-E humana. Además hemos reproducido otros dos sistemas regulados por inflamación previamente descritos: el promotor híbrido formado por el promotor de la IL-6 humana unido a la región potenciadora del promotor humano de la IL-1 y el promotor quimera basado en seis sitios de unión para el factor de transcripción NF-κB en tándem. Hemos validado estos sistemas lentivirales inducibles por inflamación *in vitro* demostrando su inducibilidad en diferentes tipos celulares (células del sistema inmune, endoteliales, de la glía y tumorales) en respuesta a estímulos inflamatorios como TNF-α, IL-1 y LPS.

Se han empleado vectores lentivirales ya que representan la mejor estrategia para obtener una expresión del transgén estable y duradera *in vivo*. Este estudio demuestra que la combinación de vectores lentivirales con promotores inducibles por inflamación permite la monitorización de procesos inflamatorios como los que tienen lugar en los modelos de inflamación aguda o durante el desarrollo de tumores sólidos. Además nuestros resultados indican que la expresión de citoquinas anti o pro-inflamatorias (IL-10 e IL-12 murinas) bajo el control de promotores regulados por inflamación empleando vectores lentivirales, constituye una estrategia eficiente para conseguir la expresión de la molécula regulada por la propia enfermedad, produciendo un efecto terapéutico en el modelo correspondiente y reduciendo los potenciales efectos secundarios. Estos promotores inducibles pueden representar excelentes sistemas para la expresión regulada por inflamación de diferentes agentes terapéuticos en futuras aplicaciones clínicas.

Index

1 Abbreviations

AAV	Adeno-associated virus
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein 1
APC	Antigen-presenting cell
CARs	Chimeric antigen receptors
CD	Cluster of differentiation
CLRs	C-type lectin receptors
CMV	Cytomegalovirus
COX2	Cyclooxygenase 2
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
ESEL	E-selectin
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBV	Hepatitis virus B
HCC	Hepatocellular carcinoma
HCV	Hepatitis virus C
HIF1α	Hypoxia-inducible factor 1 α
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP10	Interferon-inducible protein 10
IRES	Internal ribosomal entry sites
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharide
LTR	Long terminal repeat
LV	Lentiviral vector or lentivector
MALT	Mucosa-associated lymphoid tissue
MIG	Monokine induced by interferon γ
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
NF-κB	Nuclear factor-κB
NK	Natural killer
NLRs	NOD-like receptors
OEC	Olfactory ensheathing cell
OM	Olfatory mucosa
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
RIG	Retinoic acid-inducible gene
RLU	Relative light unit
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor-1
SFFV	Spleen focus-forming virus
SOD-1	Superoxide dismutase-1

STAT	Signal transducer and activator of transcription
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1

2 Introduction

2.1 Inflammation

Inflammation is a protective response to damage produced by noxious stimuli such us pathogens, chemicals, or physical injury that is intended to recover tissue functionality and homeostasis. Classically, inflammatory processes have been classified in acute and chronic inflammation[1]. Acute inflammation is characterized by rapid onset and short duration promoting stimulus neutralization followed by resolution of the inflammatory process and tissue reparation. In the case of chronic inflammation the duration is longer (from days to years) and frequently associated with severe and progressive tissue injury. Acute inflammation may progress to chronic when it is not properly resolved; however some forms of injury produce chronic inflammation response from the outset.

2.2 Inflammation components

Initially infection or tissue injury signals are detected by local cells, mainly macrophages, dendritic cells, mast cells, and others non-professional immune cells such as epithelial cells, endothelial cells, and fibroblasts, that express receptors designed to sense the presence of infectious pathogens and substances released from dead cells. These receptors are called pattern recognition receptors (PRRs) which include transmembrane proteins, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors (NLRs)[2]. They recognize structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs). Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed damage associated molecular patterns (DAMPs). The sensing of PAMPs or DAMPs (with the exception of some NLRs) by PRRs upregulates the transcription of genes involved in the inflammatory responses. These genes encode pro-inflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial proteins that promote and regulate the subsequent inflammatory response. Vascular changes are induced such as vasodilation, increased vascular permeability and endothelium activation allowing migration of leukocytes from blood stream and their accumulation in the focus of injury together with plasma proteins[3,4]. Recruited leukocytes are then activated to perform their functions, mainly phagocytosis and intracellular destruction of microbes and dead cells through reactive oxygen species (ROS) and lysosomal enzymes, liberation of effector substances (antimicrobial peptides and elastase) to destroy extracellular

pathogens and dead tissues and production of mediators (including cytokines) to amplify the inflammatory reaction [5,6].

Chemical mediators responsible for these processes are numerous and diverse[1] **(Table I)**. Mediators can be produced by cells located at the site of inflammation or can derive from circulating inactive precursors (being the liver the main source) which are activated at the site of inflammation (complement proteins, kinins and proteases activated during coagulation). Cell-derived mediators of inflammation are produced by tissue macrophages, mast cells, activated endothelial cells and recruited leukocytes. Some of them are accumulated in intracellular granules and secreted upon cellular activation (e.g., histamine and serotonin) and others are *de novo* synthetized upon stimulation (e.g., prostaglandins, leukotrienes or cytokines).

2.3 Inflammatory cytokines

Cytokines are polypeptide products of many cell types that are key mediators of inflammation and immune responses. The major cytokines in acute inflammation are tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and chemokines (group of chemoattractant cytokines for different subsets of leukocytes). TNF-α and IL-1 are produced by activated macrophages, mast cells, endothelial cells and some other cell types in response to microbial products, immune complexes and T lymphocytes products during adaptive immune response. The main role of these cytokines during inflammation is activation of endothelium[7,8]. Activated endothelial cells promote inflammation by displaying different combinations of adhesion molecules for leukocyte recruitment including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). In combination with the release of chemokines[9] they promote leukocyte adhesion and extravasation to the inflammation site. Both TNF-α and IL-1 are activators of the classical nuclear factor-κB (NF-κB) pathway[10]. IL-6 plays an important role on acquired immune response by stimulation of both antibody production and effector T-cell development [11].

Although these cytokines are secreted locally, they may enter into circulation and cause or participate in systemic reactions such us fever (TNF-α, IL-1, and IL-6), hepatic synthesis of acute-phase proteins (IL-6 and IL-1) and leukocyte proliferation and release from bone marrow (TNF-α, IL-1, and IL-6).

Other important cytokines during chronic inflammation are IFN-γ and IL-12. Classical macrophage activation by either microbe endotoxins or cytokines such as IFN-γ induces Th1 cell activation and release of cytokines, particularly IFN-γ, involved in activation of

microbicide activities. IL-12 is primarily produced by classically activated macrophages and crucial for Th1 differentiation and cytotoxic responses, ultimately leading to the production of IFN-γ[12].

Table I. Actions and sources of the principal mediators of inflammation

Mediator	Source (s)	Actions
Cell-Derived		
Histamine	Mast cells, basophils, platelets	Vasodilation, increased vascular permeability, endothelial activation
Serotonin	Platelets	Vasoconstriction
Prostaglandins	Mast cells, leukocytes	Vasodilation, pain, fever
Leukotrienes	Mast cells, leukocytes	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Platelet-activating factor	Leukocytes, mast cells	Vasodilation, increased vascular permeability, leukocyte adhesion, chemotaxis, degranulation, oxidative burst
Reactive oxygen species	Leukocytes	Killing of microbes, tissue damage
Nitric oxide	Endothelium, Macrophages	Vascular smooth muscle relaxation; killing of microbes
Cytokines (TNF, IL-1, IL-6)	Macrophages, endothelial cells, mast cells	*Local*: endothelial activation (expression of adhesion molecules). *Systemic*: fever, metabolic abnormalities, hypotension (shock)
Chemokines	Leukocytes, activated macrophages	Chemotaxis, leukocyte activation
Plasma Protein-Derived		
Complement	Plasma (produced in liver)	Leukocyte chemotaxis and activation, direct target killing (MAC) vasodilation (mast cell stimulation)
Kinins	Plasma (produced in liver)	Increased vascular permeability, smooth muscle contraction, vasodilation, pain
Proteases activated during coagulation	Plasma (produced in liver)	Endothelial activation, leukocyte recruitment

MAC= Membrane attack complex
Adapted from *Robbins Basic Pathology 9th Edition*

2.4 Anti-inflammatory mechanisms

Several mechanisms counteract inflammatory mediators and function to limit or terminate an inflammatory response that otherwise could be uncontrolled and harmful. Activated macrophages and other cells secrete interleukin-10 (IL-10), whose major functions are to down-regulate the responses of activated macrophages, providing a negative feedback loop and supress antigen presenting cell (APC) activation[13]. Transforming growth factor β (TGF-β) is another anti-inflammatory cytokine which is also a mediator of fibrosis for tissue repair after inflammation[14]. In addition lipoxines and complement regulatory proteins limit the inflammatory process at different levels[15].

2.5 Para-inflammation

Recently, chronic inflammatory states that are not caused by the classical instigators of inflammation (i.e. infection and injury) have been defined as chronic systemic inflammation or para-inflammation. This kind of inflammation seems to be associated with tissue malfunctions[16]. Sustained malfunctions can result from mutations or environmental factors that generate a chronic low-grade inflammation which is partially responsible for chronic inflammatory conditions associated with modern human diseases such as cardiovascular, neurodegenerative or metabolic conditions.

2.6 Inflammatory reactions and disorders

2.6.1 Acute inflammation pathology

Acute inflammatory reactions are triggered by a variety of stimuli such as infections, trauma, tissue necrosis and foreign bodies. However, the mechanisms involved in the elimination of microbes and dead cells are also capable of damaging normal tissues, causing pathologies associated with inflammation. Once leukocytes are activated their effector mechanisms (enzymes and ROS) do not distinguish between offender and host and secrete many cytokines which further stimulate inflammation with important systemic effects.

Organ functions are often compromised as a consequence of acute responses such as glomerulonephritis or acute respiratory distress syndrome[17]. Acute inflammatory reaction plays also a role in acute transplant rejection. In addition, severe infections (sepsis), generally caused by bacteria, induce the production of several cytokines, mainly TNF-α, IL-

12 and IL-1, in response to large amounts of bacterial toxins that may cause a septic shock[18].

2.6.2 Chronic inflammation pathology

Chronic disorders can arise under different circumstances[19] and are characterized by episodes of relapse and remission that often involve superposition of acute inflammation on top of the inflammation already present. In persistent infections difficult to eradicate, such us tuberculosis, syphilis or during some viral and fungi infections, the inflammatory response contributes more to the pathologic process than does the microbe itself. In addition, prolonged exposure to toxic agents can induce chronic inflammation, tissue injury and fibrosis.

When the inflammatory response is inappropriately directed against self-tissues, as in certain autoimmune diseases[20] such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) or psoriasis generates hypersensitivity reactions. Immune responses against environmental substances are the cause of allergic diseases, such as bronchial asthma.

Finally, as already mentioned, milder forms of chronic inflammation (para-inflammation) may be important for the pathogenesis of neurodegenerative disorders (Alzheimer disease), atherosclerosis, metabolic syndrome and the associated type 2 diabetes [16]. Many of these chronic conditions (RA, IBD, atherosclerosis and others) share the activation of NF-κB pathway as a pivotal feature [21].

2.6.3 Cancer-related inflammation

In the 19th century the physician Rudolf Virchow provided the first indication of a possible link between inflammation and cancer by reporting the presence of leukocytes within tumor masses[22]. Nowadays the role of inflammation in different steps of tumorigenesis is generally accepted[23,24]. Firstly, inflammatory conditions increase the risk of developing many types of cancer in different settings. Persistent infections promote cancer development and progression such as gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma associated with *Helicobacter pylori* infection or hepatocellular carcinoma (HCC) associated with Hepatitis virus B or C (HBV or HCV) infection[25]. Autoimmune diseases are another type of chronic inflammation that precedes tumor development, as in the case of IBD which increases the risk of colorectal cancer [26].

Second, inflammation caused by exposure to noxious environmental or dietary substances is a tumor promoter as well. Obesity promotes tumorigenesis in liver and pancreas[27] and it

has been shown that tobacco smoke in addition to its high carcinogenic content promotes cancer due to its ability to trigger chronic inflammation [28].

Third, inflammatory microenvironment associated to tumors (called tumor-promoting inflammation) has been revealed as an essential component that enhances neoangiogenesis, promotes tumor progression and metastatic spread, causes local immunosuppression and augments genomic instability. The generation of a cancer-related inflammation can follow two pathways [24], an extrinsic pathway in which inflammatory or infectious conditions promote cancer development, and an intrinsic pathway activated by genetic events that cause neoplasia. Signaling pathways involved in inflammation operate downstream of oncogenic mutations, such as RAS and MYC family members, and induce transcriptional programs leading to tumor microenvironment remodeling, leukocytes recruitment, tumor-promoting chemokines and cytokines expression and angiogenic switch induction[29,30]. The two pathways converge, resulting in the activation of transcription factors in tumor cells, mainly NF-κB [31] and signal transducer and activator of transcription 3 (STAT3) [32,33], but also others such as hypoxia-inducible factor 1 α (HIF1α) [34] or activator protein 1 (AP-1) [35]. These transcription factors coordinate the production of inflammatory mediators including cytokines, chemokines and cyclooxygenase 2 (COX2). These factors recruit and activate leukocytes, mainly cells of the myelomonocytic lineage, and activate the same key transcription factors in inflammatory cells, stromal cells and tumor cells, creating a cancer-related inflammatory microenvironment. Pro-inflammatory cytokines IL-1, IL-6, and TNF-α[36-39] have been recognized as key elements in these processes.

2.7 Inflammatory disease treatments

Traditionally, treatment for inflammatory diseases consisted in the administration of broad-spectrum immune regulators, such as glucocorticoids, methotrexate, azathioprine or 6-mercaptopurine that block leukocyte function systemically but causes important side effects. However, the therapeutic success obtained by targeted inhibition of TNF-α in RA patients in the early 1990s [40] pushed research efforts towards the development of selective inhibitors or blocking agents rather than broad immune-suppressors. Given the fundamental roles of cytokines in the development and pathogenesis of many inflammatory diseases, treatments based on the selective targeting of cytokine activities have shown beneficial for patients suffering inflammatory diseases; **Table II** summarizes the cytokine inhibition-based strategies for TNF-α, IL6-R, IL-1 and combined inhibition of IL-12/IL-23 and the responsiveness level of each chronic inflammatory disease treated[41];

it highlights how different inflammatory pathologies respond to inhibition of the same cytokine (being the most prominent example inhibition of TNF-α) and how each cytokine inhibition independently works for a particular inflammatory disease. Several approaches can be used to inhibit a given cytokine signaling: the cytokine can be blocked by specific monoclonal antibodies or sequestered by adding soluble cytokine receptors; the cytokine receptor can be also blocked by monoclonal antibodies or by receptor antagonists that compete with the ligand for the receptor binding site [42]. Another strategy to regulate and control chronic inflammatory processes is the administration of immune modulating cytokines, being the use IFN-β for the treatment of multiple sclerosis the most successful example [43-45]. However important limitations still exist as these therapies do not abrogate the concomitant use of steroids or immunosuppressive drugs, and a significant percentage of patients do not respond to these new therapies. Moreover, keeping the balance between blocking the pathological inflammatory responses while simultaneously maintaining the ability to control infections is extremely important; for instance, it has been reported an increased susceptibility to infections by the systemic blockade of soluble and membrane-bound TNF-α using neutralizing antibodies such as infliximab [46,47].

Table II. Efficacy of cytokine inhibition in chronic inflammatory diseases.

The chart shows strong clinical efficacy of inhibition of each cytokine confirmed in randomized clinical trials of various chronic inflammatory diseases (CIDs) (or several independent observational studies in case of low prevalence diseases) (dark green), preliminary data on clinical efficacy (light green), no or mild clinical efficacy or no data on efficacy (gray) and disease-aggravating effect (red). IL-12/23, combined inhibition of interleukin-12 and interleukin-23 by targeting p40.

CID	TNF	IL-6R	IL-1	IL-12/23
Rheumatoid arthritis				
Giant cell arthritis				
JIA/AID				
Gout				
Crohn's disease				
Ulcerative colitis				
Psoriasis				
Psoriatic arthritis				
Ankylosing spondylitis				
Multiple sclerosis				
Drugs	*Adalimumab* *Certolizumab* *Etanercept* *Golimumab* *Infliximab*	*Tocilizumab* *Sarilumab**	*Anakinra* *Canakinumab* *Rilonacept*	*Ustekinumab* *Briakinumab**

IL-6R=IL-6 receptor; JIA=juvenile idiopathic arthritis; AID=auto-inflammatory disease including Still's disease. Asterisks indicate drugs that are not approved to date.
Adapted from Schett G. et al. *Nat Med*. 2013.

2.8 Interleukin-10

Altering the cytokine network is a common therapeutic strategy for treatments of inflammatory diseases as it has been explained. One way to regulate the inflammatory cytokine activity is to administer anti-inflammatory cytokines, such as IL-10. The IL-10 cytokine family together with the closely related IFN family forms the larger class II cytokine family[13]. IL-10 was identified by Mosmann and colleagues in 1989 [48]. The human IL-10 is a homodimer with a molecular mass of 37 KDa, each monomer consisting of 160 amino acids. The human IL-10 gene is located on chromosome 1 and encodes for 5 exons (5.1 kb) [49]. Murine and human *IL-10* exhibit a nucleotide homology of about 80% and their structures and functions have been extensively studied [50,51].

IL-10 is expressed by cells of the innate and adaptive immune system including dendritic cells (DC), macrophages, mast cells, natural killer (NK) cells, eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T cells, and B cells [52,53]. IL-10 targets various cells types in the body by binding to a heterodimeric receptor formed by two chains, IL-10R1 and IL-10R2. The IL-10R2 chain is ubiquitously expressed whereas the IL-10R1 chain is mainly expressed on leukocytes [54], making IL-10 the only family member that primarily target these cells. IL-10R1 and IL-10R2 are associated with Jak1 and Tyk2 respectively[55,56]; although STAT3 is the key downstream transcription factor **(Figure 2.1)**, STAT1 and sometimes STAT5 can be activated[57,58].

Most studied function of IL-10 is the prevention of tissue damage caused by infections and inflammation; it regulates and represses the expression of pro-inflammatory cytokines during the recovery phase of infections and reduces the tissue damage caused by these cytokines [59,60]. IL-10 inhibits inflammatory responses from innate and adaptive immunity, and prevents the lesions in tissues caused by exacerbated adaptive immune responses; it is thus a central cytokine during the resolution phase of inflammation.

IL-10 interferes with the production of inflammatory mediators by polymorphonuclear neutrophils, monocytes, and macrophages, such as inflammatory cytokines (IL-6, IL-1 and TNF-α) and chemokines as well as upregulates the expression of molecules that amplify the anti-inflammatory effect of IL-10 (IL-1 receptor agonist and soluble TNF-α receptor) [61]. This anti-inflammatory role is illustrated by IL-10-deficient mice that develop chronic IBD owing to an inappropriate innate immune response to intestinal bacterial antigens[62].

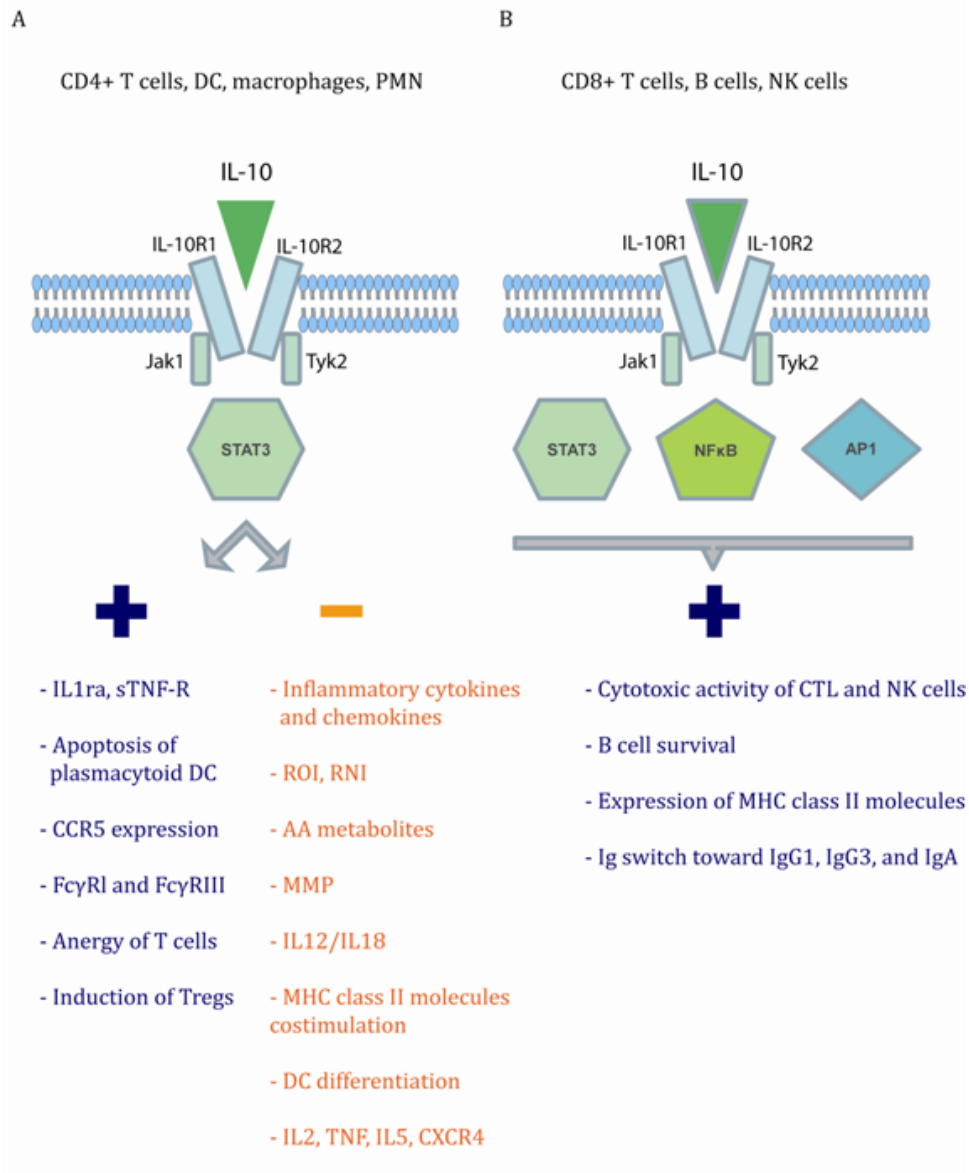

Figure 2.1
Immunoregulatory and stimulatory effects of interleukin 10
(A) IL-10 interacts with IL10R expressed on monocytes, macrophages, dendritic cells, $CD4^+$ T cells, and polymorphonuclear neutrophils (PMN). STAT3 is the transcriptional factor largely involved in the inhibitory action of IL-10 including the inhibition of NF-κB. IL-10 inhibits numerous inflammatory and antigen-presenting functions and stimulates other responses in immune cells. (B) IL-10 interacts with $CD8^+$ T cells, B cells and NK cells, in which they stimulate NF-κB and AP-1, in addition to STAT3. This leads to cytotoxic activity of $CD8^+$ T cells and NK cells, and to B cell activation. AA=arachidonic acid. DC=dendritic cell. MMP=matrix metalloproteinases. RNI=reactive nitrogen intermediates. ROI=reactive oxygen intermediates. Adapted from Mege JL. et al. *Lancet*, 2006.

IL-10 regulates adaptive immune responses through its effects on APCs and T cells. In APCs, IL-10 can directly or indirectly suppress TLR signaling [63,64] impeding APC activation. IL-10 inhibits production of IL-12 and IL-18, as well as reduces expression of MHC class II molecules and costimulatory molecules, therefore inhibiting their ability to present antigens to T cells[65]. Additionally, IL-10 impairs differentiation of monocyte-derived

dendritic cells, induces plasmacytoid dendritic cell apoptosis, and alters the migration of dendritic cells by modulating surface expression of chemokine receptors. IL-10 directly affects the function of $CD4^+$ T cells by inhibiting the expression of IL-2, TNF-α, IL-5, chemokine receptor CXCR4, and the response to stromal cell-derived factor-1 (SDF-1). Activation of T cells in the presence of IL-10 can induce non-responsiveness or anergy that cannot be reversed by IL-2 stimulation[66].

In addition of the profound anti-inflammatory effects of IL-10, it can promote humoral immune responses by enhancing the expression of MHC class II molecules on B cells, inducing immunoglobulin production (Ig) and increasing B-cell survival [67-69]. This effect on B cells may account for the presence of high circulating levels of antibodies such as IgA, associated with overproduction of IL-10 in clinical situations. Furthermore, IL-10 activates mast cells *in vitro*[70] and enhances $CD8^+$ T and NK cell activities *in vivo* [71-74]. It is interesting to note that IL-10 activates NF-κB and AP-1 in $CD8^+$ T cells but inhibits these transcription factors in monocytes and $CD4^+$ T cells[65] **(Figure 2.1).**

2.8.1 IL-10-based therapies for inflammatory diseases

Based on the comprehension of IL-10 role in inflammation and its effective reduction of pro-inflammatory cytokine activity in animal models of diseases such as sepsis, stroke, multiple sclerosis and other chronic inflammatory disorders[75-78], a variety of clinical studies were undertaken using systemic administration of recombinant IL-10 to treat psoriasis, Crohn´s disease or RA [79]. Despite of being well tolerated and the encouraging early results observed[80-82], larger blinded studies showed only modest therapeutic benefits [79]. Interestingly, it has been reported immune stimulatory effects when high doses of IL-10 were employed[80,83,84]. On the other hand, it has been shown that high or dysregulated levels of IL-10 may result in chronic infection [61,69,85,86]. Globally these results suggest that local IL-10 administration at the right site, dose and time might be effective for inflammatory disease therapies, avoiding or reducing potential side effects derived from systemic treatments.

2.9 Cancer immunotherapy

Although nowadays is accepted the role of tumor-promoting inflammation, on the other hand, immunosuppression allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages and NKs, and is considered a cancer hallmark[87]. This highlights the dual role of immune system in cancer that both antagonizes

and enhances tumor development and progression, unified in a conceptual framework called “cancer immunoediting” [88].

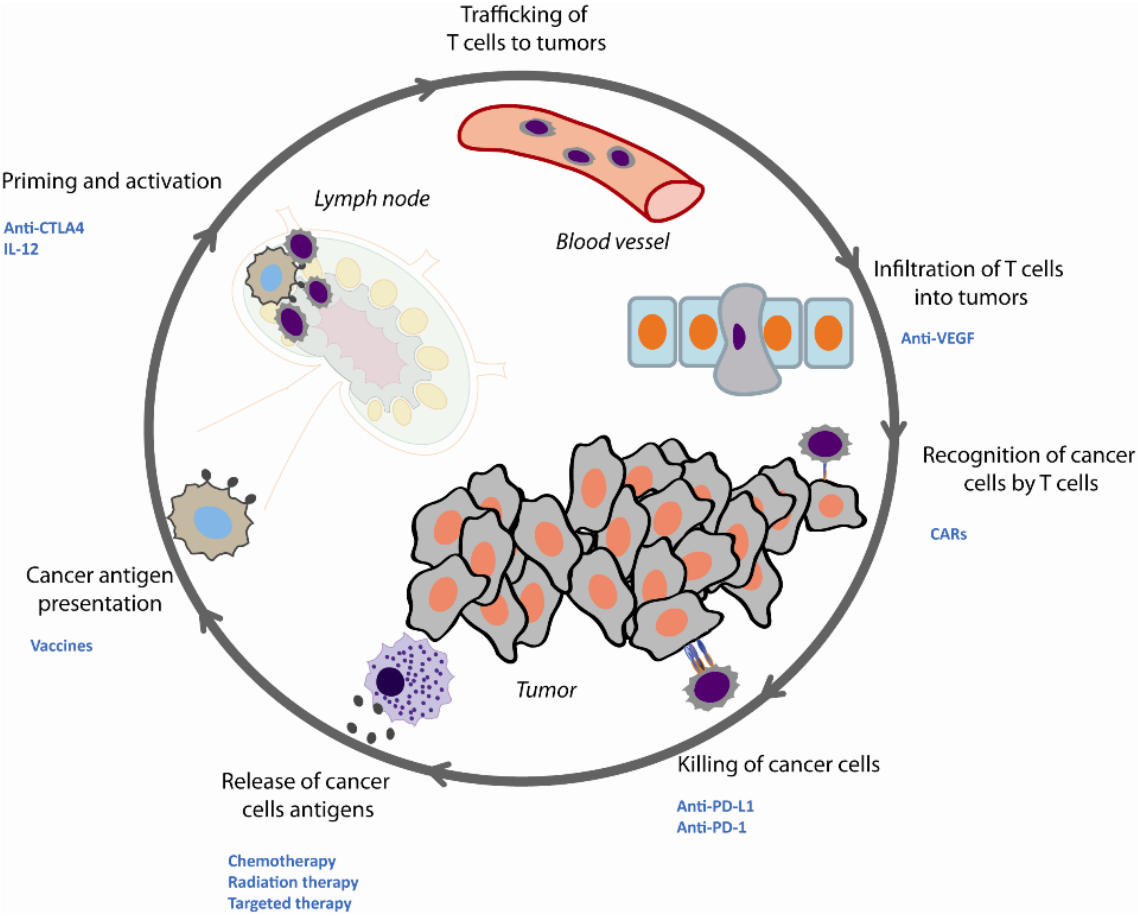

Figure 2.2
Steps and potential therapies along the cancer-immunity cycle
The numerous factors that come into play in the cancer-immunity cycle provide a wide range of potential therapeutic targets. This figure highlights examples of some of the therapies currently under preclinical or clinical evaluation. Key highlights include vaccines to improve cancer antigen presentation, anti-CTLA4 and IL-12 therapies that can primarily promote T cell priming and activation, anti-VEGF therapies to favor T cell infiltration, improved recognition of cancer by T cells using CARs and anti-PD-L1 or anti-PD-1 antibodies that can primarily promote killing of cancer cells. Targeted therapies are aimed to promote the release of cancer cell antigens. Although not developed as immunotherapies, chemotherapy and radiation therapy also produce this effect.
Anti-CTLA4=anti-cytotoxic T-lymphocyte-associated protein 4 antibodies, anti-PD-L1 and anti-PD-1=anti-programmed cell death ligand 1 and anti-programmed cell death 1 antibodies, anti-VEGF=anti-vascular endothelial growth factor, CAR = chimeric antigen receptor.
Adapted from Chen DS. et al. *Cell* 2013.

Initially cancer treatment has made use exclusively of relatively nonspecific cytotoxics and cytostatics (chemotherapies). These drugs remain the backbone of current treatments, but they are limited by a narrow therapeutic index, significant toxicities and frequently acquired resistance. More recently, an improved understanding of cancer pathogenesis has made possible new approaches resulting in mechanism-based therapeutics. They can be classified in targeted agents, which aim to inhibit molecular pathways that are crucial

for tumor growth and maintenance, and cancer immunotherapy, consisting in stimulation of the host immune response to allow a long-lived tumor recognition and destruction [89].
Anti-cancer immunity can be enhanced throughout the different steps of interaction between immunity and cancer development [90], such as cancer antigens recognition and presentation, priming and activation of effector T cell responses, T cell tumor infiltration and finally, T cell recognition and killing of cancer cells **(Figure 2.2)**. Two examples have shown the potential of cancer immunotherapy. First, the successful treatment of patients with metastatic melanoma by employing the anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) monoclonal antibody [91]. CTLA-4 is a key T cell negative regulator that prevents activation and expansion of T cells after APC interaction; thereby it controls the immune response progress and attenuates the chances of chronic autoimmune inflammation [92]. This negative regulation can be overcome by using this blocking antibody. The second example are the anti-programmed cell death ligand 1 (PD-L1) and anti-programmed cell death 1 (PD-1) antibodies. PD-L1 is a distal immune modulator expressed in many tumors (in cancer and tumor-infiltrating T cells) that binds to PD-1 expressed on activated effector T cells and blocks the secretion or production of cytotoxic mediators required for cell killing [93,94]. A number of immunotherapies that target different PD-1 or PDL-1 interactions are under development [95] and they are reporting encouraging results in a broad range of cancer types.

2.10 Interleukin-12

IL-12 is a heterodimeric cytokine composed of two subunits (p35 and p40) covalently linked by a disulfide bridge [96,97]. Both subunits are encoded by to independently regulated genes on the human chromosomes 3 and 5 respectively and co-expression yields to the formation of the biologically active p70 heterodimer[98]. IL-12 is primarily produced by macrophages, dendritic cells, neutrophils and B cells[12]. When IL-12 is secreted, it engages the IL-12 receptor (IL12R), also formed by two subunits (β1 and β2) and mostly expressed on activated T and NK cells, dendritic cells and macrophages [99-102]. 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 IL-12 function [103].
IL-12 mainly targets T and NK cells and induces IFN-γ production by these cells, therefore enhancing both innate and adaptive responses. Macrophages activated by IFN-γ produce nitric oxide synthase and subsequently release active nitrogen species. This results in an enhancement of phagocytic function and local inflammation[104]. IL-12 crucially favors the

differentiation of naïve T cells into Th1 cells and impedes Th2 differentiation[105,106]. Additionally, IL-12 increases NK cells and CD8 cytotoxic T lymphocytes (CTLs) proliferation and cytotoxic activity. Moreover, in the presence of IL-12 B cells are prompted to secrete antibody isotypes associated with Th1 responses [107] **(Figure 2.3).**

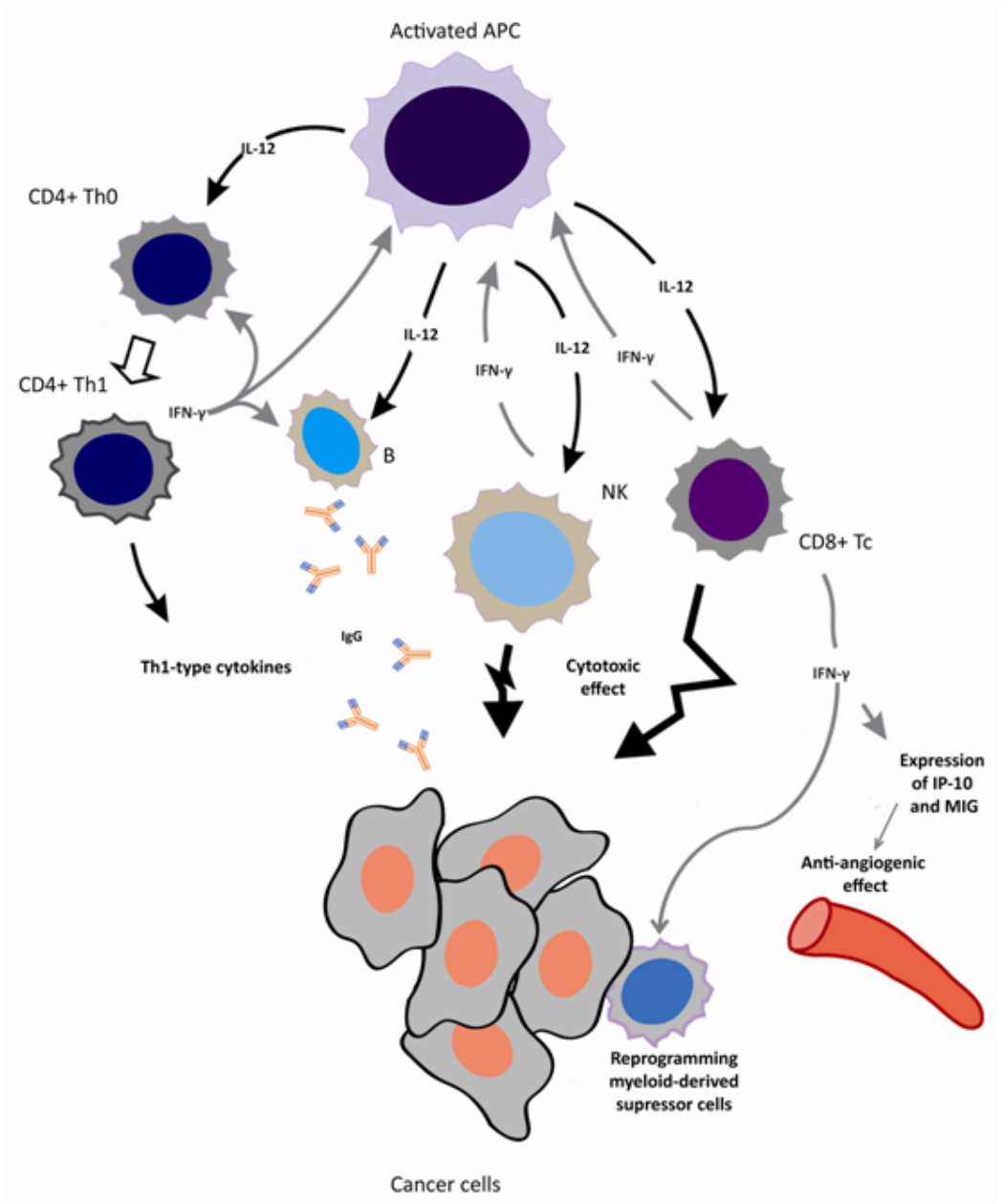

Figure 2.3
IL-12 role in cancer therapy
Overview of the biological properties of IL-12 contributing to its anti-tumor activity. Activated APCs produce IL-12 that induces $CD8^+$ T cells and NK cytotoxic effect. B cells are prompted to secrete antibodies. IL-12 crucially contributes to the skew of naïve T cells in Th1 cells. Subsequent IFN-γ production enhances both innate and adaptive responses in addition to its anti-angiogenic effect and myeloid-derived suppressor cells reprogramming. APC=antigen presenting cell, NK=natural killer cell, Tc=cytotoxic T lymphocyte, Th=T helper lymphocyte, IP-10=interferon-inducible protein 10, MIG=monokine induced by interferon γ.
Adapted from Lasek W. et al. *Cancer Immunol Immunother*, 2014.

2.10.1 IL-12 cancer therapy

The anti-tumor effect of IL-12 [108] has been known for many years. IL-12 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 interferon-inducible protein 10 (IP10) [109] and monokine induced by interferon-γ (MIG). In addition, IL-12 promotes reprogramming of myeloid-derived suppressor cells within the tumor[110] **(Figure 2.3)**. Several studies have demonstrated the anti-tumor and anti-metastatic activities of IL-12 in rodent preclinical models, including melanomas, mammary carcinomas, colon carcinoma, renal carcinoma and sarcomas[111], suggesting its therapeutic use as an anti-cancer agent. However, most clinical trials involving IL-12, with the exception of results obtained in cutaneous T cell lymphoma variants, non-Hodgkin´s lymphoma and in AIDS-related Kaposi sarcoma, have been unsuccessful and reported unimpressive results[112]. Importantly, IL-12 systemic administration is associated with marked toxic side effects, such as flu-like symptoms, toxic effects on the bone marrow and liver and hematologic toxicity (neutropenia and thrombocytopenia); this prompted the investigation into new methods of IL-12 delivery to avoid unacceptable toxicity, and enhance IL-12 anti-tumor activity[113]. As an alternative, 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 in some cases it is followed by tumor regression.

2.11 Gene therapy with lentiviral vectors

Gene therapy involves the use of nucleic acids (DNA or RNA) for the treatment, cure or prevention of human diseases, using different tools including naked oligonucleotides, viral and non-viral vectors. Viral vectors represent highly effective means for the delivery of therapeutic genes, and the scientific knowledge acquired in the field have allowed the generation of new improved vector versions, already tested in clinical trials [114]. Lentiviral vectors (also called lentivectors, LVs) which are derived from lentiviruses[115-117] are currently employed in both basic and clinical research. Lentiviruses belong to the Retroviridae family, and LVs as other retroviral vectors can integrate into the host cell genome (up to 10 kb); however and contrary to other oncoretroviral vectors, LVs allow gene delivery into not only dividing but also quiescent cells. Retroviral vectors do not integrate viral sequences that encode for packaging proteins (gag and pol), thus minimizing the risk of cytotoxic T lymphocyte activity onto vector-transduced cells. In

addition LVs (mainly derived from HIV-1) cause very low inflammatory response *in vivo*. In summary all these features make them a powerful and reliable tool for gene therapy.

Several safety concerns have been improved in the latest generation of HIV-1-based vectors. All accessory genes (among them 6 of 9 genes encoding for virulence factors) and the regulatory *tat* gene are deleted[118,119] ensuring that the parental virus cannot be reconstituted. The remaining viral sequences essential for virion production are split into three different helper vectors, to maximize the number of recombination events required to generate a replication-competent virus. Altogether these features reduce greatly the putative generation of replication-competent lentiviruses. Finally, the 3´ long terminal repeat (LTR) sequence has been partially deleted to eliminate the viral LTR enhancer/promoter activity, creating the so-called self-inactivating (SIN) LTR [120] reducing the risk of host gene activation. As a consequence of all these modifications, these lentiviral particles can undergo only one single round of transduction and the integrated proviral DNA will express only the transgene of interest. These safety improvements in LV engineering have encouraged employing them in clinical trials. LVs have been employed in *ex vivo*[121-130], and more recently, *in vivo*[131] strategies with excellent clinical results. In addition, their low immunogenicity compared to other commonly used viral vectors, such as adenoviral or adeno-associated viral vectors (AAV), make them ideal vectors for the treatment of inflammatory diseases [132-135].

2.11.1 IL-10 gene therapy

Studies in animal models have shown that gene therapy is an alternative for the local treatment of chronic inflammatory diseases as it has the potential to be more effective, better tolerated, and more specific than conventional pharmacological treatments [136]. As discussed previously, IL-10 is a good candidate to regulate inflammatory cytokine activity due to its broad spectrum of anti-inflammatory activities. As a consequence, the therapeutic effect of IL-10 expression has been tested in preclinical models under different gene therapy strategies, both *in vivo* and *ex vivo*. IL-10 has been expressed by employing different viral vectors (AAV, γ-retroviral vectors and LVs) and in different animal models mimicking inflammatory disorders such as autoimmune syndromes[137], RA[138], neuroinflammation[139,140] and allograft rejection[141]. The results have confirmed its anti-inflammatory potential and its putative use for the treatment of inflammatory diseases.

However, future gene therapy treatments for chronic inflammatory diseases must overcome one important limitation, the relapse and remission alternation that characterize these pathologies. Transgene expression should parallel the course of the

disease itself, matching the varying pathological conditions and thereby avoiding undesirable secondary effects.

2.11.2 IL-12 gene therapy

Gene therapy could also be a good alternative strategy for IL-12-based cancer therapy, as gene transfer methods can be designed to confine IL-12 production to the tumor environment, preventing systemic toxicity[142]. Several vectors derived from viruses, such as adenovirus[143], AAV[144], retrovirus[145] and herpes simplex virus[146], have been developed to transfer IL-12 genes locally to the tumor site. These preclinical experiments have shown encouraging results, not only by IL-12 expression by itself but also when it is used in combination with other anti-tumor strategies. In addition, the virus-mimicking effect of these viral vectors should trigger an IFN-mediated response, which has been proven to be absolutely required for the efficient anti-tumoral effect of IL-12 [147].

Again, 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, as is the case of the known IL-12-based therapies. Thus, a major challenge in the treatment of cancer using IL-12 is the development of expression systems restricted to and tightly regulated by the tumor environment, in order to confine transgene expression to the tumor site.

2.11.3 Disease-regulated promoters for gene therapy

Currently, viral promoters are the most frequently used for transgene expression. Initially they produce very high and constant expression of the transgene and as a consequence the therapeutic molecule can be released and spread throughout the organism increasing the risk of potential side effects. Moreover, these strong viral promoters are commonly silenced inside the cell, causing transient expression *in vivo* [148-151]. In addition, even if high expression of the transgene were successful, there might be an adaptive response to the constant high concentration of transgene protein, counteracting its therapeutic effect.

Thus, there is a need of developing accurate regulation systems for gene therapy. Several vectors driven by drug-controlled promoters have been developed to achieve regulated transgene expression such as those based on the tetracycline-regulated (Tet)[152] and the rapamycin-regulated dimerizer systems[153,154]; however, these approaches require continuous monitoring of the disease to achieve optimal efficacy, and this is further

complicated in the case of the unpredictable, relapsing course of inflammatory conditions. An ideal vector system would therefore be disease-regulated, expressing high levels of therapeutic agent during relapses and lower levels during remission phases of the disease, allowing the confinement of the therapeutic molecule and avoiding systemic toxic side effects.

Several viral expression systems that respond to inflammatory stimuli *in vivo* have been devised[155]. The first was based on a two component adenoviral expression system in which the promoter of complement factor 3 drives transcription of the HIV transactivator of transcription (Tat)[156]. However, the immunogenicity of Tat has been implicated in central nervous system disorders. Subsequently, van de Loo *et al.* [157] developed a hybrid inflammation-inducible adenoviral expression system, consisting of the human IL-6 promoter fused to the enhancer region of the human IL-1 promoter. Additionally, an inflammation-inducible expression system based on serotype 5 adeno-associated virus (AAV) has been developed by employing a chimeric promoter based on NF-κB-binding sites[158]. More recently, different inducible promoters by tissue-specific transcription factors have been developed to employ LVs for *in vivo* bioimaging [159].

Concluding, inflammation-inducible systems combined with the long term transgene expression capacity of LVs might represent an important improvement that would fulfil the requirements for an effective and safe gene therapy agent for clinical applications.

2.12 Development and *in vivo* validation of inflammation-regulated lentiviral expression systems

As has been explained, cytokines are key mediators of inflammation and immune responses, being TNF-α, IL-1 and IL-6 the main cytokines involved in the first steps of inflammation, thus we hypothesized that they could be expression-inductors for inflammation-regulated lentiviral expression systems.

Antonio Rodríguez laboratory have previously 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 pro-inflammatory cytokines (TNF-α, IL-1), and is not expressed under basal conditions, making its promoter a good candidate for the design of inflammation-regulated gene therapy vectors[160]. During my thesis work I have also generated LVs incorporating other previously described inflammation-inducible promoters, such as the human IL-6 promoter fused to the enhancer region of the human IL-1 promoter (IL1-

IL6p)[157] and a chimeric promoter based on six NFκB-binding sites (NFκBp) and the minimal cytomegalovirus (CMV) promoter[158]. I have studied *in vitro* the inducibilty of all these promoters in different primary cultures, cell lines and tumor cell lines, in response to inflammatory stimuli (TNF-α, IL-1 or LPS). In addition I have cloned murine *IL-10* gene under the control of these promoters to generate inflammation-inducible lentivectors to produce IL-10 and I studied its anti-inflammatory effect in a zymosan-induced inflammation model.

Besides, our inflammation-responsive lentivectors were employed to study the activation of inflammatory responses when olfactory mucosa (OM) of amyotrophic lateral sclerosis (ALS) patients is co-cultured with human spinal cord neurons[161].

Finally I have studied the expression pattern of our inflammation-inducible lentivector sytems in a syngeneic tumor model[162], showing that murine IL-12 expressed is able to trigger anti-tumor responses (data not published).

3 Objectives

There are three main objectives in this project:

i. Generation of inflammation-inducible lentivector systems to express luciferase, and murine IL-10 and IL-12.

ii. Analysis of promoter activity and cytokine expression *in vitro*.

iii. Study of inflammation-regulated transgene expression and therapeutic potential *in vivo*.

4 Materials, methods and results

4.1 IL-10 released by a new inflammation-regulated lentiviral system efficiently attenuates zymosan-induced arthritis

4.1.1 Introduction

Chronic inflammatory diseases are characterized by episodes of relapse and remission. Altering the cytokine network is a common therapeutic strategy in inflammatory diseases. One way to regulate inflammatory cytokine activity is to administer anti-inflammatory cytokines, such as interleukin-10 (IL-10). Compared with other anti-inflammatory molecules such as IL-4 or IL-13, IL-10 has a broader spectrum of anti-inflammatory activities, inhibiting the production of several pro-inflammatory cytokines (e.g. IL-1, IL-2, IL-6, IL-8 and TNF-α) and inducing the production of anti-inflammatory agents (such as IL-1 receptor antagonist). IL-10 also suppresses nitric oxide release in lymphocytes[163]. Local administration of recombinant IL-10 effectively reduces pro-inflammatory cytokine activity in animal models of diseases such as sepsis, stroke, multiple sclerosis and chronic inflammatory disorders[75-78].

Studies of inflammatory flare-up reactions in animal models have shown the applicability and viability of local gene therapy in arthritis[164]. Ideally, treatments for chronic inflammatory diseases should parallel the course of the disease itself, matching the varying pathological conditions and thereby avoiding undesirable secondary effects. An ideal vector system would therefore be disease-regulated, expressing high levels of anti-inflammatory agents during relapses and lower levels during remission phases of the disease.

Several viral expression systems that respond to inflammatory stimuli *in vivo* have been devised[155]. Van de Loo *et al.*[157] developed a hybrid inflammation-inducible adenoviral expression system, consisting of the human IL-6 promoter fused to the enhancer region of the human IL-1 promoter. The therapeutic efficacy of adenoviral systems is, however, compromised by the induction of an adenovirus-mediated immune response and by the rapid loss of transgene expression due to the episomal localization of the viral genomes. More recently, an inflammation-inducible expression system based on serotype 5 adeno-associated virus has been developed by employing a chimeric promoter based on NF-κB binding sites[158]. However, although the non-pathogenicity of adeno-associated viruses makes them promising candidates for long term gene therapy, under some circumstances they too can cause an inflammatory response in the host[133-135]. A potentially more appropriate system for gene therapy in chronic inflammatory processes would be lentiviral vectors, since they can infect both dividing and quiescent cells, provide long term expression, and display low immunogenicity[165].

Currently, the most frequently used promoters for transgene expression are viral, but these strong promoters are usually silenced, and result in only transient expression *in*

vivo. Moreover, even if high expression of the transgene were successful, the constant high levels of anti-inflammatory molecules might increase the risk of infection, as already observed with anti-TNF-α and anti-IL-1 treatment of patients with rheumatoid arthritis[166-169]. In addition, there might be an adaptive response to the constant high concentration of transgene protein, counteracting its therapeutic effect.

TNF-α and IL-1 are early-induced pro-inflammatory cytokines that act locally on vascular endothelium to induce the expression of adhesion molecules, including selectins and ligands for leukocyte integrins, which participate in the capture and recruitment of blood cells[170]. The selectins are a family of three type-I cell surface glycoproteins (E-, L- and P-selectin) involved in chronic and acute inflammation. E-selectin (ESEL) is rapidly and transiently expressed in response to inflammatory cytokines such as TNF-α and IL-1, and is not expressed under basal conditions except in skin microvessels[171]. The promoter of human ESEL has been used for *in vitro* gene delivery in recombinant retroviral and adenoviral vectors[172,173] and for *in vivo* delivery in non-viral vectors[174-176]. Here we report the development of a lentiviral expression system based on the ESEL promoter (ESELp). We show that ESELp-driven transgene expression is induced in response to pro-inflammatory cytokines in cell culture, and is regulated *in vivo* during chronic paw inflammation. This long term expression system shows low basal activity during remission and high expression during the acute inflammatory response. The lentiviral vector system drives *in vivo* expression of the anti-inflammatory cytokine IL-10 at levels sufficient to efficiently attenuate repetitive local acute inflammation episodes induced by yeast cell wall glucans (zymosan) injection. This attenuation is also observed when the lentiviral vector system is administered after zymosan injection. Therefore, this new expression system fulfils the requirements for a disease-regulated on/off system, suggesting potential use for auto-regulated treatment of chronic inflammatory diseases.

My personal contribution in this work consisted in:

First, the generation of the following pHRSIN contructs: Spleen focus-forming virus promoter (SFFVp)-mIL10, ESELp-mIL10, 6xNFκBp-Luc and IL1IL6p-Luc. Second, the analysis of the *in vitro* response of these promoters upon pro-inflammatory stimulation (TNF-α plus IL-1 or LPS). Third, *in vivo* local transduction followed by zymosan-induced acute inflammation model, determination of luciferase activity in tissues, comparative study of the different inflammation-regulated systems by *in vivo* imaging determination of luciferase activity, quantification of local and serum IL-10 expression levels and therapeutic effect determination. And forth, *in vivo* inflammation model in response to zymosan injection followed by local lentiviral transduction, luciferase activity monitoring by *in vivo* imaging and therapeutic effect determination.

IL10 Released by a New Inflammation-regulated Lentiviral System Efficiently Attenuates Zymosan-induced Arthritis

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Administration of anti-inflammatory cytokines is a common therapeutic strategy in chronic inflammatory diseases. Gene therapy is an efficient method for delivering therapeutic molecules to target cells. Expression of the cell adhesion molecule E-selectin (ESEL), which is expressed in the early stages of inflammation, is controlled by proinflammatory cytokines, making its promoter a good candidate for the design of inflammation-regulated gene therapy vectors. This study describes an ESEL promoter (ESELp)-based lentiviral vector (LV) that drives localized transgene expression during inflammation. Mouse matrigel plug assays with ESELp-transduced endothelial cells showed that systemic lipopolysaccharide (LPS) administration selectively induces ESELp-controlled luciferase expression *in vivo*. Inflammation-specific induction was confirmed in a mouse model of arthritis, showing that this LV is repeatedly induced early in acute inflammation episodes and is downregulated during remission. Moreover, the local acute inflammatory response in this animal model was efficiently blocked by expression of the anti-inflammatory cytokine interleukin-10 (IL10) driven by our LV system. This inflammation-regulated expression system has potential application in the design of new strategies for the local treatment of chronic inflammatory diseases such as cardiovascular and autoimmune diseases.

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INTRODUCTION

Chronic inflammatory diseases are characterized by episodes of relapse and remission that often involve superposition of acute inflammation on top of the inflammation already present. Altering the cytokine network is a common therapeutic strategy in inflammatory diseases. Therapies based on natural cytokines are very promising as they are more effective, better tolerated, and more specific than pharmacological treatments.[1] One way to regulate inflammatory cytokine activity is to administer anti-inflammatory cytokines, such as interleukin-10 (IL10). Compared with other anti-inflammatory molecules such as IL4 or IL13, IL10 has a broader spectrum of anti-inflammatory activities, inhibiting the production of several proinflammatory cytokines [*e.g.*, IL1, IL2, IL6, IL8, and tumor necrosis factor-α (TNF-α)] and inducing the production of anti-inflammatory agents (such as IL1 receptor antagonist). IL10 also suppresses nitric oxide release in lymphocytes.[2] Local administration of recombinant IL10 effectively reduces proinflammatory cytokine activity in animal models of diseases such as sepsis, stroke, multiple sclerosis, and chronic inflammatory disorders.[3–6]

Studies of inflammatory flare-up reactions in animal models have shown the applicability and viability of local gene therapy in arthritis.[7] Ideally, treatments for chronic inflammatory diseases should parallel the course of the disease itself, matching the varying pathological conditions and thereby avoiding undesirable secondary effects. An ideal vector system would therefore be disease-regulated, expressing high levels of anti-inflammatory agents during relapses and lower levels during remission phases of the disease.

Several viral expression systems that respond to inflammatory stimuli *in vivo* have been devised.[8] The first was based on a two-component adenoviral expression system in which the promoter of complement factor 3 drives transcription of the HIV transactivator of transcription (Tat).[9] However, the immunogenicity of Tat has been implicated in central nervous system disorders. Subsequently, van de Loo *et al.*[10] developed a hybrid inflammation-inducible adenoviral expression system, consisting of the human IL6 promoter fused to the enhancer region of the human IL1 promoter. The therapeutic efficacy of adenoviral systems is, however, compromised by the induction of an adenovirus-mediated immune response and by the rapid loss of transgene expression due to the episomal localization of the viral genomes. More recently, an inflammation-inducible expression system based on serotype 5 adeno-associated virus (AAV) has been developed by employing a chimeric promoter based on NF-kB-binding sites.[11] However, although the nonpathogenicity of AAVs makes them promising candidates for long-term gene therapy, under some circumstances they too can cause an inflammatory response in the host.[12–14] A potentially more appropriate system for gene therapy in chronic inflammatory processes would be lentiviral vectors

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(LVs), since they can infect both dividing and quiescent cells, provide long-term expression, and display low immunogenicity.[15]

Currently, the most frequently used promoters used for transgene expression are viral, but these strong promoters are usually silenced, and result in only transient expression *in vivo*. Moreover, even if high expression of the transgene were successful, the constant high levels of anti-inflammatory molecules might increase the risk of infection, as already observed with anti-TNF-α and anti-IL1 treatment of patients with rheumatoid arthritis (RA).[16–19] In addition, there might be an adaptive response to the constant high concentration of transgene protein, counteracting its therapeutic effect. Several vectors driven by drug-controlled promoters have been developed to achieve regulated transgene expression; however, this approach requires constant monitoring of the disease to achieve optimal efficacy, and this is further complicated by the unpredictable, relapsing course of the disease.

TNF-α and IL1 are early induced proinflammatory cytokines that act locally on vascular endothelium to induce the expression of adhesion molecules, including selectins and ligands for leukocyte integrins, which participate in the capture and recruitment of blood cells.[20] The selectins are a family of three type-I cell surface glycoproteins (E-, L-, and P-selectin) involved in chronic and acute inflammation. E-selectin (ESEL) is rapidly and transiently expressed in response to inflammatory cytokines such as TNF-α and IL1, and is not expressed under basal conditions except in skin microvessels.[21] The promoter of human ESEL has been used for *in vitro* gene delivery in recombinant retroviral and adenoviral vectors[22,23] and for *in vivo* delivery in nonviral vectors.[24–26] Here, we report the development of a lentiviral expression system based on the ESEL promoter (ESELp). We show that ESELp-driven transgene expression is induced in response to proinflammatory cytokines in cell culture, and is regulated *in vivo* during chronic paw inflammation. This long-term expression system shows low basal activity during remission and high expression during the acute inflammatory response. The LV system drives *in vivo* expression of the anti-inflammatory cytokine IL10 at levels sufficient to efficiently attenuate repetitive local acute inflammation episodes induced by zymosan injection. This attenuation is also observed when the LV system is administered after zymosan injection. Therefore, this new expression system fulfills the requirements for a disease-regulated on/off system, suggesting potential use for autoregulated treatment of chronic inflammatory diseases.

RESULTS

ESELp-driven transgene expression is efficiently activated by proinflammatory cytokines in lentivirus-transduced endothelial cells

To assess the ability of lentivectors to efficiently transduce endothelial cells, we infected mouse or human primary endothelial cell cultures [mouse lung endothelial cells (MLEC) and human umbilical vein endothelial cells] with a LV encoding green florescent protein (GFP) under the control of the constitutive SFFV viral promoter (LV-SFFVp-GFP). In addition, we infected immortalized MLEC (iMLEC).[27] GFP expression was analyzed after 48 hours, and the efficiency of transduction was close to 100% in all cases (**Supplementary Figure S1**).

Since ESEL is the earliest endothelium-specific adhesion molecule induced by proinflammatory cytokines, we tested whether the ESELp might be a useful tool for achieving targeted transgene expression at sites of inflammation. We generated a LV encoding GFP under the control of ESELp (LV-ESELp-GFP; **Supplementary Figure S2**) and infected iMLEC and human umbilical vein endothelial cells. Treatment of infected cells with TNF-α strongly increased GFP expression in both cell types, paralleling the expression of endogenous ESEL (**Figure 1a,b**). In contrast, GFP expression from the constitutively active LV-SFFVp-GFP vector was not modified by TNF-α treatment (**Supplementary Figure S3**). The potent induction by TNF-α of endogenous ESEL *in vitro* is greatly enhanced by preincubation with the proangiogenic factor vascular endothelial growth factor (VEGF).[28] We therefore preincubated infected cells for 24 hours with VEGF and then with TNF-α for different periods. As in the case of endogenous ESEL, VEGF pretreatment potentiated TNF-α-induced ESELp-driven expression of GFP; induction of GFP expression peaked at 6 hours both in VEGF and in vehicle pretreated cells, and declined after 12 hours (**Supplementary Figure 4a**).

As VSV-pseudotyped LVs have a broad host range, we tested the ESELp activity in non endothelial immortalized and primary cells susceptible to be infected after intraplantar administration by employing a lentivector containing the luciferase reporter gene under the control of the ESELp (LV-ESELp-Luc; **Supplementary Figure S2**). Treatment of infected cells with proinflammatory cytokines increased luciferase expression in all cell types assayed (**Figure 1c–f**). However, only in the case of stimulated LV-ESELp transduced endothelial cells the level of luciferase was similar to that observed in LV-SFFVp transduced cells (**Figure 1c**). In the rest of cell types we found a low basal level of luciferase expression and modest induction upon stimulation (**Figure 4d–f**).

We next investigated whether LV-ESELp could be activated by an inflammatory milieu. Injection of mice with lipopolysaccharide (LPS) induces the release of proinflammatory cytokines, resulting in activation of the endothelium and the expression of additional proinflammatory factors and cell-specific adhesion molecules that participate in the inflammatory response.[27] To test the effect of these inflammatory mediators on LV-ESELp, we transduced endothelial cells with LV-ESELp-Luc and treated these cells with culture supernatant from LPS-activated macrophages.[29] The cytokine-containing supernatant increased luciferase activity in iMLEC transduced with LV-ESELp-Luc but had no effect on cells transduced with LV-SFFVp-Luc (**Supplementary Figure 4b**). LV-ESELp-Luc driven luciferase activity was also increased by treatment with VEGF plus TNF-α, but direct treatment with LPS did not modify luciferase activity in endothelial cells driven by either SFFVp or ESELp (data not shown).

ESELp-driven transgene expression is induced by proinflammatory cytokines in a mouse subcutaneous matrigel model

The inducibility of LV-ESELp-Luc by inflammatory mediators was further investigated by subcutaneous matrigel experiments in mice. iMLEC were transduced with LV-SFFVp-Luc or LV-ESELp-Luc, and, after 24 hours, cells were embedded in VEGF-containing matrigel and injected subcutaneously into syngenic mice. Forty-eight

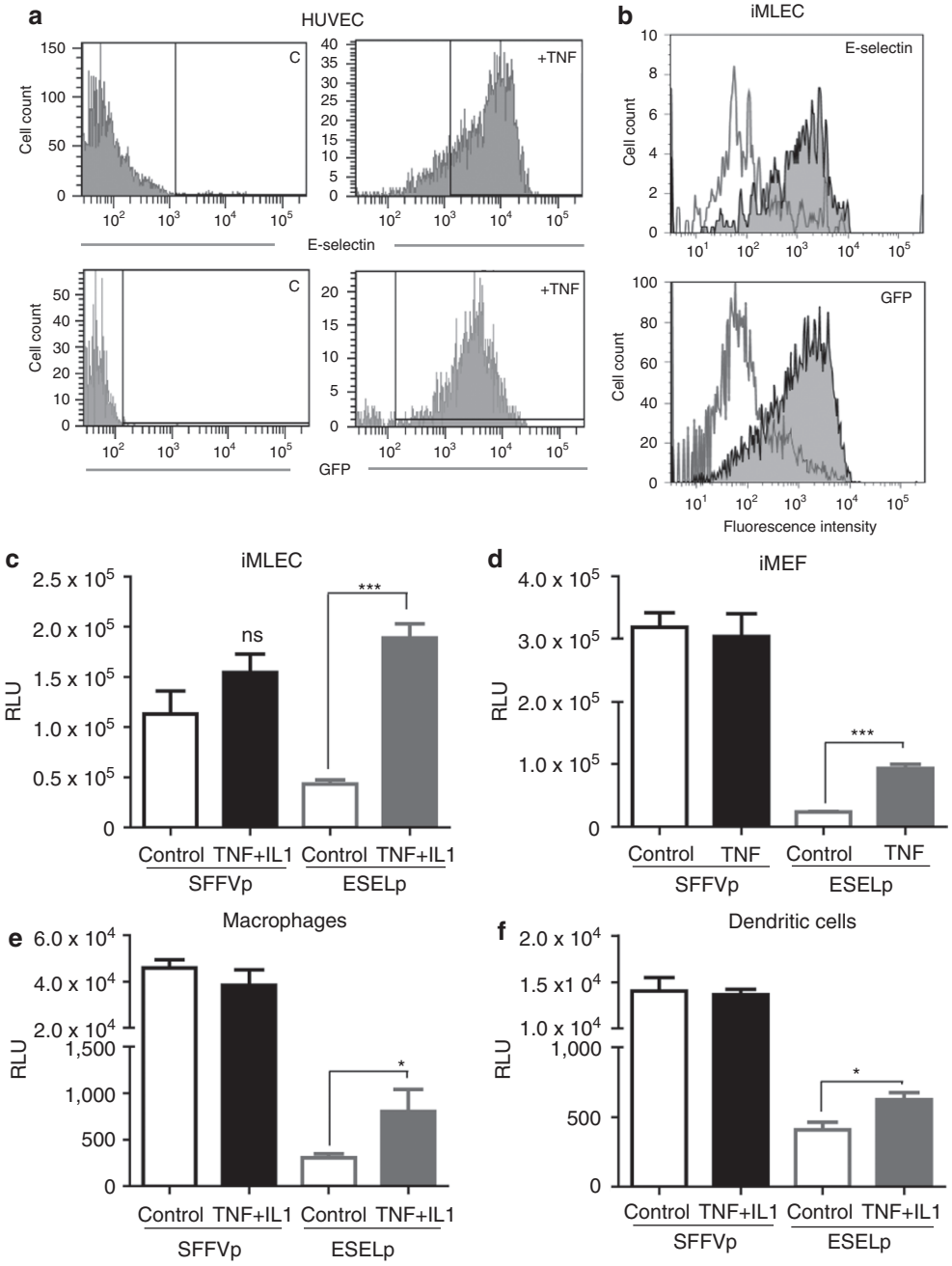

Figure 1 Inducibility of the E-selectin promoter (ESELp)-based lentiviral system *in vitro*. (**a**) Human umbilical vein endothelial cells (HUVEC) were infected with the LV-ESELp-GFP and left untreated (left) or incubated with 30 ng/ml tumor necrosis factor-α (TNF-α) (right) for 6 hours. Flow cytometry histograms show the expression of endogenous E-selectin (top panels) and green florescent protein (GFP) (bottom panels). (**b**) LV-ESELp-GFP-infected iMLEC were treated as in (**a**). Histograms show the fluorescence intensity of untreated cells (gray line) and TNF-treated cells (filled). (**c**,**d**) Immortalized and (**e**,**f**) primary cells were transduced for 5 hours with either LV-SFFVp or LV-ESELp, serum-starved for 12 hours and stimulated with the indicated cytokines for 6 hours. The MOI employed was 5 for iMLEC and iMEF, 50 for peritoneal macrophages and 100 for bone-marrow derived dendritic cells. Three experiments were performed and one representative is shown (mean +SEM; $n = 3$). *$P < 0.05$ and ***$P < 0.001$ versus untreated cells. LV, lentiviral vector; RLU, relative light units.

hours later, mice were treated with LPS [intraperitoneal (i.p.)], and luciferase activity in matrigel plugs was monitored by *in vivo* optical bioluminescence imaging. LPS administration led to similar increases in serum IL6 levels in all mice, but luciferase activity was increased only in LV-ESELp-Luc matrigel implants, thus confirming the selectivity of ESELp induction by inflammatory cytokines *in vivo* (**Figure 2** and **Supplementary Figure S5**).

ESELp-driven transgene expression is modulated by proinflammatory cytokines in a mouse model of chronic inflammation

We next addressed the inducibility of the LV system in a mouse model of RA, a disease characterized by chronic inflammation of the joints which leads to destruction of cartilage and bone. We used a zymosan-induced arthritis (ZIA) model, in which intraplantar

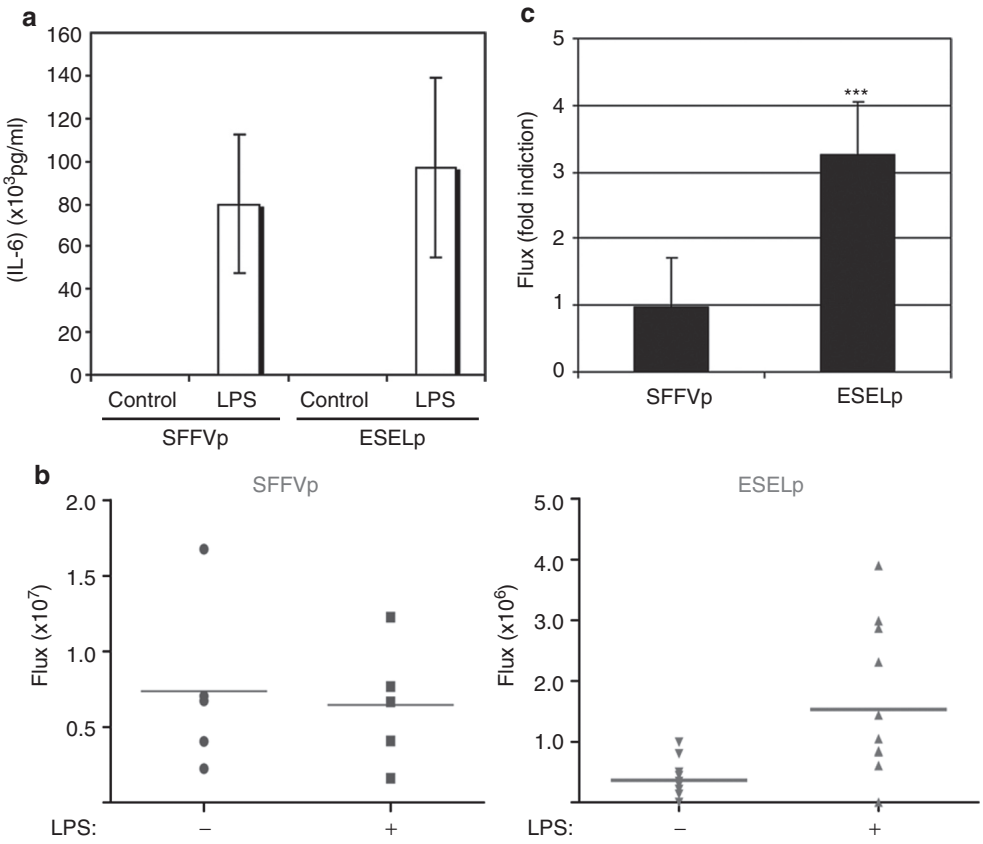

Figure 2 Proinflammatory cytokines induce the E-selectin promoter (ESELp)-based lentiviral system in vivo. Mice were injected subcutaneously with matrigel containing mouse lung endothelial cells (MLEC) infected with either LV-SFFVp-GFP or LV-ESELp-GFP, and were subsequently injected [intraperitoneal (i.p.)] as indicated with 40 mg/kg lipopolysaccharide (LPS). (**a**) Graph showing serum IL6 levels (mean ± SD) from a representative experiment (n = 9). (**b**) Scatter plots showing *in vivo* luciferase activity (flux) of mice from a representative experiment (ESELp: P = 0.0090; n = 9). (**c**) Graph showing *in vivo* luciferase activity (flux) as fold induction of LPS-treated versus control mice (mean± SD of three independent experiments;***P < 0.001 versus untreated mice). GFP, green florescent protein; LV, lentiviral vector.

administration of zymosan, a glucan obtained from yeast cell wall, induces the secretion of inflammatory ILs.[30] Intraplantar injection of zymosan causes pronounced, dose-dependent edema. This inflammatory response is time-dependent and can last up to 14 days following zymosan administration. LV-SFFVp-Luc or LV-ESELp-Luc was injected subcutaneously into both hind paws 1 week before zymosan administration to allow sufficient time for integration of the vector genome. After 1 week the right paw was injected with zymosan and the left paw with physiological saline solution. Luciferase expression was monitored by imaging of luciferase bioluminescence every 3–4 days over the first 2 weeks, when the signal intensity started to decrease, with additional measurements after 20 and 30 days. Compared with control paws, the inflamed paws showed a notable induction of ESELp-driven luciferase expression 4 days after zymosan injection, reaching a maximum after 7 days and decreasing from this point (**Figure 3a–c**). In contrast, paws injected with SFFVp-Luc showed no differences in luciferase expression upon injection with zymosan (**Figure 3a–c**). Comparable progression of ZIA in LV-SFFVp-Luc and LV-ESELp-Luc-injected paws was confirmed by measuring paw diameter (**Figure 3d**). In addition, inflammation was measured by bioluminescence after i.p. administration of luminol, which allows quantitative longitudinal monitoring of myeloperoxidase (MPO) system activity[31] (**Figure 3e**). LV injection by itself produced no inflammatory reaction that could contribute to ZIA, since luminol reactions were only observed in zymosan-treated paws.

It has been demonstrated that the majority of transduced cells after lentivector foot pad administration are dendritic cells (DCs) (90% CD11c⁺ cells) which can migrate to the draining lymph nodes and spleen.[32] To test whether locally infected cells were migrating and luciferase was expressed in these organs, lymph nodes, spleen and liver were extracted from LV-transduced animals 5 days after zymosan administration. Total tissue homogenates from these organs were employed to measure the luciferase activity. The results showed that only very low-luciferase activity was detected in the popliteal lymph node draining the paw infected with the luciferase-bearing LV (**Figure 3f,g**). Although we cannot rule out that LV particles may reach these and other organs, this result suggests that transgene expression remains restricted to the paw after zymosan injection.

To identify the uncharacterized 10% of transduced cells, we performed double immunostaining in cross-sections of paraffin-embedded transduced paws. Our preliminary data indicate that transgenes are expressed in transduced endothelial cells *in vivo* (data not shown); however further experiments would need to be

performed to confirm these data and investigate whether other cell types are contributing to the overall transgene expression *in vivo*.

ESELp-driven transgene expression *in vivo* responds to inflammation flare-ups

An important aim in gene therapy is the development of expression systems which can be switched on and off on demand. Such vectors would allow cessation of transgene expression upon resolution of the pathological process, and its restoration should the disorder reactivate. We therefore wanted to determine whether our lentiviral ESELp-driven expression system is modulated by the inflammatory conditions induced by zymosan. We monitored the inflamed paws after the first injection of zymosan by weekly measurement of the bioluminescence produced in response to i.p. administration of luminol. After one month, no detectable bioluminescence signal was generated in the paws, and correspondingly control and zymosan-injected paws showed no differences in ESELp-driven luciferase activity (day 30, **Figure 4a**). At this point, we reactivated the inflammation by administering a second zymosan injection to the same paw, and monitored SFFVp- and ESELp-controlled luciferase expression by bioluminescence. The new inflammatory process again led to an increase in ESELp-driven transgene expression in the zymosan-injected paws, whereas no apparent changes were observed in paws infected with LV-SFFVp-Luc (**Figure 4a,b** and **Supplementary Figure S6a**). The acute inflammatory reaction induced by the second zymosan injection was similar in LV-SFFVp-Luc and LV-ESELp-Luc-infected mice, as estimated by paw diameter and luminol bioluminescence (**Figure 4c** and **Supplementary Figure S6b**). These data indicate that the ESELp-driven lentiviral expression system has the potential to selectively target inflammatory tissues and can be reinduced by acute inflammatory episodes.

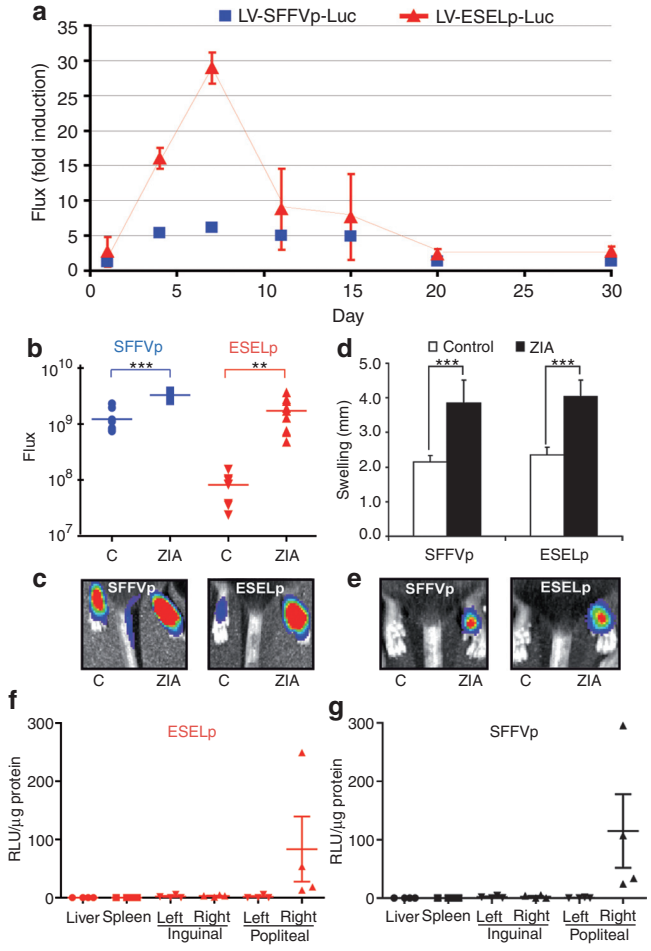

Figure 3 The E-selectin promoter (ESELp)-based lentivector drives local transgene expression at inflammatory sites. Mice received an intraplantar injection of either LV-SFFVp-Luc or LV-ESELp-Luc in both hind paws. After 1 week, mice received intraplantar injections of 180 µg zymosan in the right paw and saline solution in the left paw (as a negative control for inflammation). *In vivo* luciferase activity was determined by bioluminescence at different times after zymosan injection. (**a**) Fold induction of *in vivo* luciferase activity (flux) in zymosan- and saline-injected paws at the indicated times. (**b**) Scatter plot showing *in vivo* luciferase activity (flux) of control and zymosan-induced arthritis (ZIA)-treated paws at day 7 ($P < 0.0016$; $n \geq 8$). (k) Representative bioluminescence images of paws infected with the indicated vectors and treated with saline (**c**) or zymosan (ZIA) for 7 days. (**d**) Graph showing the diameters (mm) of mouse hind paws infected with the indicated vectors and treated with saline (control) or zymosan (ZIA) for 7 days (mean ± SEM; $n \geq 8$). $^{**}P < 0.01$ and $^{***}P < 0.001$. (**f**,**g**) Luciferase activity detected in tissues from mice locally transduced with the indicated lentiviral vector (LV). Total tissue homogenates from liver, spleen, and the indicated lymph nodes were employed to measure luciferase activity ($n = 4$), expressed as RLU (relative light units) per microgram of protein (µg). LV, lentiviral vector.

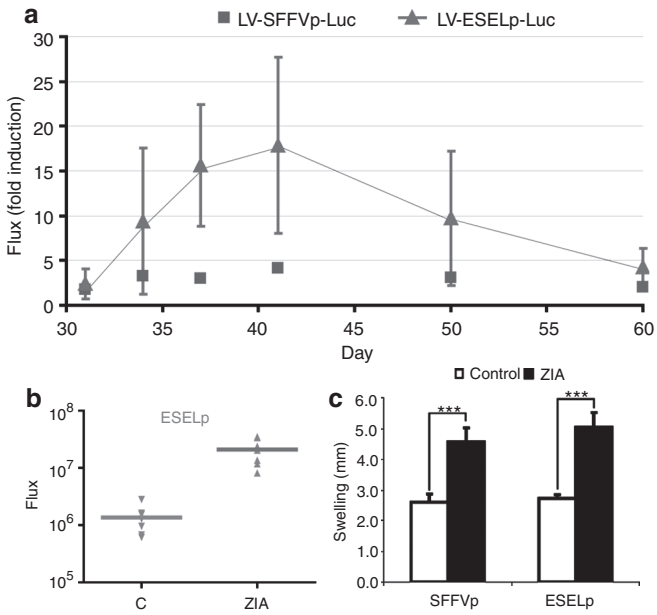

Figure 4 Expression of the E-selectin promoter (ESELp)-based lentiviral system is regulated by the level of inflammation *in vivo*. (**a**) One month after the first zymosan injection, when the inflammation had receded, hind paws were reinjected with saline or 180 µg zymosan to reactivate inflammation. The graph shows fold induction of *in vivo* luciferase activity measured by bioluminescence in zymosan and saline reinjected paws of mice at the indicated times. (**b**) Scatter plot showing *in vivo* luciferase activity (flux) of control and zymosan-induced arthritis (ZIA)-treated paws 7 days after the second zymosan boost ($P = 0.0027$; $n = 7$). (**c**) The graph shows the diameters (mean ± SEM; $n \geq 8$) of mouse hind paws infected with the indicated vectors, measured 7 days after the second treatment with saline (control) or zymosan (ZIA). $^{**}P < 0.01$ and $^{***}P < 0.001$.

Comparative study of transgene expression under different inflammation-inducible expression systems

As other viral expression systems that respond to inflammatory stimuli have been described, we decided to compare the transcriptional inducibility of ESELp with two other inflammation-inducible promoters: a NF-kB responsive promoter (NFkBp) and a hybrid promoter based on the human IL6 promoter fused to the enhancer region of the human IL1 promoter (IL1-IL6p).[10,11] After replacing ESELp with either NFkBp or IL1-IL6p and generating the corresponding luciferase-containing LVs, we employed them to transduce different cell lines. We found that LV-NFkBp displayed the highest luciferase activity in non endothelial cells (**Figure 5a,b**); however, in endothelial cells LV-ESELp and LV-NFkBp showed similar basal luciferase activity (**Figure 5a,c**). Upon stimulation, LV-ESELp was induced in all the cell types tested (**Figure 6a–c**), displaying the highest luciferase activity in endothelial cells (**Figure 5c**). In the case of LV-IL1-IL6p, it showed the highest induction in RAW cells after treatment with LPS, displaying luciferase units similar to those already published.[10]

Next, we compared these inflammation-inducible systems *in vivo*. For this, LV-ESELp-Luc, LV-NFkBp-Luc or LV-IL1-IL6p-Luc was injected subcutaneously into both hind paws and 1 week later the right paw was injected with zymosan and the left paw with physiological saline solution. Luciferase expression was monitored by imaging of luciferase bioluminescence 4 days after zymosan injection. As shown in **Figure 5d**, LV-NFkBp displayed very high-luciferase basal activity that was further increased after zymosan injection. LV-IL1IL6p displayed the lowest luciferase activity and the modest induction observed has no statistical significance. Once again, LV-ESELp showed a low basal activity which was significantly increased after zymosan administration.

ESELp-driven IL10 expression attenuates local acute inflammation *in vivo*

Since IL10 shows potential as an anti-arthritic agent which counteracts the actions of proinflammatory molecules, we used the inflammation-regulated LV-ESELp system to express this cytokine. Cells infected with LV-driving expression of IL10 (LV-ESELp-IL10) released this IL into the culture medium (**Supplementary Table S1**). To test the efficacy of lentiviral-driven IL10 expression *in vivo*, we injected LV-ESELp-IL10 subcutaneously into the right hind paw and LV-ESELp-Luc into the left hind paw of the same animal, so that each mouse served as its own control. Another group of animals was similarly administered with LV-SFFVp-

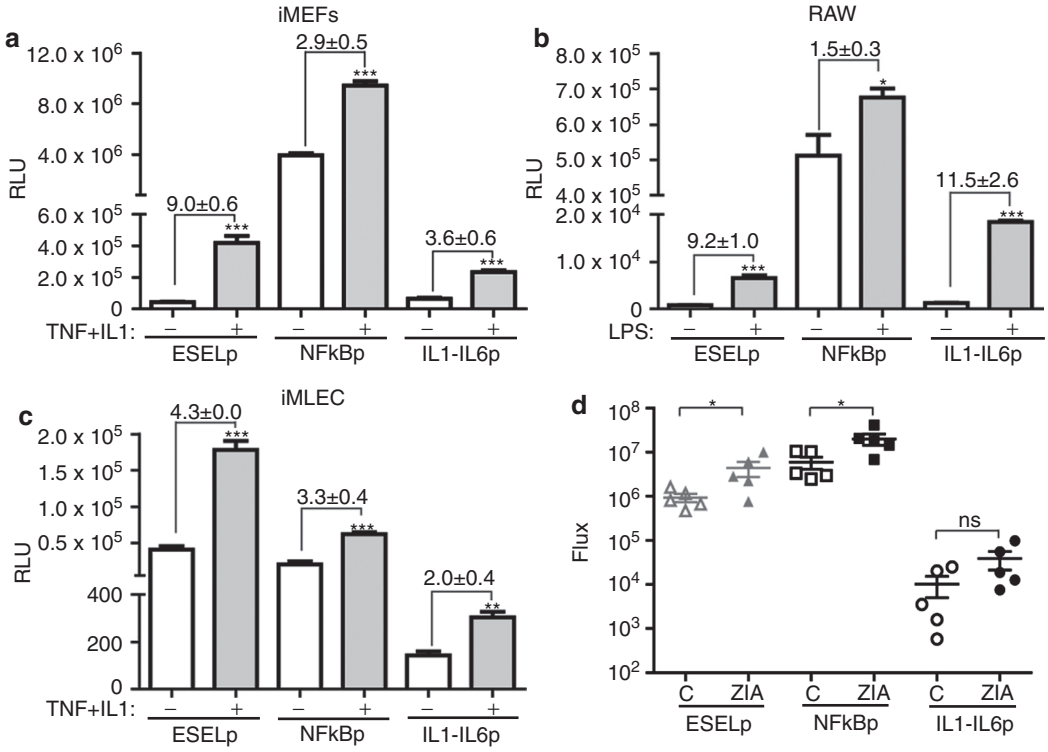

Figure 5 Comparative study of different inflammation-regulated systems. The E-selectin promoter (ESELp)-based system was compared with two other inflammation-inducible systems, the chimeric NFkB promoter (NFkBp) and the human IL1-IL6 hybrid promoter (IL1-IL6p), (**a**–**c**) in cell cultures and (**d**) *in vivo*. (**a**–**c**) The indicated lentivector vector (LV) was employed to transduce (**a**) iMEF (MOI = 5), (**b**) RAW (MOI = 10), and (**c**) iMLEC (MOI = 5) for 5 hours. After serum starvation, cells were incubated with (**a**,**c**) tumor necrosis factor (TNF) (100 ng/ml) plus IL1 (10 ng/ml) for 6 hours or (**b**) with lipopolysaccharide (LPS) (2 µg/ml) for 24 hours. Three experiments were performed and one representative is shown (mean + SEM; $n = 3$). Numbers indicate the fold induction (mean + SD; $n = 3$). (**d**) Mice received an intraplantar injection of either LV-ESELp, LV-NFkBp-Luc, or LV-IL1-IL6p-Luc in both hind paws. After 1 week, mice received intraplantar injections of zymosan (180 µg) in the right paw and saline solution in the left paw (C = control). *In vivo* luciferase activity was determined by bioluminescence after zymosan injection. Scatter plot shows *in vivo* luciferase activity (flux) of control and zymosan-induced arthritis (ZIA)-treated paws at day 5 ($n = 5$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus untreated cells or control hind paw; P value. ns, not significant.

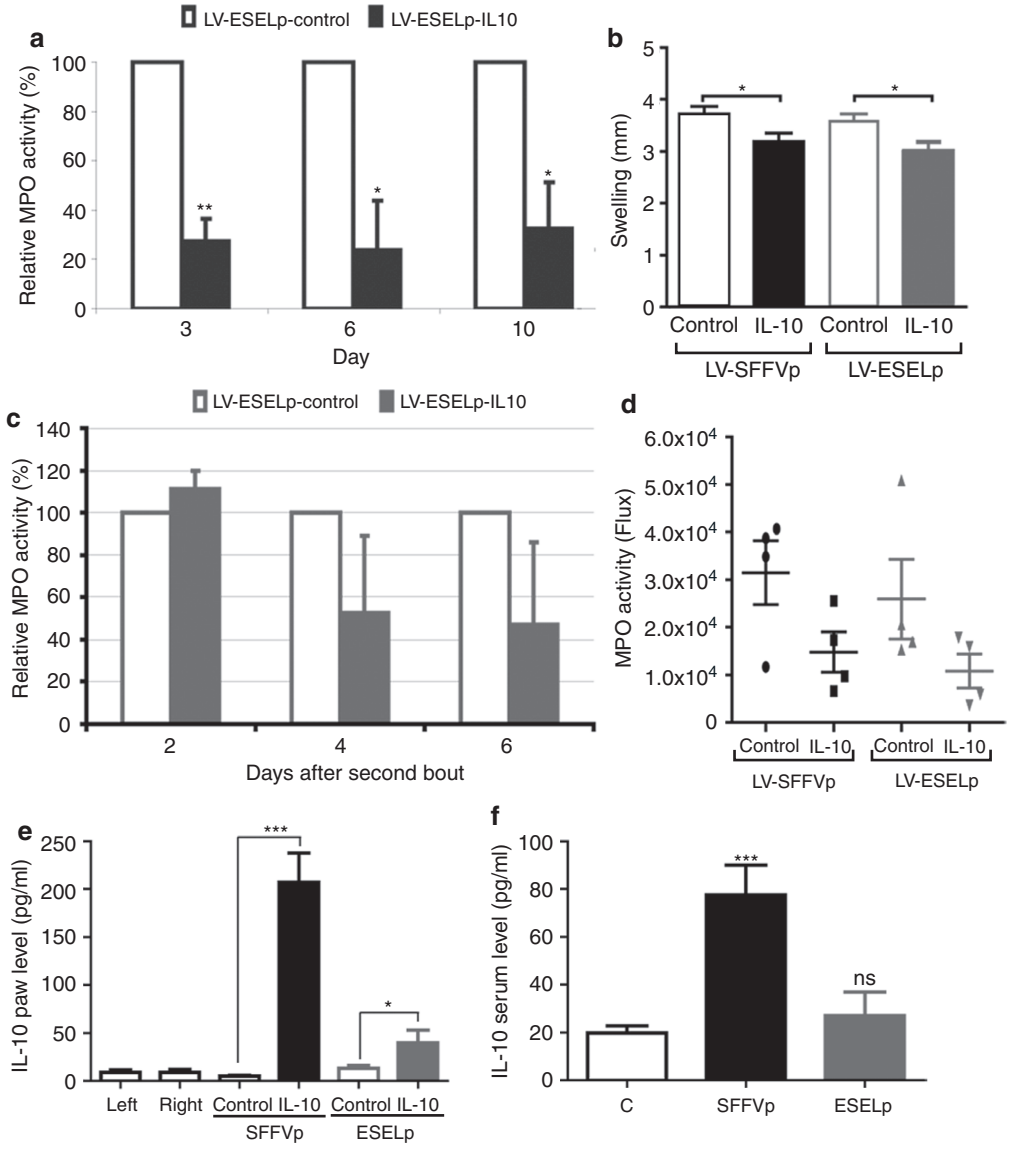

Figure 6 Interleukin-10 (IL10) release from the E-selectin promoter (ESELp) lentiviral system attenuates zymosan-induced inflammation. One group of mice received an intraplantar injection of LV-ESELp-Luc in the left hind paw and LV-ESELp-IL10 in the right one. A second group received similar intraplantar injections of LV-SFFVp-Luc and LV-SFFVp-IL10. After 1 week, mice received intraplantar injections of 180 µg zymosan in both hind paws. (**a**) Myeloperoxidase (MPO) activity was measured by bioluminescence at different times after zymosan injection, and the percentage of relative MPO activity was calculated; 100% = MPO activity in paws injected with LV-ESELp-Luc or LV-SFFVp-Luc (*$P < 0.05$ and **$P < 0.01$; $n > 5$). (**b**) Paw diameters measured 3 days after the first zymosan injection (*$P < 0.05$; $n > 5$). (**c**) After remission of the first acute inflammation, hind paws were reinjected with zymosan (180 µg per paw) to reactivate inflammation, and MPO activity was measured by bioluminescence at different times ($n = 4$). (**d**) The scatter plot shows *in vivo* MPO activity (flux) of zymosan-induced arthritis (ZIA)-treated paws 6 days after the second bout. (**e**,**f**) IL10 level present in (**e**) footpad supernatant and (**f**) serum from the indicated mice 5 days after the first zymosan bout was measured by ELISA (mean ± SEM; $n \geq 4$). *$P < 0.05$ and ***$P < 0.001$ versus control; ns, not significant P value. LV, lentiviral vector.

IL10 and LV-SFFVp-Luc. One week later, ZIA was induced in both hind paws, and MPO (luminol) and luciferase activities were monitored by imaging of bioluminescence every other day until inflammation remission began (10 days). Compared with the luciferase-expressing inflamed paws, the IL10-expressing paws showed a notable reduction in MPO activity, by 90% and 70% 3 days after zymosan injection in the case of LV-SFFVp and LV-ESELp, respectively (**Figure 6a** and **Supplementary Figure S7**). This was accompanied by corresponding reductions in paw swelling (**Figure 6b**).

To test the reactivation of the system, we administered a second zymosan injection after remission of the first acute episode, and monitored luciferase and MPO activity by bioluminescence. The second acute inflammatory episode was again attenuated in the IL10-expressing paws (**Figure 6c,d**). The smaller attenuation observed correlated with the lower degree of inflammation (MPO activity) after a repeated zymosan administration (**Figure 4**). These results show that the anti-inflammatory effect obtained by low and transient level of IL10 expressed under LV-ESELp was similar to that observed after the high and constant level of IL10

released by the LV-SFFVp. Therefore, the expression of IL10 under the LV-ESELp would avoid the side effects associated with a prolonged release of IL10.

Locally released IL10 might reach the blood stream, raising its serum level and therefore increasing the risk of infection. To test this, we measured IL10 paw and serum levels in the two animal groups 5 days after the first zymosan bout. We found that both LV-SFFVp-IL10 and LV-ESELp-IL10 increased the local amount of IL10 (**Figure 6e**); however, only the injection of LV-SFFVp-IL10 significantly raised IL10 serum level which might cause immunosuppression and put the animals at risk of opportunistic infections (**Figure 6f**). Therefore, these results suggest that LV-ESELp-IL10 local administration does not increase susceptibility to infections.

As the local environment in the animal paw is different after zymosan injection, we wondered whether our lentiviral system was also effective when it was administered after zymosan injection. We injected LV-ESELp-IL10 subcutaneously into the right hind paw and LV-ESELp-Luc into the left hind paw of the same animal 1 day after zymosan administration. Another group of animals was similarly administered with LV-SFFVp-IL10 and LV-SFFVp-Luc. We monitored luciferase activity and found the peak at day 7 after zymosan injection (**Figure 7a**). At that time there was a clear swelling reduction in the IL10-expressing paws in both groups which was maintained at least for two more days (**Figure 7b**). These results showed that LV-ESELp also works when it is administered after the initiation of inflammation; therefore, it might be useful as a therapeutic tool after detecting a new inflammatory episode.

DISCUSSION

The inflammatory response is precisely controlled by the expression of cytokines whose local levels are directly related to the severity of the process. A major challenge in the treatment of chronic inflammatory diseases is the development of an expression system that is tightly regulated by the variable levels of these cytokines. This study describes a long-term lentiviral expression system based on the ESELp, and which is locally induced by inflammatory stimuli in direct correlation with the intensity and duration of the inflammatory response.

Studies in animal models have shown that gene therapy is an alternative for the local treatment of chronic inflammatory diseases. One of the critical factors in gene transfer is the type of vector employed. Nonviral vectors commonly yield low gene transfer efficiency.[33,34] Among the viral vectors, adenoviruses are the most widely used, but they are poor candidates for the treatment of chronic inflammatory diseases because of the immune response associated with their application and the rapid loss of transgene expression due to lack of persistence of the viral genomes.[35,36] AAV have emerged as a very promising alternative, since although AAV vectors have limited cargo capacity, they stably transduce host cells and show low immunogenicity. However, recent studies have reported an inflammatory response after AAV application.[12–14] In addition, technical limitations limit the scalability of AAV

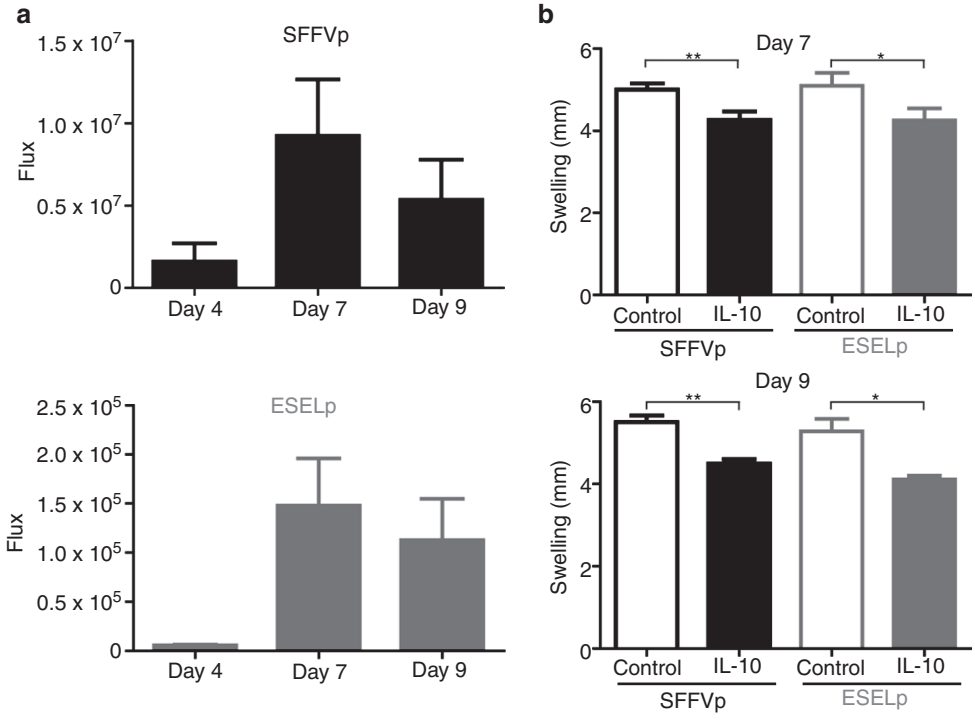

Figure 7 Therapeutic effect of E-selectin promoter (ESELp)-IL10 local administration. Mice received intraplantar injections of 180-µg zymosan in both hind paws. The next day, one group of mice received an intraplantar injection of LV-ESELp-Luc in the left hind paw and LV-ESELp-IL10 in the right one. A second group received similar intraplantar injections of LV-SFFVp-Luc and LV-SFFVp-IL10. (**a**) *In vivo* luciferase activity was determined by bioluminescence after zymosan injection in both animal groups. (**b**) Paw diameters measured (top) 7 and (bottom) 9 days after the zymosan injection. The graph shows the diameters (mean ± SEM; $n = 5$) of mouse hind paws infected with the indicated vectors. *$P < 0.05$ and **$P < 0.01$ versus control. LV, lentiviral vector.

vectors, making it difficult to produce adequate viral titers.[37–39] As an alternative, lentivirus-derived expression systems have been employed in animal models of neuroinflammation.[40] Lentivectors not only infect dividing and quiescent cells, but they also provide long-term expression and show low immunogenicity. In addition, the biosafety profile of LVs has been improved significantly by minimizing the regions of homology between vector and helper sequences (split configuration), and by using heterologous promoters.[41] Furthermore, the use of vesicular stomatitis virus glycoprotein confers efficient transduction in a wide range of cell types from many species, and allows high titers of the lentiviral particles for clinical applications.[42,43] Our study suggests that lentivectors may also be a valuable alternative in the treatment of chronic inflammatory diseases.

Several inflammation-inducible systems have been described recently, all of which are based on chimeric promoters. The precise *in vivo* regulation of these tailored promoters is still unknown. Our expression system is based on the proximal promoter region that controls the expression of the *ESEL* gene. This gene is particularly attractive as it is induced early and transiently upon inflammation and its promoter region contains the binding sites for transcription factors induced by the early induced proinflammatory cytokines TNF-α and IL1. Compared to other described expression systems, the ESELp-based system shows the highest transcriptional activity in endothelial cells. In addition, our expression system is highly induced in endothelial cells by the early induced proinflammatory cytokines TNF-α and IL1. These results are important as activated endothelium plays an important role in inflammation initiation. We tested our expression system in an experimental model of chronic inflammation by administering repeated local injections of zymosan. ESELp-driven transgene expression is rapidly induced after zymosan administration, coinciding with the peak of inflammation 7 days after the first treatment, and is maintained until inflammation recedes. Compared to other inflammation-inducible systems, the ESELp-based is characterized by a low basal activity that after zymosan administration increased to levels similar to those observed with a strong viral promoter. The other expression systems analyzed showed either very high basal activity (NFkB-based system) or very low inducibility after inflammation (IL1-IL6p-based system). Our results show that ESELp-dependent transgene expression increases several fold, correlating with the severity of inflammation in the animal system tested. Future experiments will enable more detailed comparison of how these expression systems perform in gene transfer models.

Since chronic inflammatory diseases are characterized by flare-ups and remission phases, it was important to test whether the promoter was silenced *in vivo* and whether transgene expression could be reinduced after a second zymosan boost. Transgene expression again correlated with inflammatory status after a second zymosan boost, showing no evidence of promoter silencing. We therefore consider the ESELp a valuable tool for the development of gene expression systems for the treatment of chronic inflammatory diseases. The use of ESELp-based gene delivery systems to selectively express anti-inflammatory agents in arthritis-affected joints might eliminate some of the problems of tolerability and compliance associated with systemic drug therapies.

One important issue in locally applied gene therapy is to study the putative migration of transduced cells *in vivo*. In this regard, it has been demonstrated that LV injection into the mouse footpad transduces DCs which are able to migrate to the draining lymph nodes and spleen.[32] However, we only detected residual luciferase activity in the popliteal lymph nodes draining the infected paw suggesting that transduced DCs remain in the local inflammatory focus (**Figure 3f,g**). This is in agreement with previously published results showing that IL10 transgene expression modulates DC maturation.[44,45] The authors observed that DCs transduced with adenoviral vectors expressing IL10 maintained an immature state characterized by low MHC class II, CD86, and IL12 expression. The immaturity might affect to their migratory ability which would support our *in vivo* results. Further experiments should be performed to further characterize the impact of IL10 expression on DC migration *in vivo*.

The occurrence of unpredictable relapses complicates the treatment of chronic inflammatory diseases. RA, the most frequent inflammatory rheumatic disorder, is a paradigm of chronic inflammatory diseases characterized by an imbalance of pro- and anti-inflammatory molecules. Although systemic administration of anti-inflammatory agents is beneficial to patients with chronic RA, these treatments are limited by loss of efficiency and relapse after treatment cessation. There are also significant side effects associated with a prolonged systemic imbalance of the natural inflammatory response.[46,47] Viral vectors are promising candidates for gene therapy for local treatment of RA, and several clinical trials are underway.[48] However, there is still a need to develop new therapeutic approaches that provide prolonged remission from disease with limited side-effects by targeting anti-inflammatory mediators to the diseased joints. The use of disease-regulated promoters to drive transgene expression might provide therapeutic levels of the anti-inflammatory agent exclusively during flare-ups. In addition, administration of virus directly into arthritic joints should avoid the side-effects associated with systemic administration and increase the site-specific effects of the therapeutic agent.

It has been shown that local administration of recombinant IL10 effectively reduces proinflammatory cytokine activity in several animal models of human diseases; however, constant high levels of anti-inflammatory molecules might increase the risk of infection, therefore prolonged administration of IL10 is limited by associated side-effects. Our study demonstrates that activation of the LV-ESELp lentiviral expression system is regulated by the local level of inflammation. Moreover, the LV-ESELp system drives inflammation-regulated IL10 expression at levels sufficient to reduce acute inflammation induced by zymosan with no effect in the IL10 serum levels. Although the LV-ESELp system releases considerably lower local concentrations of IL10 than the constitutive expression vector LV-SFFVp, it is noteworthy that regulated IL10 expression is as effective as constant expression in reducing inflammation *in vivo*. Furthermore, the inducible system is switched off during remission of the inflammation, thus avoiding the risks associated with a sustained release of IL10. Altogether, these results suggest that local administration of LV-ESELp-IL10 should not increase animal susceptibility to opportunistic infections. As expected, the levels of inflammation and transgene induction are lower after the second bout than observed at disease

onset. This endogenously regulated system for the local expression of anti-inflammatory molecules provides a potential new approach for the local treatment of chronic inflammatory diseases.

MATERIALS AND METHODS

Plasmid constructs. The human ESELp (–940;+40) (ESELp) was amplified from human genomic DNA by PCR. The PCR product was cloned into the pHRSIN HIV-derived transfer vector to replace the ubiquitously expressed spleen forming Focus virus promoter, SFFVp. Firefly luciferase cDNA was PCR amplified from a commercially available plasmid (pGL3 Basic).

The NFkB chimeric promoter (NFkBp) was generated by annealing sense and antisense oligonucleotides and direct cloning into pBlueScript in the following order: *Hind*III-*EcoR*I minimal CMV promoter (sense 5′ AGCTTGTAGGCGTGTACGGTGGAGGTCTATATAAGCAGAGCTCG 3′; antisense 5′ AATTCGAGCTCTGCTTATATAGACCTCCACCGTACA CGCCTACA 3′), *Xho*I-3xNFkB-*Hind*III (sense 5′ TCGAGGGACTTTCC ACAAGGGGACTTTCCACAAGGGGACTTTCC3′;antisense5′AGCTGG AAAGTCCCCTTGTGGAAAGTCCCCTTGTGGAAAGTCCC 3′) and *Kpn*I-*Mlu*I-3xNFkB-*Xho*I (sense 5′ CACGCGTGGGACTTT CCACAAGGGGACTTTCCAC AAGGGGACTTTCC 3′; antisense 5′ TCGAGGAA AGTCCCCTTGTGG AAAGTCCCCTTGTGGAAAGTCC CACGCGTGGTAC 3′). The IL1-IL6 hybrid promoter (IL1-IL6p) was generated by cloning the PCR products containing the human IL1 enhancer (–3,690; –2,720) and the human IL6 promoter (–172;+12) into pBlueScript. The PCR primers employed were MluI-IL1-fwd (5′ CCACGCGTGATCCAAGAGGGAGAAGAAGC 3′), XbaI-IL1-rv (5′ GGTCTAGACTGATGCTTTCGCTCGAGGG 3′), IL6-fwd (5′ GGCTTAGCGCTAGCCTCAATGAC 3′) and *BamH*I-IL6-rv (5′ GGGGATCCGAGACTCTAATATTGAGAC TCATGGG 3′). The ESELp sequence was removed from pHRSIN-ESELp-Luc-IRES-GFP by *Mlu*I and *BamH*I digestion and replaced by either NFkBp or IL1-IL6p.

The IL10 coding sequence was amplified by PCR using cDNA generated from total RNA extracted from LPS-treated RAW cells. The PCR products were directly cloned into the SIN-BX plasmid to generate the bicistronic cassettes. The luciferase-IRES-GFP and IL10-IRES-GFP inserts were cloned into the pHRSIN-SFFVp and pHRSIN-ESELp transfer plasmids. The sequences of all plasmids were confirmed and are available in our lab websites (http://www.rodriguezlab.com or http://www.lablife.org/labs/947).

Lentivirus production and titration. HEK-293 cells were transiently transfected by the calcium phosphate method. For viral particle production, the indicated pHRSIN transfer plasmid was cotransfected with two helper plasmids (8.9 and pMD2-G). Supernatants were collected 48 hours after transient transfection and cell debris was removed by centrifugation (10 minutes, 740 x *g*, 4 °C). Viral particles were concentrated by ultracentrifugation in a swing bucket rotor for 2 hours at 121,986 x *g*, 4 °C (Ultraclear Tubes, SW28 rotor and Optima L-100 XP Ultracentrifuge; Beckman Coulter, Fullerton, CA). After removal of the supernatant, viral particles were resuspended in phosphate-buffered saline (PBS) and stored at –80 °C until use.

Total viral content was determined by quantitative PCR.[49] The concentration of transducing units was calculated by infecting 50,000 Jurkat cells (in a p96 plate), with 1, 0.1, and 0.01 µl (duplicates) of concentrated viral supernatant. After 12 hours, viruses were removed and cells were suspended in PBS and analyzed by flow cytometry (FACS Canto HTS; Beckton Dickinson, Franklin Lakes, NJ); the number of infective particles/µl was calculated from the percentage of GFP^+ cells.

Cell culture, transient transfection, and transduction. HEK-293 (ATCC #CRL-1573), RAW 264.7 (ATCC #TIB-71) cells were grown in Dulbecco's modified Eagle's medium (Bio-Whittaker; Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO) and L-glutamine plus antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Jurkat cells were cultured in RPMI medium (RPMI; Bio-Whittaker, Lonza) containing 10% FBS and L-glutamine plus antibiotics. Mouse embryonic fibroblasts (MEFs) were derived from wild-type mice, immortalized with SV40 T large antigen using standard protocols (iMEFs), and grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS plus antibiotics. Elicited peritoneal macrophages were obtained from male mice 4 days after intraperitoneal administration of 1 ml of 10% thioglycolate broth. Peritoneal macrophages were seeded at densities of $0.5–1.0 \times 10^6$ cells in 24-multiwell plates in RPMI supplemented with 10% FBS and antibiotics for 12 hours and transduced. Mouse granulocyte-macrophage colony-stimulating factor bone marrow-derived DCs were generated using granulocyte-macrophage colony-stimulating factor-containing media by standard protocols. DCs cultured in the presence of granulocyte-macrophage colony-stimulating factor were transduced at day 5 of culture. Primary cultures of human umbilical vein endothelial cells and MLEC were isolated and maintained as described.[50] iMLEC were grown in Dulbecco's modified Eagle's medium F-12 (Bio-Whittaker, Lonza) supplemented with 10% FBS, L-glutamine plus antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 50 µg/ml ECGF.[27] Cells were transduced for 5 hours at the indicated multiplicity of infection, washed with PBS and serum-deprived for 12 hours before stimulation with the indicated reagents. After stimulation, cells were trypsinized and harvested for further analysis.

Flow cytometry, ELISA, and luciferase assay. GFP expression was analyzed by flow cytometry (FACScanto, BD Biosciences, Franklin Lakes, NJ) of harvested cells washed with PBS. For footpad cytokine extraction, each one was cut into small pieces in 0.2 ml RPMI with 10% FBS and were incubated for 45 minutes at 37 °C to allow cytokine release. Cytokine-containing media were collected and centrifuged at 2,000*g* for 20 minutes. Footpad or cell culture supernatants were collected for cytokine detection by employing commercial ELISA kits (Quantikine immune assays; R&D Systems, Minneapolis, MN) and measured in a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). To determine luciferase activity, transduced cells were collected after stimulation, washed with PBS, lysed, and analyzed in an AutoLumat LB953 luminometer (Berthold Technologies, Bad Wildbad, Germany). Results are expressed in relative light units. For tissue luciferase assays, 1 ml of reporter lysis buffer was added to 100 mg of each sample and homogenized at 4 °C using a tissue grinder. Tissue homogenates were centrifuged for 10 minutes at 12,800*xg* at 4 °C. Supernatants were saved and employed for luciferase (20 µl) and protein quantification (Bradford; Bio-Rad). Reporter gene expression is shown as relative light units/µg of protein.

Animals. Four-week-old male and 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 CNIC Animal Facility guidelines for the care and use of laboratory animals.

In vivo *optical bioluminescence imaging.* Bioluminescent imaging analysis was conducted with the IVIS 200 *in vivo* imaging system (Caliper, Hopkinton, MA). Mice were i.p. injected with 150 mg/kg firefly luciferin (Promega, Madison, WI) 15 minutes before imaging and anesthetized with isoflurane during the procedure. Photons emitted from live mice were acquired as photons per s/cm^2 per steradian, using Living Imaging 3.0 (Caliper). For photon quantification, a region of interest was manually selected and kept constant within each experiment.

In vivo *matrigel plug assay.* iMLEC transduced with lentiviral particles containing luciferase cDNA were embedded in matrigel (Sigma) which was implanted subcutaneously in the chest of mice. After 24 hours, lipopolysaccharide (40 mg/ml LPS from *Escherichia coli*; Sigma) was intraperitoneally injected and blood samples were obtained 4-6 hours later to determine serum cytokine levels (IL6) by ELISA. Bioluminescence due to luciferase activity was monitored (see above) 24, 48, and 72 hours after LPS treatment.

ZIA animal model. Viral particles (2×10^7 transducing units) were injected into each hind paw of the mouse. After 1 week, the left paw was injected with saline solution (30 µl) and arthritis was induced in the right paw by intraplantar administration of 180 µg zymosan.[30] Luciferase expression was monitored over one month using the IVIS 200 system (Caliper). Inflammation was determined by paw diameter and by monitoring the luminescence after i.p. administration of luminol (Sigma).[31]

Statistical analysis. Data were analyzed for statistical significance using GraphPad Prism (version 5.01). Data shown in **Figure 2** were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test. Data from ZIA experiments were analyzed by *t*-test. Statistical significance was assigned at $P < 0.05$. SEM, standard error of the mean; SD, standard deviation.

SUPPLEMENTARY MATERIAL

Figure S1. Endothelial cells are efficiently infected by lentivectors *in vitro*.

Figure S2. Lentiviral vectors containing the human E-selectin promoter sequence.

Figure S3. Constitutive GFP expression driven by the SFFVp viral promoter is not affected by TNF-treatment.

Figure S4. ESELp-based lentiviral system is induced by an inflammatory milieu in endothelial cells.

Figure S5. *In vivo* optical bioluminescence images of luciferase activity from a representative experiment of the ESELp-driven transgene expression induced by proinflammatory cytokines in a mouse subcutaneous matrigel model.

Figure S6. Inflammation induced by the second zymosan administration.

Figure S7. IL10 release from the SFFVp lentiviral system *in vivo*.

Table S1. IL10 release by LV-transduced cells.

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4.2 Patient-derived olfactory mucosa for study of the non-neuronal contribution to amyotrophic lateral sclerosis pathology

4.2.1 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder of unknown origin characterized by progressive degeneration of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and the spinal cord, initiating in mid-age life[177]. It results in muscle paralysis and ultimately death due to respiratory failure, most commonly within 3 to 5 years of diagnosis[178]. Approximately 90% of ALS patients are considered sporadic (sALS) as they appear to occur randomly throughout the community, whereas the remaining 10% are familial (fALS) cases[179], showing autosomal dominant inheritance in the majority of instances. Of these, ~12% are associated with mutations in the Cu/Zn superoxide dismutase (SOD-1) gene[180], which seem to confer toxic gain of an unknown function rather than loss of normal SOD1 activity[181]. Recently, a large GGGGCC repeat expansion in the first intron of the C9orf72 gene has been reported to be the most common genetic cause of ALS, accounting for approximately 40% of fALS and 7% of sALS[182-184]. While several pathogenic processes for the motor neuron degeneration observed in ALS have been proposed, one of the most prevailing hypotheses supports a non cell-autonomous process: neighbouring astrocytes are thought to play a major role in disease progression[185-190]. The molecular mechanisms underlying neuronal death in ALS are presently not fully determined and appear to be multifactorial and among others, neuroinflammation[191] have been proposed to be involved. Evidence of inflammation is observed in post mortem tissue[192-195], in cerebrospinal fluid (CSF)[196,197] and in blood samples[198] from fALS and sALS patients. These observations are in agreement with previous observations of neuroinflammation in rodent models of ALS[199,200]. In transgenic mice expressing mutant SOD-1, increased levels of Toll-like receptors (TLRs) are detected[201]. Microglial neurotoxic inflammatory responses have been suggested to be facilitated via TLR2 [202]. In addition, it has been shown that mutant SOD-1 binds to CD14, which is a co-receptor of TLR2 and TLR4, and that the microglial activation mediated by mutant SOD-1 can be attenuated using TLR2, TLR4 and CD14 blocking antibodies[203]. In accordance, Casula *et al*. have reported an upregulation of TLR2 and TLR4 as well as other pro-inflammatory molecules such as Receptor for Advanced Glycation End products (RAGE) and High Mobility Group protein B1 (HMGB1) in reactive glia in the spinal cord of sALS victims[194]. One unanswered question, however, is whether these correlative observations reflect a causal relationship, with inflammatory activation provoking ALS, or alternatively, ALS pathology triggering inflammatory responses.

There is a need for patient-derived cell models that are relevant and robust enough to produce the cells required for molecular and functional analyses. In this study we have

explored an alternative patient-derived neural model for the study of sALS disease: primary olfactory mucosa (OM) cultures derived from sporadic ALS patients. The OM is easily accessible for non-invasive biopsy in human adults[204]. Either biopsied tissue or derived primary cultures have been shown to exhibit alterations in Rett's syndrome, Alzheimer's disease, fragile X syndrome, schizophrenia, Parkinson's disease and bipolar disorder with respect to healthy donors[205-210]. OM derived primary cultures contain several types of extraepithelial cells including multipotent stem cells[211-214] as well as olfactory ensheathing glia[215] which normally support the growth of primary olfactory axons from the neuroepithelium in the nasal cavity to the brain[216]. These glia are present both in the peripheral as well as the central nervous system, and share characteristics of both astrocytes as well as Schwann cells. Moreover, olfactory mucosa-derived ensheathing glia have been used for therapeutic purposes in spinal cord injury[217,218] and even in ALS[219]. The fact that olfactory mucosa transplants exhibit regenerative capacity in spinal cord[218], led us to speculate that the interaction of OM derived cells with spinal cord-derived motor neurons may recapitulate the non-cell autonomous properties described for ALS. It is conceivable that olfactory ensheathing cells (OECs) may share some similar characteristics of inflammatory cells, providing a degree of immunological protection against infections in the olfactory system. Indeed microarray analysis has revealed that, relative to astrocytes and Schwann cells, OECs express higher levels of a number of innate immune factors, including lysozymes, chemokines and monocyte chemotactic proteins, suggestive of functions in modulating neuroinflammation[220]. OECs have also been demonstrated to express Toll-like receptors and possess the cellular machinery to respond to certain bacterial ligands[221,222].

Another interesting aspect of using olfactory mucosa cells to model ALS is that it has been proposed that chemosensory impairment is an early symptom of many neurodegenerative diseases[223,224], including ALS[225,226]. Notably, disease-specific alterations in gene expression, protein expression and cell function have previously been found in primary olfactory mucosa cultures derived from patients with schizophrenia and Parkinson's disease[227] as well as spastic paraplegia[228]. In this study, we propose the use of olfactory mucosa to model ALS, demonstrating their negative effect over motor neuron survival and morphology as well as the activation of inflammatory responses as a consequence of this deleterious interaction.

My personal effort in this work has been the expertise with the inflammation-responsive LVs which were employed to detect the inflammatory response when OM cells from ALS patients are co-cultured with human spinal cord neurons.

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Patient-derived olfactory mucosa for study of the non-neuronal contribution to amyotrophic lateral sclerosis pathology

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Abstract

Amyotrophic lateral sclerosis (ALS) is a degenerative motor neuron disease which currently has no cure. Research using rodent ALS models transgenic for mutant superoxide dismutase 1 (SOD1) has implicated that glial–neuronal interactions play a major role in the destruction of motor neurons, but the generality of this mechanism is not clear as SOD1 mutations only account for less than 2% of all ALS cases. Recently, this hypothesis was backed up by observation of similar effects using astrocytes derived from post-mortem spinal cord tissue of ALS patients which did not carry SOD1 mutations. However, such necropsy samples may not be easy to obtain and may not always yield viable cell cultures. Here, we have analysed olfactory mucosa (OM) cells, which can be easily isolated from living ALS patients. Disease-specific changes observed when ALS OM cells were co-cultured with human spinal cord neurons included decreased neuronal viability, aberrant neuronal morphology and altered glial inflammatory responses. Our results show the potential of OM cells as new cell models for ALS.

Keywords: olfactory mucosa • amyotrophic lateral sclerosis • non-cell autonomous toxicity • SOD-1 neurotoxicity • inflammation-responsive promoter

Introduction

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is a neurodegenerative disorder of unknown origin characterized by progressive degeneration of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and the spinal cord, initiating in mid-age life [1]. It results in muscle paralysis and ultimately death because of respiratory failure, most commonly within 3–5 years of diagnosis [2].

The classical division of ALS into familial or sporadic types has been made depending on patient family history. Approximately, 90% of ALS patients are considered sporadic (sALS) as they appear to occur randomly throughout the community, whereas the remaining 10% are familial (fALS) cases [3], showing autosomal dominant inheritance in the majority of instances. Of these, ~12% are associated with mutations in the Cu/Zn superoxide dismutase (SOD-1) gene [4], which seem to confer toxic gain of an unknown function rather than loss of normal SOD1 activity [5]. Other genes, including TARDBP (TAR DNA-binding protein 43) [6], FUS (fused in sarcoma) [7, 8] and angiogenin (ANG) [9] have also been implicated in fALS. Recently, a large GGGGCC repeat expansion in the first intron of the C9orf72 gene has been reported to be the most common genetic cause of ALS, accounting for approximately 40% of fALS and 7% of sALS [10–12]. Sporadic and familial ALS cases are however, clinically indistinguishable, suggesting similar underlying pathophysiological mechanisms of neurodegeneration.

While several pathogenic processes for the motor neuron degeneration observed in ALS have been proposed, one of the most prevailing hypotheses supports a non-cell autonomous process: neighbouring astrocytes are thought to play a major role in disease progression [13–18]. Nevertheless, the basis for the specificity of astrocytes and other glial cells in the selective degeneration of motor neurons remains unclear. Indeed, the molecular mechanisms

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underlying neuronal death in ALS are presently not fully determined and appear to be multifactorial. Glutamate excitotoxicity, mitochondrial dysfunction [19], accumulation of intracellular protein aggregates [20], oxidative stress [21], hypoxia [22, 23], alterations in RNA metabolism [24], impaired axonal transport [25], growth factor deficiency [26] and neuroinflammation [27] have all been proposed to be involved.

Evidence of inflammation is observed in post-mortem tissue [28–31], in cerebrospinal fluid [32, 33] and in blood samples [34] from fALS and sALS patients. These observations are in agreement with previous observations of neuroinflammation in rodent models of ALS [35, 36]. In transgenic mice expressing mutant SOD-1, increased levels of toll-like receptors (TLRs) are detected [37]. Microglial neurotoxic inflammatory responses have been suggested to be facilitated *via* TLR2 [38]. In addition, it has been shown that mutant SOD-1 binds to CD14, which is a co-receptor of TLR2 and TLR4, and that the microglial activation mediated by mutant SOD-1 can be attenuated using TLR2, TLR4 and CD14 blocking antibodies [39]. In accordance, Casula *et al.* have reported an up-regulation of TLR2 and TLR4 as well as other pro-inflammatory molecules such as Receptor for Advanced Glycation End products and High Mobility Group protein B1 in reactive glia in the spinal cord of sALS victims [30]. One unanswered question, however, is whether these correlative observations reflect a causal relationship, with inflammatory activation provoking ALS, or alternatively, ALS pathology triggering inflammatory responses.

A large amount of ALS research has made use of *in vitro* and *in vivo* models based on overexpression of genes with mutations linked to familial ALS. However, as 90% of ALS cases are sporadic, it is difficult to ascertain how representative these models are with respect to the human disease. This highlights one of the main obstacles currently limiting the study also of other neurological diseases: the need for patient-derived cell models that are relevant and robust enough to produce the large quantities of cells required for molecular and functional analyses. Current efforts on developing sALS models have used patient-derived samples from post-mortem brain, spinal cord and muscle [28, 40]. More recently, sALS patient-derived cell cultures have been developed including astrocytes differentiated from neural progenitor cells obtained from spinal cord necropsies [41], and induced pluripotent stem cells [42, 43]. All these models represent various degrees of compromise between relevance for understanding disease aetiology and suitability for diagnostics and drug discovery.

In this study, we have explored an alternative patient-derived neural model for the study of sALS disease: primary olfactory mucosa (OM) cultures derived from sporadic ALS patients. The OM is easily accessible for non-invasive biopsy in human adults [44]. Either biopsied tissue or derived primary cultures have been shown to exhibit alterations in Rett's syndrome, Alzheimer's disease, fragile X syndrome, schizophrenia, Parkinson's disease and bipolar disorder with respect to healthy donors [45–50]. OM-derived primary cultures contain several types of extraepithelial cells including multipotent stem cells [51–54] as well as olfactory ensheathing glia [55] which normally support the growth of primary olfactory axons from the neuroepithelium in the nasal cavity to the brain [56]. These glia are present both in the peripheral as well as the central nervous system, and share characteristics of both astrocytes as well as Schwann cells. Moreover, OM-derived ensheathing glia have been used for therapeutic purposes in spinal cord injury [57, 58] and even in ALS [59–61]. The fact that OM transplants exhibit regenerative capacity in spinal cord [58], led us to speculate that the interaction of OM-derived cells with spinal cord-derived motor neurons may recapitulate the non-cell autonomous properties described for ALS.

It is conceivable that olfactory ensheathing cells (OECs) may share some similar characteristics of inflammatory cells, providing a degree of immunological protection against infections in the olfactory system. Indeed, microarray analysis has revealed that, relative to astrocytes and Schwann cells, OECs express higher levels of a number of innate immune factors, including lysozymes, chemokines and monocyte chemotactic proteins, suggestive of functions in modulating neuroinflammation [62]. OECs have also been demonstrated to express TLRs and possess the cellular machinery to respond to certain bacterial ligands [63, 64].

Another interesting aspect of using OM cells to model ALS is that it has been proposed that chemosensory impairment is an early symptom of many neurodegenerative diseases [65, 66], including ALS [67, 68]. The mucus covering olfactory epithelium has been shown to contain reduced levels of growth factors that may be related to this reduction in olfactory capacity [68]. Thus, this olfactory dysfunction suggests that mucosa neuroepithelium components might also be affected in neurodegenerative disorders, supporting their use as disease models. Notably, disease-specific alterations in gene expression, protein expression and cell function have previously been found in primary OM cultures derived from patients with schizophrenia and Parkinson's disease [69] as well as spastic paraplegia [70].

In this study, we propose the use of OM to model ALS, demonstrating their negative effect over motor neuron survival and morphology as well as the activation of inflammatory responses as a consequence of this deleterious interaction.

Materials and methods

Reagents and antibodies

All media such as Hank's balanced salt solution (HBSS), HBSS supplemented with Ca^{2+} and Mg^{2+}, DMEM, DMEM:F12 and Neurobasal medium as well as other cell culture reagents including L-Glutamine, TrypLE, bovine pituitary extract, B-27 supplement, N-2 supplement were purchased from Gibco, Life Technologies (Barcelona, Spain).

Other special reagents used were: trypsin, penicillin, streptomycin, bovine serum albumin, trypan blue, retinoic acid and lipopolysaccharide (LPS), all purchased from Sigma-Aldrich (St. Louis, MO, USA); DNase-I (Roche, Mannheim, Germany); foetal calf serum (FCS, Hyclone, Logan, UT, USA); forskolin (Alomone, Jerusalem, Israel); primocin (InvivoGen, San Diego, CA, USA); carboxyfluoresceinsuccinimidyl ester (CFSE, Molecular Probes/Life Technologies, Eugene, OR, USA); brain-derived neurotrophic factor (BDNF, Santa Cruz Biotechnology, Santa Cruz, CA, USA), forskolin (Alomone Labs, Jerusalem, Israel); Smoothened agonist (SAG, Merck/Calbiochem, Darmstadt, Germany); Fluoromount-G (Southern Biotech, Birmingham, AL, USA); matrigel (BD Biosciences/Pharmingen, Madrid, Spain) and DAPI (Calbiochem, Nottingham, UK).

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The antibodies against the following proteins were used: βIII-tubulin [71]; S100β, SOD-1 and GAPDH (from Sigma-Aldrich); GFAP and nestin (from Chemicon, Temecula, CA, USA, now part of Merk-Millipore, Darmstadt, Germany); vimentin (Boehringer, Ingelheim, Germany); p75 and neuroligin (from Santa Cruz Biotechonology). The secondary antibodies used were anti-rabbit, antimouse and anti-goat Alexa 555, and Alexa 488 (Molecular Probes/Life Technologies) for immunofluorescence and peroxidase-labelled secondary antibodies (Sigma-Aldrich) for Western blot.

Culture of patient-derived OM

Human OM primary cultures were obtained and cultured as previously described [72]. OM primary cultures were derived from human nasal endoscopic biopsies that were carried out by otolaryngologists (E. Scola and J. Medina) at the Gregorio Marañón Hospital, Madrid. Written informed consent was obtained from all healthy or ALS patients, and the study was approved by the Gregorio Marañón Hospital ethics committee. Pre- and post-nasal biopsy olfaction was evaluated in ALS patients and controls, according to the Barcelona Smell Test-24 (BAST-24), previously validated for the Spanish population [73]. No significant changes were found between the ALS patient group and controls, either before or after nasal biopsy. Briefly, tissue samples were kept at 4°C in HBSS until processing by disaggregation with 0.05% trypsin in HBSS for 20 min, inactivation with one volume of FCS, followed by a 5 min. treatment with 0.01% DNAse-1 in HBSS supplemented with Ca^{2+} and Mg^{2+}, after which the cell pellet was finally resuspended in ME medium: DMEM:F12 (1:1), 10% FCS, 2 mM glutamine, 20 μg/ml bovine pituitary extract, 2 μM forskolin, 50 μg/ml primocin. Unless specified, OM cells were grown as adherent monolayer cultures in ME medium.

Immunofluorescence assay

Immunofluorescence analysis was performed as previously described [68] using the primary and secondary antibodies detailed above and followed by 10 min. incubation with DAPI (1/500). Coverslips were mounted with Fluoromount-G according to the provider's guidelines. Representative images were taken with an Axiovert200 (Zeiss, Oberkochen, Germany) inverted microscope coupled to a CCD camera using Metavue 5.07 software (Universal Imaging, Bedford Hills, NY, USA).

Neuronal survival assay

Preparation of frozen stocks of differentiated neurons from human foetal spinal cord neural precursors was as previously described [76]. For co-cultures with patient-derived glia, OM samples were first seeded in six-well plates (10^5 cells per well). After 24 hrs, differentiated neurons were thawed out and labelled with 25 μM CFSE in PBS containing 1% bovine serum albumin, for 15 min. at 37°C. After labelling, cells were diluted with 5 ml of neuronal medium [DMEM:F12 supplemented with B-27 (20 μl/ml), N-2 (10 μl/ml), streptomycin (100 U/ml) and penicillin (100 μg/ml)]. The cells were then centrifuged and resuspended in 3 ml of neuronal medium at 37°C. The percentage of live neurons was determined by trypan blue exclusion and the labelled cells were seeded in neuronal medium over OM monolayers at a neuron:OM ratio of 1:6. Culture medium was changed partially three times per week. After 2 weeks of co-culture cells were detached with TrypLE, washed with PBS and resuspended in 200 μl PBS for flow cytometry analysis. The total number of CFSE-positive cells was counted in 110 μl of each sample using a FACS CantoII flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) and the results analysed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Olfactory mucosa survival assay

Olfactory mucosa cells were seeded in six-well plates (10^5 cells/well). After 24 hrs, ME culture medium was substituted for neuronal medium (DMEM:F12 supplemented with 20 μl/ml B-27, 10 μl/ml N-2, 100 U/ml streptomycin and 100 μg/ml penicillin) after which the medium was renewed partially three times per week. Twenty-three days after seeding, cells were collected and the total number of surviving cells was counted in a FACS CantoII flow cytometer (Becton Dickinson Biosciences) and the results analysed using FlowJo software (TreeStar Inc.).

Neuronal morphology assay

Differentiation of human foetal spinal cord neural precursors along the motor neuron lineage was carried out by culturing them in Neurobasal medium supplemented with N-2 (5 μl/ml), BDNF (50 ng/ml), retinoic acid (100 nM), Forskolin (5 μM) and streptomycin/penicillin (100 U/ml and 100 μg/ml, respectively) during the first 4 days and Neurobasal medium similarly supplemented with N-2, BDNF, retinoic acid, streptomycin/penicillin, and SAG (100 nM) for up to 3 weeks.

A subconfluent culture of OM cells was grown on round coverslips in 24 multi-well plates and differentiated neurons were seeded on top (5×10^3 neurons per well) in Neurobasal medium supplemented with N-2 (5 μl/ml) and streptomycin/penicillin (100 U/ml and 100 μg/ml, respectively). The medium was changed partially three times per week. After 2 weeks of co-culture, cells were fixed with paraformaldehyde and immunofluorescence analysis was performed as previously described [75] using 195 antiserum specific for βIII-tubulin [71] diluted 1:3000 during 1 hr followed by incubation with anti-rabbit Alexa 555 and DAPI. Representative images were taken and morphological quantification was performed by scoring a minimum of 15 βIII-tubulin-positive cells in at least nine random fields per condition into different morphologies:neuronal, when the cells exhibited filamentous βIII-tubulin staining and the length of the longest neurite was at least four times that of the longest axis of the nucleus; non-neuronal, when the βIII-tubulin stain was diffuse, cytoplasm was more expanded and its longer part was smaller than four times the size of longest axis of its corresponding nucleus. Cells not matching either morphology were discarded from the analysis.

Lentivector production and titration

The viral vectors used to express wild-type and mutant SOD-1 were LentiSOD1^{wt} and LentiSOD1^{G37R} [18]. The inflammation-responsive lentivectors LV-NFκBp-luc, LV-ESELECp-luc, LV-IL1-IL6p-luc encode the luciferase-IRES-GFP reporter construct under the control of 6× NFκB, E-selectin and IL1-IL6 hybrid promoters, respectively; the LV-SFFVp-luc lentivector encodes the same reporter under the control of the SFFV constitutive promoter [74]. Lentiviral stocks were produced as previously described [75]. Vector titre was determined in OM cells by

infection with serial dilutions of the viral supernatants and the number of transduced cells determined 48 hrs post-infection by flow cytometry (FACSCalibur, BD Biosciences) using GFP expression.

fALS cell model

The foetal human primary astrocyte cell line, HA1800, was obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and was cultured according to the provider's instructions. These cells were infected with LentiSOD1^{wt} and LentiSOD1^{G37R} [18] before each experiment.

Innate immune response assay

Astrocytes and OM cells were infected with the indicated lentivectors 2 days after the treatment under study. For LPS stimulation, 24 hrs before treatment, cells were subjected to serum starvation by incubation overnight in DMEM containing 2% FCS to minimize interference by serum-borne factors. Cells were then cultured in the presence of LPS (500 ng/ml) in DMEM containing 2% FCS for the indicated period.

For co-culture, subconfluent spinal cord neural precursors were seeded in 96 multi-well plates coated with matrigel and differentiated along the motor neuron lineage by culture in Neurobasal medium supplemented with N-2 (5 μl/ml), BDNF (50 ng/ml), retinoic acid (100 nM), Forskolin (5 μM) and streptomycin/penicillin (100 U/ml and 100 μg/ml, respectively) during first 4 days and Neurobasal medium similarly supplemented with N-2, BDNF, retinoic acid, streptomycin/penicillin, and SAG (100 nM) during the last 5 days. Thereafter, OM cells and human astrocytes expressing SOD-1 (where indicated) and harbouring luciferase reporter constructs, were seeded on top of the neurons at a density of 7000 per well in Neurobasal medium supplemented with N-2 (5 μl/ml) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Medium was changed every 2 days and luciferase assays were performed at the time indicated for each experiment.

Luciferase assay

Cells were washed with PBS and frozen at −80°C. Luciferase activity was measured with an AutoLumat LB953 luminometer (Berthold Technologies, Bad Wildbad, Germany) using a commercially available assay system (E1501; Promega, Madison, WI, USA) following the manufacturer's instructions. All treatments were performed in triplicate. Normalized luciferase activity represented in the graphs was the result of dividing the relative light unit (RLU) values obtained for each inflammation-responsive vector by the mean RLU value obtained for the constitutive SFFV promoter vector under the same conditions.

Western blot assay

Cells were collected, washed and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5; 300 mM NaCl; 0.5% sodium dodecyl sulphate and 1% Triton X-100) and incubated for 15 min. at 95°C. Protein concentration of the extracts was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA, USA) and 30 μg of each cell extract was resolved by electrophoresis in 12% polyacrylamide gels in the presence of sodium dodecyl sulphate. After electrophoresis, proteins were transferred to nitrocellulose membranes which were blocked with 10% low-fat milk in PBS-T (0.3% TWEEN 20 in PBS) and incubated overnight at 4°C with specific primary antibody against SOD-1 (1:1000) and anti-GAPDH antibody (1:5000). After washing, membranes were incubated with peroxidase-labelled secondary antibodies and the immunoreactive proteins were visualized using the enhanced chemiluminescence detection kit Western Lightning Plus-ECL (PerkinElmer, Waltham, MA, USA) following the supplier's instructions.

Statistical analysis

Statistical comparison of the data sets was performed with Student's *t*-test. The differences are given with their corresponding *P*-value, which is the probability that the observed result could occur merely by chance under the null hypothesis.

Results

Co-culture of neurons with ALS mucosa cells diminishes their survival

As a result of the fact that the majority of ALS cases are not associated with a known mutation [21], our first goal was to generate an ALS cell bank based on sporadic cases. Numerous studies have highlighted the relevance of glial cells in ALS pathogenesis [13–18] and therefore we concentrated on OM as it is a source of glia that can be obtained from living patients *via* a small nasal endoscopic biopsy [72]. We generated a bank of OM from eight healthy donors and seven ALS patients (Table 1). In this study, we used culture conditions optimized to enrich for the growth of glia cells [72]; we successfully obtained primary cultures from all of the patients and did not detect any difference in the efficiency, survival, growth rates, life span or antigenic markers in samples derived from ALS patients compared to those from healthy donors (Figs S1 and S2, and Table 1). All cells were similar in their expression of the antigenic markers GFAP, vimentin, S100β, neuroligin, nestin and the low affinity NGF receptor p75 (Fig. S2), all of which are characteristic of OECs. However, as a cautionary note, our previous work has demonstrated that these immunocytochemical properties do not clearly distinguish OECs from other cell types such as fibroblasts or gliomas [72]. To test whether these biopsy-derived glia exhibit disease-specific neurotoxicity similar to that observed for spinal cord astrocytes derived from ALS patient cadavers, we co-cultured them with differentiated post-mitotic human spinal cord neurons [76] which we pre-labelled with CFSE to facilitate quantification. The neurons were plated over OM monolayers at a neuron:OM ratio of 1:6 (Fig. 1A) and after 2 weeks of co-culture, cells were harvested and the total number of surviving neurons was counted by flow cytometry. We observed that neuronal survival was significantly higher when the neurons were co-cultured with OM derived from healthy donors

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Table 1 Information about olfactory mucosa donors

Patient	Gender	Age	Maximum cell passage	Number of cells/ mm^2 for confluence
C1	Male	26	10	260
C2	Male	36	>10	364
C3	Female	51	>10	260
C16	Male	20	>10	260
C17	Female	25	>10	260
C18	Female	22	>10	260
C19	Male	29	>10	260
C20	Female	30	>10	364
ALS1	Male	57	>10	208
ALS3	Male	72	9	364
ALS4	Male	48	>10	208
ALS5	Male	36	10	208
ALS6	Female	61	>10	260
ALS7	Male	40	>10	364
ALS8	Male	39	>10	364

compared to when they were co-cultured with OM from ALS patients (Fig. 1B).

Neuron morphology is aberrant in co-cultures with OM from ALS patients

In addition to diminished survival when spinal cord neurons were co-cultured with OM cells from ALS patients, we observed that the morphology of the surviving cells was significantly affected (Fig. 2). While neurons grown over control OM cells preserved their typical morphology with a small cell body and long neuritis positive for strong fibrillar βIII-tubulin staining (Fig. 2A, upper row), neurons grown over OM cells from ALS patients often displayed aberrant morphologies, exhibiting wider cell bodies and retracted neurites with less fibrillar and more diffuse βIII-tubulin staining (Fig. 2A, lower row). As OM cells may express low levels βIII-tubulin [72], we optimized the βIII-tubulin staining sensitivity to exclusively label CFSE-positive cells. For morphometric analysis, cells were scored as neuronal if the length of the longest neurite was at least four times that of the longest axis of the nucleus. Based on this criterion, the data showed a significant reduction in the percentage of cells with neuronal morphology when the neurons were co-cultured with OM from ALS patients compared to when they were co-cultured with OM from healthy donors. We thus concluded that co-culture with OM derived from ALS patients not only reduced neuronal survival but also modified the morphology of surviving cells.

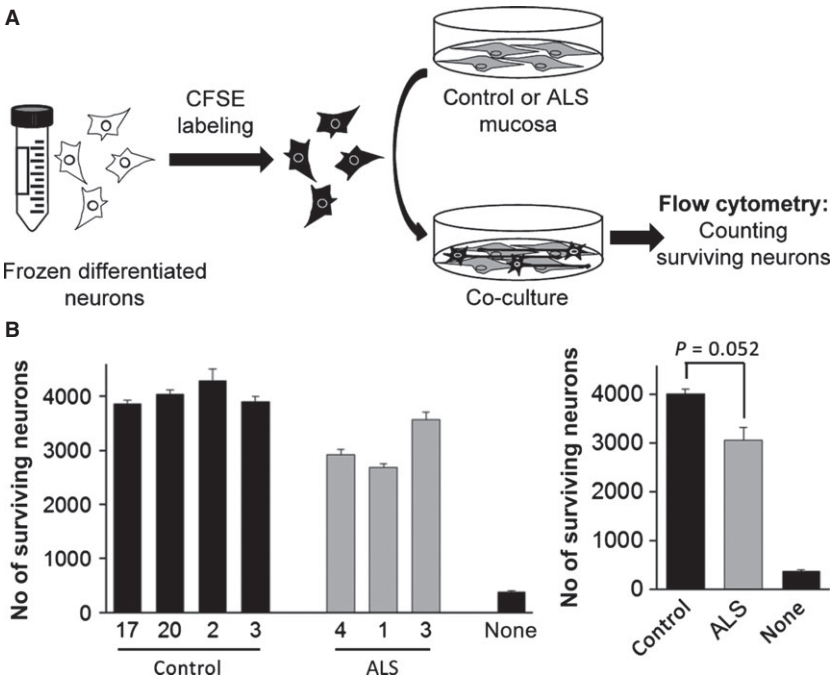

Fig. 1 Spinal cord neuron survival after co-culture with olfactory mucosa (OM). (**A**) Scheme of the procedure using flow cytometry to study the survival of CFSE-labelled spinal cord neurons after co-culture with OM cells from healthy control or ALS donors. (**B**) Flow cytometry quantification of the number of CFSE-labelled surviving neurons after 2 weeks of co-culture with OM cells or in the absence of these cells (none). The left graph shows means and standard errors of the mean (SEM) of three independent assays of each OM sample. Samples are ordered by ascending patient age within each group (control and ALS). The right graph represents the combined mean and SEM of the control or ALS groups with the *P*-value for comparison of the means.

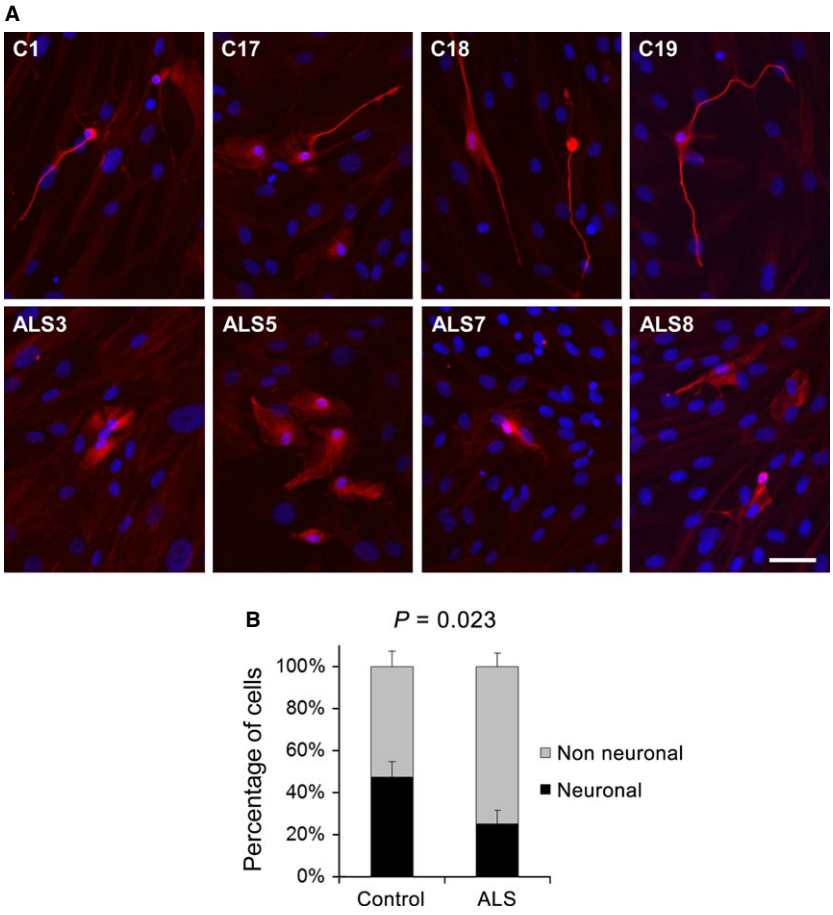

Fig. 2 Morphology of the surviving spinal cord neurons after co-culture with olfactory mucosa (OM). (**A**) Representative immunofluorescence images of the surviving spinal cord neurons labelled for βIII-tubulin after 2 weeks of co-culture over monolayers of OM from four different healthy control donors (upper row) and four different ALS patients (lower row). Nuclei were labelled with DAPI. (**B**) Morphometric quantification of spinal cord neurons after co-culture over OM cells. CFSE-labelled cells were classified as: neuronal if they exhibited filamentous βIII-tubulin staining and the length of the longest neurite was at least four times the length of the longest axis of the nucleus; non-neuronal if they exhibited diffuse βIII-tubulin staining and all neurites were shorter than four times the length of the longest axis of the nucleus. A minimum of 15 neurons in at least nine random fields were scored for each OM sample. Graphs represent the percentage of cells exhibiting each morphology, showing means and standard errors of the mean of eight different controls and seven different ALS OM samples with the *P*-value for comparison of the means. The scale bar represents 50 μm.

Glial inflammatory response to LPS is not altered in ALS

Mounting evidence suggests that neuroinflammation plays an important role in the degeneration of motor neurons in ALS [77]. It has been demonstrated that reactive astrocytes and microglia can release pro-inflammatory factors such as cytokines and chemokines, which are harmful to neighbouring neurons [78]. To test whether OM cells from ALS patients have an altered pro-inflammatory state and/or a modified response to inflammatory stimuli, we transduced OM cells with three different inflammation-regulated lentivector systems [74]. As a control, OM cells were transduced with the same reporter cassette (luciferase-IRES-GFP) under the control of a constitutively promoter (SFFVp). We first transduced OM cells from healthy donors and challenged them with a pro-inflammatory stimulus, LPS. We observed efficient activation of the inflammation-regulated promoters (NFκBp, IL1/IL6p and ESELp) after LPS treatment for 6 and 27 hrs (Fig. 3A). As the largest responses to LPS were obtained with the NFκB and E-selectin promoters, we selected these reporters for subsequent experiments.

Our next goal was to ascertain if abnormal pro-inflammatory innate responses in ALS glia play a role in their toxicity to motor neurons. For this, we used the inflammation-inducible expression systems to first analyse human astrocytes overexpressing $SOD1^{G37R}$, a previously published genetic model of ALS; in this model, the authors showed that co-culture with these modified astrocytes was deleterious to mouse motor neurons [18]. We thus compared astrocytes expressing either wild-type SOD1 or the mutant $SOD1^{G37R}$ to control astrocytes (non-transduced) after LPS treatment for 6 hrs (Fig. 3B and C). Luciferase activities were lower in astrocytes than in OM cells, indicating less pro-inflammatory response in this cell type. In addition, NFκBp and ESELp reporter activity did not increase in astrocytes expressing either wt SOD1 or mutant $SOD1^{G37R}$; overall luciferase activity was actually lower in transduced astrocytes compared to that observed in control astrocytes. In the case of NFκBp, reporter activity was significantly higher in the presence of mutant SOD1 than in the presence of wild-type SOD1. In addition, LPS challenge induced a significant increase in luciferase activity in presence of mutant SOD1, but not in the presence of wild-type SOD1 (Fig. 3B). A similar effect was observed using the ESELp

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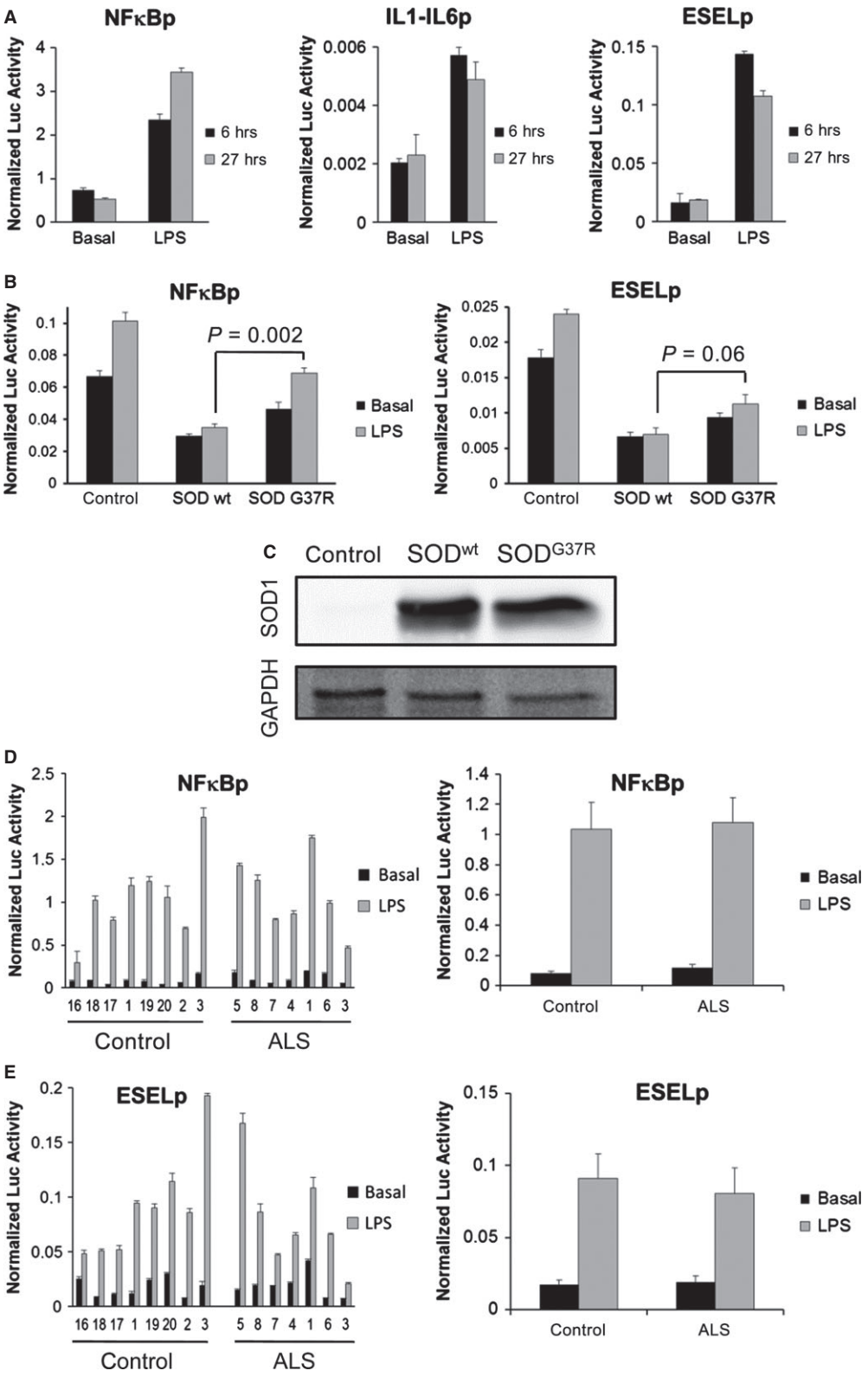

Fig. 3 Study of the inflammatory response in ALS cell models. Cells were transduced with reporter lentivectors encoding the luciferase gene under the control of three inflammation-responsive promoters (NFκBp, an artificial promoter containing multiple NF-κB binding sites; IL1/IL6p, consisting of the human IL-6 promoter fused to the enhancer region of the human IL-1 promoter; or ESELp, the human E-selectin promoter) or the constitutive spleen focus-forming virus promoter (SFFVp). Normalized luciferase activity was obtained by dividing the luciferase activity measured in relative light units (RLU) for each inflammation-responsive promoter by the mean activity of the SFFVp reporter in the same conditions. Graphs represent means and standard errors of the mean (SEM) of three independent samples. (**A**) Reporter activities in untreated control olfactory mucosa (OM) cells (Basal) or after challenge with LPS (500 ng/ml) for either 6 or 27 hrs. (**B**) Reporter activities in control human astrocytes or those overexpressing $SOD1^{wt}$ or $SOD1^{G37R}$ either without (Basal) or with LPS challenge for 6 hrs. Comparison of mean luciferase activities (corresponding *P*-values are shown) after LPS treatment of astrocytes overexpressing SOD^{G37R} with those overexpressing SOD^{wt} revealed a significant increase using the NFκBp reporter; this effect was similar using the ESELp reporter, although not statistically significant. (**C**) Western blot analysis of SOD1 expression in the human astrocyte ALS cell models used in B. (**D**) The left graph shows luciferase activities of the NFκBp reporter in OM cells from eight control donors and seven ALS patients either without (Basal) or with LPS challenge for 6 hrs. Samples are ordered by ascending patient age within each group (control and ALS). The right graph represents the combined mean and SEM of the control or ALS groups. (**E**) Similar study to that shown in D but using the ESELp reporter.

reporter although the differences were not statistically significant (Fig. 3B).

We next used these inflammation-inducible reporter systems to study the inflammatory response in OM cells from healthy donors and ALS patients (Fig. 3D). Although we observed efficient activation of both NFκBp (Fig. 3D) and ESELp (Fig. 3E) reporters in response to LPS treatment, no significant differences between ALS and healthy samples were observed, either in basal conditions or after LPS exposure. Thus, our results indicate that there is no alteration of the inflammatory response in OM cells from ALS samples.

Olfactory mucosa from ALS patients shows increased inflammatory response in co-cultures with spinal cord neurons

We next posed the question if the altered inflammatory response described for ALS might be a consequence, rather than the cause, of the abnormal interaction between neurons and glia. To address this issue, we co-cultured human astrocytes, either unmodified (control), or overexpressing wild-type SOD1 or mutant $SOD1^{G37R}$ with differentiated human spinal cord neurons (Fig. 4A and B). As we had observed the largest response using the NFκBp reporter previously (Fig. 3), we employed it to study the effects of only neuronal co-culture on the astrocytes (Fig. 4A). Under basal conditions (in the absence of neurons), the SOD1-overexpressing astrocytes showed little change in reporter activity compared to unmodified astrocytes; neuronal co-culture significantly increased the luciferase activity in astrocytes overexpressing wild-type SOD1, and this increase was even greater in those overexpressing mutant $SOD1^{G37R}$ (Fig. 4A). Western blot analysis confirmed similar levels of expression of wild-type or mutant SOD1, ruling out the possibility that the different sensitivities to the neurons were because of different SOD1 levels. These results are consistent with the idea of the accumulation of wild-type SOD1 in sporadic ALS patients [79] to adopt an abnormal pathogenic conformation which may be exacerbated by overexpression or by certain point mutations.

We next applied the same methodology to measure the NFκB-mediated response in OM samples derived from healthy donors and ALS patients (Fig. 4C). Again, a significant increase in reporter activity was observed when the cells were co-cultured with spinal cord neurons, with the OM cells from ALS patients showing higher luciferase activity than those from healthy donors (Fig. 4C). As increased reporter activity in the ALS group is not observed in the absence of co-culture with neurons, we conclude that the altered glial inflammatory response in ALS is likely to be a consequence, rather than a cause, of neuronal death, which may liberate inflammatory cytokines which in turn activate NFκB-mediated responses.

Discussion

Multiple lines of evidence have shown the immune system, including astrocytes and microglia, to be deleterious for motor neurons in ALS. Reactive astrocytes and microglia may release pro-inflammatory factors such as cytokines and chemokines which are harmful for the neighbouring cells [78]. However, their role as the primary cause of the disease remains undetermined. Our data indicate that there is no increased innate immune response of glia in ALS: using the established model of SOD1 overexpression in human astrocytes, we did not observe increased pro-inflammatory response after LPS treatment and this result is similar when OM cells from healthy donors and those from ALS patients are compared. However, co-culture with motor neurons increases glial sensitivity to pro-inflammatory stimuli in ALS: we observed augmented NFκB-dependent reporter activity both in the SOD1-overexpressing astrocyte model as well as in OM cells from ALS patients. These results indicate that alterations in the innate immune response of glia in ALS might be a consequence of their interaction with damaged neurons rather than the cause of initial neuronal damage. Nevertheless, once sensitized, the modified pro-inflammatory response of glia in ALS could further worsen the state of neighbouring neurons. In agreement with this concept, previous work using a SOD1 transgenic mouse model demonstrated that microglia and T cells initially slow disease progression, but at later stages after accumulation of SOD1 protein, contribute to acceleration of the disease [80]. Moreover, it has been shown in both ALS patients as well as in mouse models that activation of microglia and astrocytes takes place only after distal axon degeneration [40].

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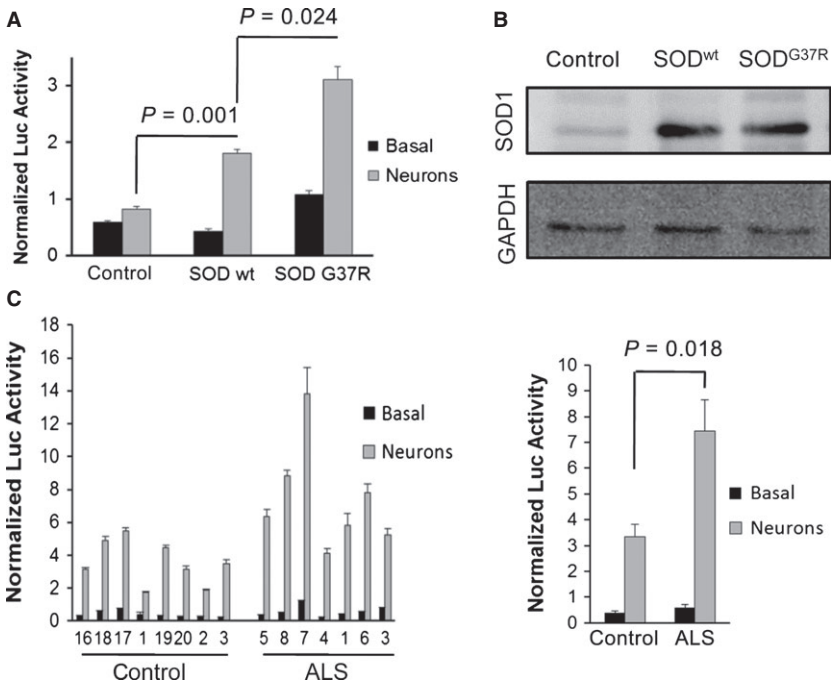

Fig. 4 Study of the inflammatory response in ALS cell models co-cultured with spinal cord neurons. Cells were transduced with reporter lentivectors encoding the luciferase gene under the control of an artificial promoter containing multiple NF-κB binding sites (NFκBp) or the constitutive spleen focus-forming virus promoter (SFFVp). Normalized luciferase activity was obtained by dividing the luciferase activity measured in relative light units (RLU) for each inflammation-responsive promoter by the mean activity of the SFFVp reporter in the same conditions. Graphs represent means and standard errors of the mean (SEM) of three independent samples. (**A**) Reporter activities in control human astrocytes or those overexpressing $SOD1^{wt}$ or $SOD1^{G37R}$ cultured either alone (Basal) or with spinal cord neurons for 5 days (Neurons). (**B**) Western blot analysis of SOD1 expression in the human astrocyte ALS cell models used in A. (**C**) The left graph shows reporter activities in olfactory mucosa cells from eight control donors and seven ALS patients cultured either alone (Basal) or with spinal cord neurons for 1 day. Samples are ordered by ascending patient age within each group (control and ALS). The right graph represents the combined mean and SEM of the control or ALS groups with the *P*-value for comparison of the means.

The use of OM to model ALS offers certain advantages over other cell models. The non-invasive and relatively simple nasal biopsy procedure provides a patient-derived source of living cells that can be easily expanded to perform molecular analysis and functional assays. Samples can be collected not only from patients showing genetic linkage but also from sporadic cases which represent the majority of ALS victims and are more difficult to model. Most importantly, in view that sporadic ALS patients may show wide variability in disease aetiology and responses to therapy, OM cells can be obtained from living patients, offering the future possibility of personalized *in vitro* drug screening prior to treatment of the patient. Presently, the majority of patient-derived sALS models originate from post-mortem tissue [28, 40, 41], placing limitations on the average cellular lifespan and not offering any benefit to the donor. The advent of induced pluripotent stem cells generated from sALS patients offers an expandable cell model that can be obtained from living patients [42], but this involves a complex, time-consuming and expensive protocol. On the contrary, OM cell culture is a relatively simple and reproducible technique which can yield long-lived cultures without the need for genetic manipulations which could generate undesired non-disease-related alterations. Furthermore, OM cultures avoid the necessity for inefficient, complex and expensive protocols to differentiate neural precursors as they provide a direct source of neural cells, which, as we have observed in the present study, recapitulate ALS-specific hallmarks. Firstly, we observed that compared to healthy donor OM cells, co-culture with those derived from ALS patients results in reduced survival and aberrant morphology of spinal cord neurons, in agreement with the deleterious effect of ALS glia on co-cultured neurons previously reported [14–18]. This may be because of the generation of a toxic factor and/or decreased trophic support by the glia [26]. The latter is particularly relevant in view of the fact that our controls show that neurons exhibit drastically reduced survival in the absence of mucosa cells (control samples 'none' in Fig. 1B and [72]). Secondly, we observed good correlation between the effects obtained with OM cells from ALS patients and those from a previously published ALS model using SOD1-expressing astrocytes [18]. While LPS challenge did not result in increased NFκB-mediated response in ALS samples, when OM cells or astrocytes were co-cultured with neurons, an augmented sensitivity was observed in ALS samples that was not detected in the absence of neurons. These data indicate that while there may be no alteration of the innate immune response in cells from ALS patients, the differential death of neurons after co-culture

with ALS-derived samples may trigger an inflammatory process including activation of NF-κB pathways. Consistent with this, NF-κB activation has previously been observed in spinal cord astrocytes in ALS patients as well as in TDP-43 animal models where it was also demonstrated that the inhibition of NF-κB with Withaferin A reduced denervation in neuromuscular junctions [81, 82].

Although important advances in ALS research have been made using mutated SOD1-expressing astrocytes, this model has the disadvantage that it only represents a small fraction of genetic cases of the disease. Furthermore, transduction by the SOD1 transgene may result in overexpression the protein at non-physiological levels or generate other disease-unrelated artefacts such as insertional mutagenesis. In the present study, to achieve a completely human model, we have examined the effect of patient OM on motor neurons derived from human foetal spinal cord. In further work, it would be interesting to compare their effect on rodent motor neuron primary cultures, which can be prepared cells that are more fully committed to the motor neuron lineage [83, 84]. To facilitate such studies, the relative homogeneity of response to different ALS OM samples with respect to controls may permit the use of pooled patient cells or perhaps even immortalized cell lines to generate more user-friendly cell models for ALS. Validation of OM as a cell model for ALS offers a new versatile tool to accelerate research and therapeutic development for this presently incurable devastating disease. Detailed characterization of OM cell models opens up the possibility of correlating genetic and functional differences which will facilitate the identification of more cellular components implicated in the disease process.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Survival of olfactory mucosa.

Figure S2 Immunocytochemistry of OM cells cultured in glial medium.

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4.3 Efficient expression of bioactive murine IL-12 as a self-processing P2A polypeptide driven by inflammation-regulated promoters in tumor cell lines

4.3.1 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 pro-tumor and anti-tumor mediators of both innate and adaptive immunity[229]. 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 pro-tumor effector mechanisms are upregulated by chronic inflammation, therefore endorsing the hypothesis of a pro-tumorigenic chronic inflammation, which seems to produce an immune suppressive and tumor-friendly environment, promoting carcinogenesis and tumor growth[230]. On the other hand, inflammation is also necessary to create an environment that promotes the induction of anti-tumor immunity by the recruitment and activation of several immune effector cells[90].

IL-12 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 IL-12 is secreted, it engages the IL-12 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 IL-12 function. IL-12 is crucial for Th1 differentiation and cytotoxic responses[12]. For many years the anti-tumor effect of IL-12 has been known[108], it 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 anti-angiogenic factors such as IP10[109].

Several studies have demonstrated the anti-tumor activity of IL-12 in preclinical models, suggesting its therapeutic use as an anti-cancer agent. However, clinical trials involving IL-12 have been unsuccessful due to the toxic side effects associated with its systemic administration, prompting investigation into new methods of IL-12 delivery designed to avoid unacceptable toxicity[113]. In this context, gene therapy seems to be a good alternative strategy, as gene transfer methods can be designed to confine IL-12 production to the

tumor environment, preventing systemic toxicity[142]. Several vectors derived from viruses[143-146], as well as non-viral vectors[231] have been developed to transfer IL-12 genes locally to the tumor site, offering encouraging results in preclinical experiences, not only by itself but also when used in combination with other anti-tumor 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 IL-12[147].

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 rapidly and transiently upon acute inflammation in response to early pro-inflammatory cytokines (TNF-α, IL1), making this promoter a good candidate for the design of inflammation-regulated gene therapy vectors[160]. We have also generated lentivectors incorporating other previously described inflammation-inducible promoters, such as the human IL-6 promoter fused to the enhancer region of the human IL-1 promoter (IL1-IL6p)[157] and a chimeric promoter based on NFκB-binding sites (NFκBp)[158]. Since bioactive IL-12 is a heterodimer composed of two subunits, p40 and p35, and it has been shown that p40 homodimers are potent IL-12 antagonists[232], it is important to develop a system that ensures equimolecular expression of both IL-12 subunits to maximize the therapeutic effects of IL-12. In this work we have cloned and expressed both murine IL-12 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, to transduce murine tumor cell lines commonly employed in syngeneic tumor models. In addition, these IL-12 genes are expressed in lentiviral vectors under the control of different inflammation-inducible promoters to confine their expression to inflammation foci. We confirmed the inducibility of these systems *in vitro* and *in vivo* and demonstrated the successful expression of both IL-12 subunits and the release of bioactive IL-12 upon pro-inflammatory stimulation.

My personal contribution has been the characterization of the transduced tumor cell lines with our inflammation-inducible LVs upon pro-inflammatory stimulation *in vitro* and after subcutaneous implantation *in vivo*.

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ORIGINAL ARTICLE

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

C Lorenzo[1,3,4], G Pérez-Chacón[2,4], G Garaulet[1,4], Z Mallorquín[1], JM Zapata[2] and A Rodríguez[1]

Interleukin 12 (IL12) is a heterodimeric proinflammatory cytokine that has shown promise as an anticancer 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.

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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 the 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]

Interleukin 12 (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 interferon (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 anticancer 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

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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 IFN-mediated response, which has proven to be absolutely required for the efficient antitumoral effect of IL12, as recently demonstrated by Melero and co-workers 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 (tumor necrosis factor-α (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′-GATCCGGAGCCACGAACTTCTCTCTGTTAAAGCAAGCAG GAGACGT GGAAGAAAACCCCGGTCCT-3′) and 2A-2 (5′-CTAGAGG ACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGA GAAGTTCGTGG CTCCG-3′) (Sigma-Aldrich, St Louis, MO, USA). 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 polymerase chain reaction (PCR) amplified from the AG250-DPmuIL12opt plasmid[19] using the oligonucleotides 5′- CGCTCTTCTAGAC**ATG**TGCCAGTCGCGCTACCT CCTCTTC-3′, which added a *XbaI* site (underlined) upstream to the start codon (bold), and 5′-GCGAGAGCGGCCGC**TT**ATCAGGCGGAACTCAGGTA GCCCATC-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, Madison, WI, USA) 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; Teddington, UK), murine LLC (ATCC #CRL-1642), murine melanoma (B16-F10; ATCC #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 (Sigma-Aldrich) and L-glutamine (2 mM) plus antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). After serum starvation (2% fetal bovine serum O/N), RAW cells were incubated with lipopolysaccharide (2 μg ml^{-1}) for 24 h.

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, Mountain View, CA, USA), 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 h, the culture media was collected and stored at − 70 °C. Fresh culture medium containing the protein transport inhibitor Brefeldin A (BD GolgiPlug, BD Biosciences, San Jose, CA, USA; cat. #555029) was added to the transfected cells. After 6 h 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 h at room temperature, the blots were incubated overnight at 4 °C with the corresponding first antibody solution: anti-mouse IL12 antibody (0.1%, v/v; R&D Systems, Minneapolis, MN, USA; cat. #AF-419-NA), rabbit polyclonal anti-2A peptide (0.05%, v/v; Millipore, Billerica, MA, USA; 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, Grand Island, NY, USA; cat. #4160 and #31430, respectively) in TBS-T with 5% skimmed milk for 1 h at room temperature. The blots were then washed again with TBS-T and membrane-bound antibody was detected with enhanced chemiluminescence detection reagent (GE Healthcare, Pittsburgh, PA, USA).

Second-generation lentivector production and titration

HEK-293 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, Cambridge, MA, USA). Supernatants were collected 48 h after transient transfection and cell debris was removed by centrifugation (10 min, 740*g*, 4 °C). Viral particles were concentrated by ultracentrifugation in a swing bucket rotor for 2 h at 121, 986*g* at 4 °C (Ultraclear Tubes, SW28 rotor and L8–70 Ultracentrifuge; Beckman Coulter, Brea, CA, USA). After supernatant removal, viral particles were resuspended in phosphate-buffered (PBS) and stored at − 70 °C. Total viral content was determined by quantitative PCR.[22]

Transduction and induction assay

LLC, B16-F10, and HEK-293 cells were seeded in six-well plates (5×10^5 cells per well) and transduced for 5 h with different lentiviral particles at the indicated multiplicity of infection. They were then passaged into 24-well plates and incubated for 12 h under serum deprivation (0.5% fetal bovine serum for LLC and 2% fetal bovine serum for B16-F10 and HEK-293) before proinflammatory stimulation with TNF-α (100 ng ml^{-1}) and IL1β (10 ng ml^{-1}) (Peprotech, London, UK). After 6 h of stimulation, supernatants were collected and cells were lysed for further analysis. Each experiment was performed at least three 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, Sunnyvale, CA, USA) and for protein quantification (Bradford; Bio-Rad, Hercules, CA, USA). Reporter gene expression is shown as relative light units 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, USA). Splenocytes were resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol (2-ME), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2 mM L-glutamine and incubated in 96-well plates (5×10^4 cells per well) with concanavalin A (ConA, 4 μg ml^{-1}) for 30 h at 37 °C. After this incubation, different amounts of codon-optimized murine IL12 were added and cell survival (proliferation) was determined 48 h 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 and subsequently incubated for 30 h in the presence of ConA (4 μg ml^{-1}), washed with PBS, and cultured in 96-well plates (5×10^4 cells per well) in triplicates with different amounts of codon-optimized murine IL12. After 48 h supernatants were harvested and stored at −20 °C 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.

Enzyme-linked immunosorbent assay

Cell culture supernatants were collected for IL12 and IFN-γ detection by employing mouse IL12 ELISA Kit (Thermo Scientific, Grand Island, NY, USA; 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 the manufacturer's recommendations.

Animals

Six-week-old female C57/BL6 mice (Charles River, Burlington, MA, USA) were fed lab chow and kept on a 12 h 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; version 5.03) was employed for statistical analysis. Data shown in Figures 1–4 were analyzed by *t*-test (unpaired, two-tailed test) compared with control or untreated samples. Data shown in Figure 5 were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test. EC50 was calculated by Gaddum/Schild EC50 shift. Statistical significance was assigned at $P<0.05$. s.d., 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.[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 HEK-293 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 enzyme-linked immunosorbent assay (Figure 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 h 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 (Figure 1b and 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

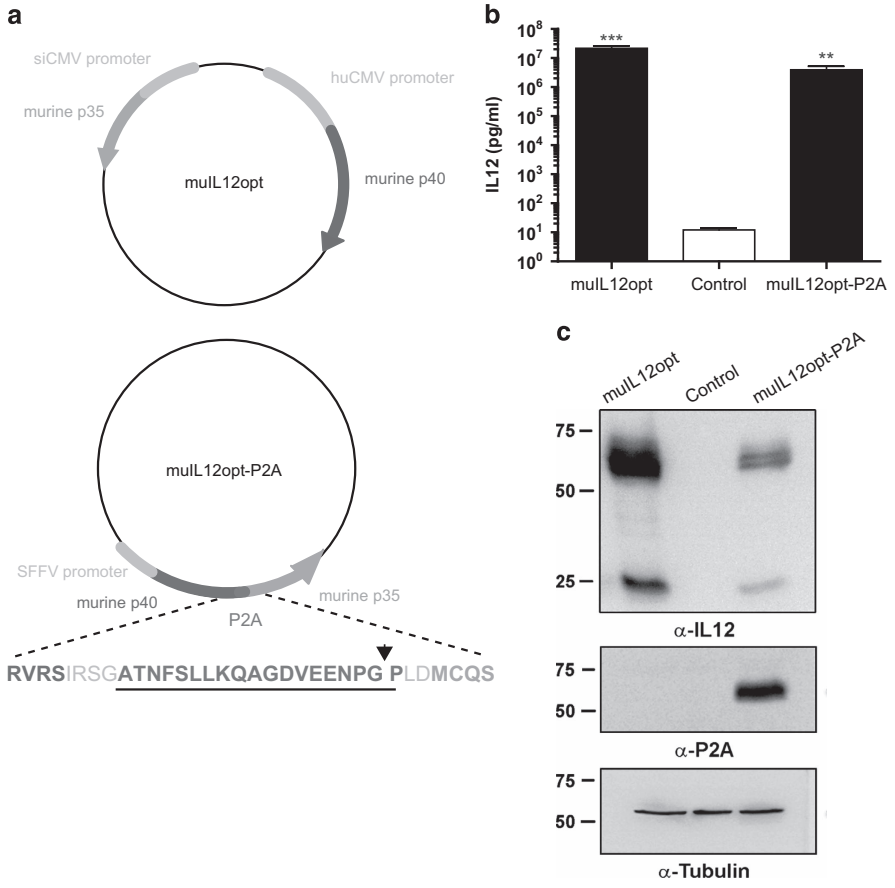

Figure 1. Murine IL12 is successfully expressed as a P2A self-processing polypeptide. (**a**) Schematic representation of the murine IL12-encoding plasmids. The murine codon-optimized sequences encoding the IL12 subunits (p35 and p40) were amplified by PCR from the muIL12opt plasmid and employed to generate 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 48 h 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 h. Cell extracts were analyzed by western blotting using anti-IL12, anti-2A peptide, anti-GFP, or anti-β-tubulin antibodies. ELISA, enzyme-linked immunosorbent assay; huCMV, human cytomegalovirus promoter; IL12, interleukin 12; SFFVp, spleen focus forming virus promoter; siCMV, simian CMV. $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control; $n = 3$.

inducibility of the different expression systems in a variety of tumor cell lines, including HEK-293 human cells, murine 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 h, harvested and cell extracts were made to measure luciferase activity and protein content. We first employed TNF plus IL1β 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). HEK-293 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 HEK-293 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 B16-F10 transduced cells with a cytokine cocktail-containing media collected from lipopolysaccharide-treated RAW cells. We

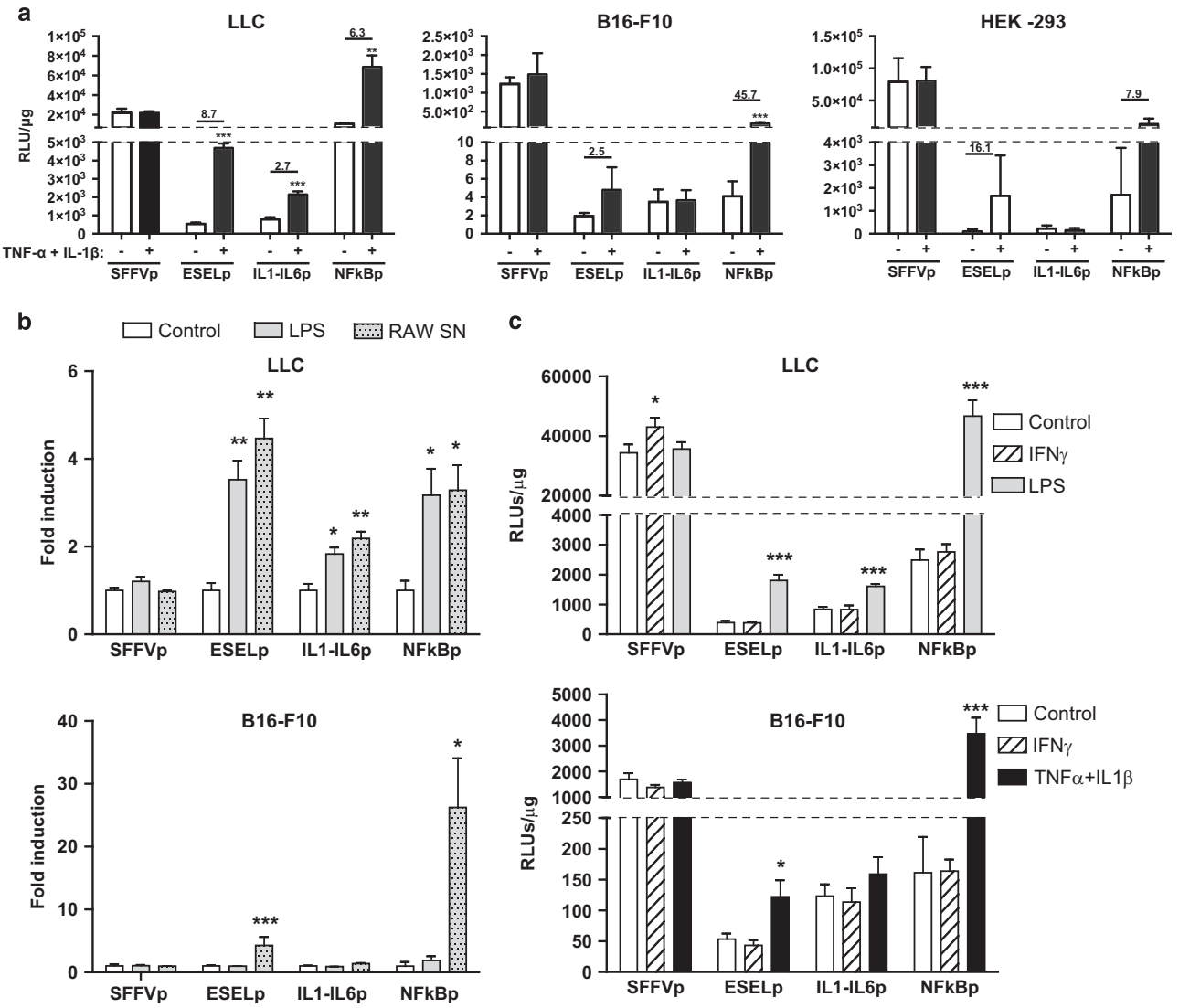

Figure 2. Inflammation-regulated promoters are induced upon proinflammatory stimulation of transduced LLC and B16-F10 tumor cells. The indicated lentiviral vectors encoding the luciferase reporter transgene under the control of the inflammation-induced promoters (ESELp, IL1–IL6p, NFkBp) or the constitutive 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) (100 ng ml^{-1}) plus IL1β (10 ng ml^{-1}) (**a**, **c**), LPS (**b**, **c**), supernatant from LPS-treated RAW cells (RAW SN) (**b**), or interferon-γ (**c**) for 6 h, 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; IL1β, interleukin 1β; IL1–IL6p, IL1 enhancer–IL6 promoter; LLC, Lewis lung carcinoma; LPS, lipopolysaccharide; NFkBp, 6xNFkB sites; SFFVp, spleen focus forming virus promoter.

found that treatment with these murine cytokine cocktails mimicked the results obtained with TNF plus IL1β (Figure 2b). As LLC express TLR4,[30] lipopolysaccharide 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 B16-F10 cells.

Induction of IFN-γ 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 IFN-γ-dependent induction would generate a problematic activation loop in terms of therapeutic applications. As shown in Figure 2c, IFN-γ 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 IFN-γ 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 lipopolysaccharide or

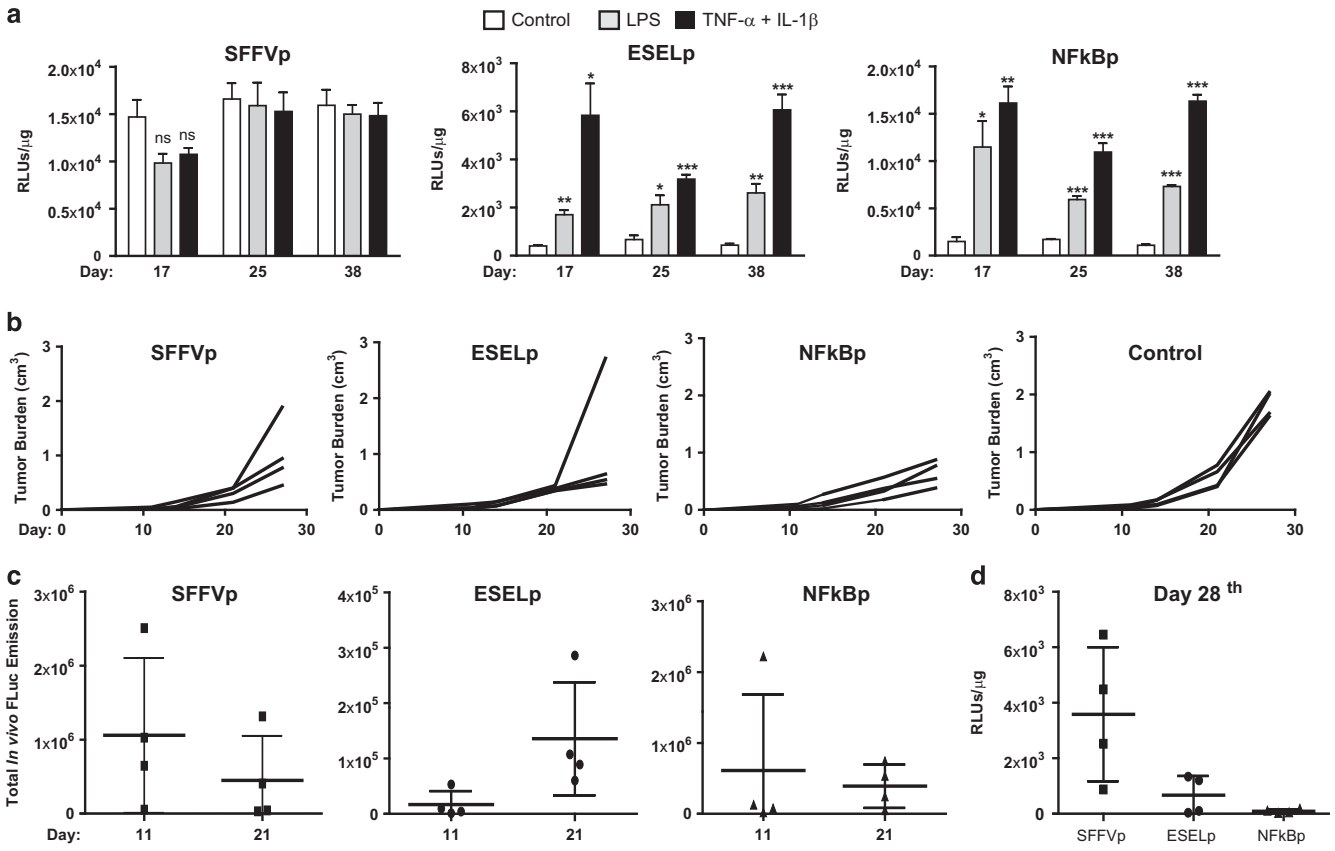

Figure 3. Intratumoral upregulation of inflammation-regulated promoters *in vivo*. The indicated lentiviral vectors encoding the luciferase reporter transgene under the control of the inflammation-induced promoters (ESELp, NFkBp) or the constitutive viral promoter (SFFVp) were employed to transduce LLC 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,[17,25,36] transduced cells were deprived of serum and incubated with either LPS or tumor necrosis factor (TNF) (100 ng ml^{-1}) plus IL1β (10 ng ml^{-1}) for 6 h, 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 (7.5×10^5) 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 euthanized and luciferase activity was measured in tumor homogenates. Scatter plots show RLU (relative light units) per microgram of protein (μg). ESELp, E-selectin promoter; LLC, Lewis lung carcinoma; LPS, lipopolysaccharide; NFkBp, 6xNFkB sites; SFFVp, spleen focus forming virus promoter.

TNF-α 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 (Figures 3b and c, 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 28), 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 proinflammatory 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 mulL12opt-P2A coding sequence in the inflammation-regulated lentivectors (Figure 4a), as inflammation is one of the main characteristics of tumor environment and IL12 has shown potential as an anticancer agent. We generated the lentiviral particles encoding the mulL12opt-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 HEK-293, LLC, and B16-F10 tumor cells. As in the previous experiments, transduced cells were treated for 6 h with TNF plus IL1β. After

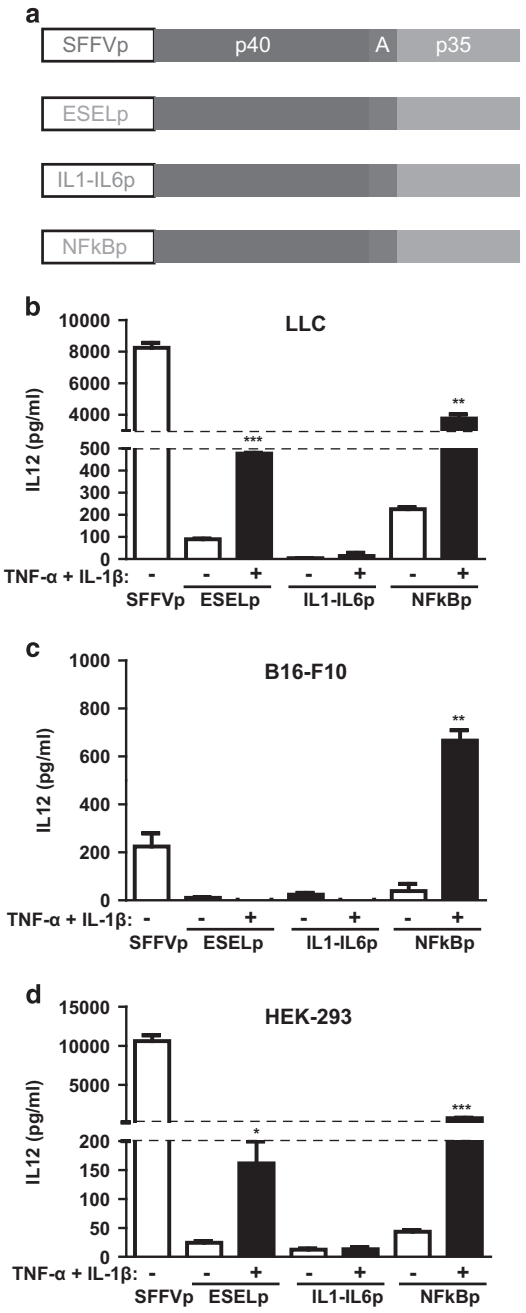

Figure 4. The P2A self-processing IL12 is released upon proinflammatory stimulation of transduced murine tumor cells. (**a**) Schematic representation of the different transfer plasmids generated. The muIL12opt-P2A coding sequence was cloned in constructs to place it under the control of the inflammation-regulated 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) (100 ng ml^{-1}) plus IL1β (10 ng ml^{-1}) for 6 h, 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; $n = 3$. ELISA, enzyme-linked immunosorbent assay; ESELp, E-selectin promoter; LLC, Lewis lung carcinoma; NFkBp, 6xNFkB sites; SFFVp, spleen focus forming virus promoter.

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 HEK-293 and LLC cell lines showed inflammation-dependent IL12 induction when ESELp and NFkBp were employed (Figure 4b and d). 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 IFN-γ 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 HEK-293 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 (EC50) of each murine IL12. As shown in Figure 5b, muIL12opt-P2A showed the lowest EC50 (0.16 ng ml^{-1}), closely followed by the muIL12opt (0.27 ng ml^{-1}). As expected, the purified muIL12R showed a significantly higher EC50 (8.07 ng ml^{-1}).

Treatment with IL12 induces IFN-γ release, which is essential for the antitumor activity of IL12. Therefore, in addition to lymphoblast proliferation, we also measured the amount of IFN-γ released by ConA-stimulated murine lymphoblasts and further treated with IL12. Freshly isolated splenocytes were first stimulated with ConA and then incubated for 48 h with increasing concentrations of IL12. As shown in Figure 5c, we found that incubation with IL12 induced IFN-γ in a dose-dependent manner. As in the case of cell proliferation, recombinant purified IL12 was less efficient and the amount of IFN-γ 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 IFN-γ 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

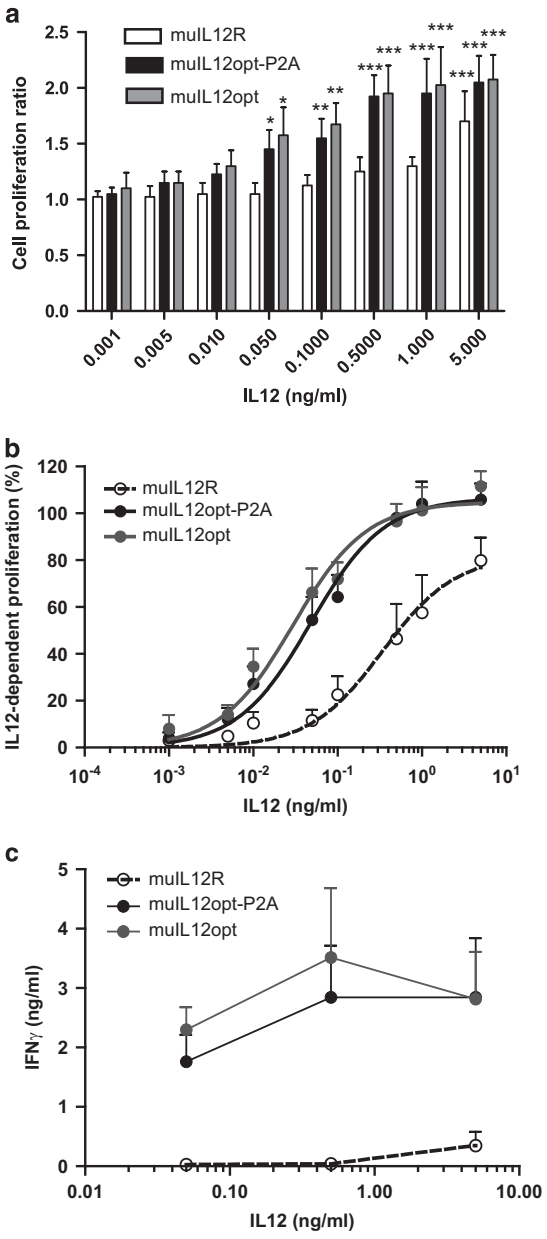

Figure 5. Murine IL12 induces murine lymphoblast proliferation and IFN-γ release. (**a**, **b**) Purified mononuclear cells (5×10^4) from spleens of C57 mice ($n = 4$) were stimulated with concanavalin A (ConA, 4 μg ml^{-1}) for 30 h, and then co-stimulated with murine IL12-containing supernatants from HEK-293 transfected cells with either mulL12opt-P2A or mulL12opt. Commercially available recombinant murine IL12 (mulL12R) was used as a positive control. Forty-eight hours after mulL12 stimulation, increase in cell number was determined using the CellTiter-Glo Luminiscent Assay from Promega. (**a**) The 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**) EC50 value determination. GraphPad Prism (version 5.01) was used for the analysis and for the EC50 value calculation. Samples were measured in triplicates. Statistical analysis shows mean ± standard error of the mean ($n = 4$). (**c**) Purified mononuclear cells (5×10^4) from spleens of C57 mice ($n = 3$) were stimulated with concanavalin A (ConA, 4 μg ml^{-1}) for 30 h, washed, and then co-stimulated with murine IL12-containing supernatants as above. Forty-eight hours later supernatants were collected and the concentration of IFN-γ measured by enzyme-linked immunosorbent assay. IFN-γ, interferon gamma; IL12, interleukin 12.

expression-optimized IL12 plasmid (mulL12opt) described by Dr Felber and co-workers,[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, 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, 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 mulL12opt was apparently successful in all the tumor cell lines tested: HEK-293, LLC and B16-F10, as transduced cells produced and released functional heterodimeric IL12 (p70), measured by enzyme-linked immunosorbent assay (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, IL12-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 IL12 therapy have been started or completed to date.[7] Recombinant IL12 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.[32] By employing viral and non-viral gene delivery methods local antitumor effects have been achieved in cats, horses, and dogs. They demonstrate that IL12-based gene therapy is an effective approach. Altogether these results support and upgrade the

antitumor IL12 features observed in preclinical experiments performed in rodents. Most recently initiated IL12-based clinical trials are focused on local tumor treatment by gene therapy.[7] These strategies are trying not only to minimize the IL12-dependent toxic effects but also to induce specific antitumor mechanisms by overcoming the strong immunosuppressive tumor microenvironment.[33] In this regard it is important to mention that the inflammation-regulated promoters ESELp and NFkBp are still induced in the presence of IL10 (Garaulet *et al*[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 IL12 expression have proven that local production of IL12 inside a tumor can stimulate tumor infiltration by effector immune cells and that in some cases this is followed by tumor regression. Therefore IL12 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^{-1} in LLC; 160 pg ml^{-1} 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^{-1} in LLC, 25 pg ml^{-1} 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^{-1}) while it was significantly increased after proinflammatory stimulation (666 pg ml^{-1}); 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 established by immune memory, to find out how many IL12-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 IL12 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 IL12 under the control of non-inducible promoters.[6,34,35] Therefore, new vectors with regulated, long-term production of IL12 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 B16-F10, 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 IL12 is that it synergizes with several other cytokines.[36] The fact that IL12 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.[7] Nevertheless, despite these results, IL12 remains as a recognized anticancer agent with a great potential for synergistic combinations with other immunotherapies and/or conventional cancer treatments.[37,38]

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.[6,36] In this regard, it has recently been shown by Melero and co-workers[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 antitumor 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 and (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 antitumor properties.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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4.4 Tumor cell transduction with a lentivector expressing a bioactive murine IL-12 as a self-processing P2A polypeptide driven by an inflammation-regulated promoter severely impairs tumor growth *in vivo*

4.4.1 Introduction

The immune system can recognize and eliminate tumor cells, however sometimes the anti-tumor immune response does not eradicate them and cancer arise. The inability to protect against tumors may be due to mechanisms of evasion, active suppression or sub-optimal activation of an immune response[233]. Cancer immunotherapy strategies try to activate or redirect the immune response against tumor cells. Cytokines are important players in both innate and adaptive immune responses and exert key effects on tumor biology[234]. In particular, cytokines involved in activation of immune effector mechanisms, including type I Interferons, GM-CSF, IL-2 and IL-12, are excellent candidates for cancer treatments.

IL-12 is a heterodimeric protein composed of two subunits, p35 and p40, joined by a disulfide bond[12]. It is a potent mediator of anti-tumor immunity[111] as it is a Th1-polarizing pro-inflammatory cytokine[235] and interferes with angiogenesis[236] and metastasis[237]. As systemic administration of IL-12 has anti-tumor activity but it is associated with toxic side effects, new IL-12 delivery methods are currently under development. In this regard, gene therapy approaches are a promising alternative to local IL-12 administration within the tumor environment[142]. In fact, viral and non-viral vectors have been employed to locally express IL-12 into the tumor mass in preclinical trials[143-146,231]. These gene therapy approaches either by themselves or in combination with other anti-tumor strategies have shown encouraging results. Viral vectors are immune-appealing tools as their virus-mimicking effect may trigger an IFN-mediated response; in the case of a Semliki Forest virus vector (SFV), the virus-mediated IFN release was absolutely required for an IL-12 efficient anti-tumoral effect[147]. Lentiviral vectors (LVs) have been reported to trigger a rapid and transient type I IFN response in mice which is dependent on functional vector particles[238].

Among the viral vectors, LVs are promising candidates for immunotherapeutic gene therapy approaches. LVs have been also extensively studied as anti-cancer vaccines due to DC activation through either TLR signaling or protein kinase R (PKR) phosphorylation at high MOIs[239]. In addition, LVs intrinsically induce Th1-polarizing immunogenicity[240,241]. In fact, IL-12-expressing LVs have been employed to transduce tumor cells and these transduced cells have been successfully used as autologous tumor vaccines[145,242]. We have cloned a codon optimized and P2A self-processing IL-12 (IL-12P2A) in LVs and transduced tumor cells commonly employed in syngeneic tumor models[162]. IL-12P2A was efficiently released by transduced cells and exerted bioactive effects on splenocytes *ex vivo* (i.e. cell proliferation and IFN-γ release).

Among other features tumor environment is characterized by inflammation. An appropriate system to control transgene expression in this pathological scenario would be an inflammation-regulated system: cytokine would be secreted upon inflammation, avoiding it constant release and minimizing side effect risk. We have developed several inflammation-regulated LV systems which are efficiently upregulated by acute inflammation *in vivo*[160]. In addition, we have found that they are also upregulated when transduced tumor cells are ectopically implanted in mice[162]. Therefore, we decided to clone IL-12P2A under these inflammation-inducible systems and test their putative immunotherapeutic effect *in vivo*. For this, we transduced Lewis Lung Carcinoma (LLC) cells with inducible LV systems and characterized isolated clones (proliferation and IL-12P2A release). The *in vitro* selected clones were further ectopically implanted in mice to monitor tumor growth, mice survival and T lymphocyte activation. Our results show that mice implanted with tumor cells transduced with IL-12P2A expressing LVs under the NFκB inducible promoter (NFκBp) have a very high survival rate. The percentage of survival was even higher than that observed when IL-12P2A was under the control of a constitutively strong promoter (SFFVp). Importantly, NFκBp-dependent IL-12P2A expression caused a 60% of tumor regression. In both cases, the survival improvement correlated with a significant $CD8^+$ T cell activation after implantation of tumor cells expressing IL-12P2A under either SFFVp or NFκBp. These results demonstrate that LLC cells transduced with the NFκBp-IL12P2A LV expression system severely impairs *in vivo* tumor growth and their implantation significantly activates $CD8^+$ T lymphocytes. This system might be useful for direct LV administration into tumor cells as well as for *in vivo* anti-tumor vaccination.

My personal contribution to this work consisted in performing the ectopic syngeneic mouse model and the subsequent analyses.

Tumor cell transduction with a lentivector expressing a bioactive murine IL-12 as a self-processing P2A polypeptide driven by an inflammation-regulated promoter severely impairs tumor growth *in vivo*

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Abstract
Interleukin 12 (IL-12) is a key cytokine for the initiation of Th1-dependent immune response and a promising anti-cancer agent. However, systemic IL-12 administration is hampered by undesired side-effects. New approaches are currently explored to reduce or minimize these adversities. We have previously shown that our inflammation-regulated lentiviral expression systems are induced in a solid tumor environment. In addition we have shown that a self-processing P2A murine IL-12 (IL-12P2A) efficiently stimulates murine lymphoblast proliferation and IFN-γ release *ex vivo*. In this work we tested the efficacy of these expression systems in a solid tumor model. For this we transduced Lewis Lung Carcinoma (LLC) cells, we isolated clones and characterized their proliferation and IL-12P2A release. We further subcutaneously injected selected clones into wild type C57/BL6 mice and analyzed the tumor cells growth and mice survival. Our results show that implantation of IL-12P2A-expressing cells reduced tumor growth. Notably cells transduced with the system that expresses IL-12P2A under the control of the inflammation-regulated system barely grew after implantation. In fact, up to 60% of tumor regression was observed, increasing significantly the survival rate of these animals. T-cell analysis showed that implantation of IL-12-producing cells activated $CD8^+$ cells. Altogether, these results demonstrate that inducible IL-12P2A release under this inflammation-regulated promoter is able to activate CTLs and impair tumor growth; as a result, the overall survival is significantly increased. Therefore, this inflammation-regulated expression system might be useful for the development of new IL-12 delivery systems with less toxicity and more secure for clinical applications.

Introduction

The immune system can recognize and eliminate tumor cells, however sometimes the anti-tumor immune response does not eradicate them and cancer arise. The inability to protect against tumors may be due to mechanisms of evasion, active suppression or sub-optimal activation of an immune response[1]. Cancer immunotherapy strategies try to activate or redirect the immune response against tumor cells. Cytokines are important players in both innate and adaptive immune responses and exert key effects on tumor biology[2]. In particular, cytokines involved in activation of immune effector mechanisms, including type I Interferons, GM-CSF, IL-2 and IL-12, are excellent candidates for cancer treatments.

IL-12 is a heterodimeric protein composed of two subunits, p35 and p40, joined by a disulfide bond[3]. It is a potent mediator of anti-tumor immunity[4] as it is a Th1-polarizing pro-inflammatory cytokine[5] and interferes with angiogenesis[6] and metastasis[7]. As systemic administration of IL-12 has anti-tumor activity but it is associated with toxic side effects, new IL-12 delivery methods are currently under development. In this regard, gene therapy approaches are a promising alternative to local IL-12 administration within the tumor environment[8]. In fact, viral and non-viral vectors have been employed to locally express IL-12 into the tumor mass in preclinical trials[9-13]. These gene therapy approaches either by themselves or in combination with other anti-tumor strategies have shown encouraging results. Viral vectors are immune-appealing tools as their virus-mimicking effect may trigger an IFN-mediated response; in the case of a Semliki Forest virus vector (SFV), the virus-mediated IFN release was absolutely required for an IL-12 efficient anti-tumoral effect[14]. Lentiviral vectors (LVs) have been reported to trigger a rapid and transient type I IFN response in mice which is dependent on functional vector particles[15].

Among the viral vectors, LVs are promising candidates for immunotherapeutic gene therapy approaches. LVs have been also extensively studied as anti-cancer

vaccines due to DC activation through either TLR signaling or protein kinase R (PKR) phosphorylation at high MOIs[16]. In addition LVs intrinsically induce Th1-polarizing immunogenicity[17,18]. In fact, IL-12-expressing LVs have been employed to transduce tumor cells and these transduced cells have been successfully used as autologous tumor vaccines[11,19]. We have cloned a codon optimized and P2A self-processing IL-12 (IL-12P2A) in LVs and transduced tumor cells commonly employed in syngeneic tumor models[20]. IL-12P2A was efficiently released by transduced cells and exerted bioactive effects on splenocytes *ex vivo* (i.e. cell proliferation and IFN-γ release).

Among other features tumor environment is characterized by inflammation. An appropriate system to control the transgene expression in this pathological scenario would be an inflammation-regulated system: cytokine would be secreted upon inflammation, avoiding it constant release and minimizing side effect risk. We have developed several inflammation-regulated LV systems which are efficiently upregulated by acute inflammation *in vivo*[21]. In addition, we have found that they are also upregulated when transduced tumor cells are ectopically implanted in mice[20]. Therefore, we decided to clone IL-12P2A under these inflammation-inducible systems and test their putative immunotherapeutic effect *in vivo*. For this, we transduced Lewis Lung Carcinoma (LLC) cells with inducible LV systems and characterized isolated clones (proliferation and IL-12P2A release). The *in vitro* selected clones were further ectopically implanted in mice to monitor tumor growth, mice survival and T lymphocyte activation. Our results show that mice implanted with tumor cells transduced with IL-12P2A expressing LVs under the NFκB inducible promoter (NFκBp) have a very high survival rate. The percentage of survival was even higher than that observed when IL-12P2A was under the control of a constitutively strong promoter (SFFVp). Importantly NFκBp-dependent IL-12P2A expression caused a 60% of tumor regression. In both cases, the survival improvement correlated with a significant $CD8^+$ T cell activation after implantation of tumor cells expressing IL-12P2A under either SFFVp or NFκBp. These results demonstrate that LLC cells transduced with the NFκBp-IL12P2A LV expression system severely impairs *in vivo* tumor growth and their implantation significantly activates $CD8^+$ T lymphocytes. This system might be useful for direct LV administration into tumor cells as well as for *in vivo* anti-tumor vaccination.

Materials and Methods

Cell culture

Human Embryonic Kidney (HEK-293; ATCC #CRL-1573) and murine Lewis Lung Carcinoma (LLC; ATCC #CRL-1642) 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).

Second generation lentivector production and titration

HEK-293 cells were transiently transfected by the calcium phosphate method [22]. For viral particle production, the corresponding pHRSIN HIV 1-derived transfer vector was co-transfected with two helper plasmids, the 8.91 packaging vector [23] 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 [24].

Cell cloning and inducibility test assay

LLC cells were transduced with the different lentiviral particles at the multiplicity of infection (MOI) of 100. After checking the transgene expression by western-blotting and/or ELISA, transduced cells were seeded onto Terasaki plates at a density of 0.3 cells per well[25]. Wells containing a single cell were identified by visual inspection under a light microscope. Isolated clones were expanded and tested for either luciferase (luciferase activity) or IL-12 expression (Western-blotting and ELISA). For ELISA assays, LLC clones ($1.7x10^4$ cells in 0.25ml) were seeded onto 24-wells at 80% confluency, incubated for 2h in low-serum containing media (0.5% FBS) and then stimulated for 6h with LPS (200ng/ml). For IL-12 analysis, supernatant was collected and IL-12 content measure by ELISA (Thermo Scientific). For IL-12 western-blotting, a protein transport inhibitor (BD GolgiPlugTM, Biosciences) was added during LPS stimulation.

Proliferation of the selected transduced LLC clones was analyzed by employing the cell proliferation reagent WST-1 (Roche) following manufacturer's recommendations. Cell viability was monitored by periodically cell counting and trypan blue staining.

Luciferase assay, Western Blotting and ELISA

To determine luciferase activity Luc-expressing clones 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 20/20N luminometer (Turner BioSystems) and for protein quantification (Bradford;

Bio-Rad). Reporter gene expression is shown as relative light units (RLU) per microgram of protein.

To determine IL-12 expression stimulated clones 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 first antibody solution: rabbit polyclonal anti-2A peptide (0.05%, v/v; Millipore) or mouse monoclonal anti-α-tubulin (0.1%, v/v; Sigma-Aldrich) 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) 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).

To measure IL-12 release by transduced cell clones, cell culture supernatants were collected for detection by employing mouse IL12 ELISA Kit (Thermo Scientific; cat. #EMIL 122). Samples were measured in a microplate spectrophotometer (xMark; Bio-Rad) following manufacturer's recommendations.

Animals

Six-week-old male 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 CNIO Animal Facility guidelines for the care and use of laboratory animals.

In vivo tumor model

Transduced LLC clones were grown in culture media, collected by centrifugation and washed with PBS. LLC cells ($7.5x10^5$ cells per animal) were resuspended in 150 µl of PBS, embedded in matrigel (BD) and implanted subcutaneously into the right flank of the mouse. Tumor growth was monitored and tumor volume calculated ($V= 4/3\ \pi r^3$). Bioluminescence images were captured with an IVIS 200 in vivo imaging system (Caliper). For this, mice were i.p. injected with firefly luciferin (150 mg/kg; Promega), and anesthetized with isoflurane during the procedure. Living Imaging 3.0 software (Caliper) was employed for imaging analysis.

Mononuclear spleen-derived population analyses

Spleens from euthanized animal were mechanically disaggregated and mononuclear cells isolated by gradient centrifugation (Lympholyte-M, Cedarlane Laboratories, Burlington, NC). Analyses of the different populations were performed by incubating with the indicated specific antibodies conjugated with fluorophores (BD Biosciences, San Jose, CA): CD4 FITC/APC, CD8 FITC/PE, CD25 FITC/PE and CD69 FIT/PE. Briefly, mononuclear cells ($0.5\text{-}1 \times 10^6$) were incubated with γ-globuline (50 µg/ml) for 10 minutes at 4ºC, washed and then incubated with the corresponding specific antibody for 40 minutes at 4ºC. After several washes with PBS cells were analyzed by flow cytometry (BD FACSCanto II) employing the BD FACSDiva software (BD Biosciences).

Statistical analysis

Prism 5 for Windows (GraphPad Software Inc.; version 5.03) was employed for statistical analysis. Data shown were analyzed by t-test (unpaired, two-tailed test) compared to control or untreated samples. Statistical significance was assigned at $P<0.05$. SD, standard deviation; SEM, standard error of the mean.

Results

Isolation and characterization of Luciferase- and IL-12P2A-producing LLC clones

We have shown that transgene expression under our inflammation-regulated lentivector systems are induced *in vitro* and *in vivo*. In particular, we have recently demonstrated that luciferase activity of LLC cells transduced with the NFκB-based promoter (NFκBp) increased upon implantation in mice[20]. After these results, we decided to further test our expression systems *in vivo*. For this we isolated and characterized luciferase and IL-12P2A-expressing LLC clones in order to achieve a better understanding of the different promoter upregulation and transgene expression in these tumor cells. As shown in **Figure 1A**, both inflammation-regulated promoters, ESELp and NFκBp, were upregulated (fold induction: 7.8 and 10.4, respectively) after LPS treatment of the selected clones. We found that proliferation of these clones was not affected by transduction and luciferase activity, as their growth curves overlap with the one from non-transduced LLC cells (**Figure 1B**). We next focused on the isolation of IL-12P2A expressing clones. Our results have shown that NFκBp displayed the highest inducibility and the levels of IL-12P2A released upon upregulation of this promoter were similar to those observed when a strong viral promoter was employed (SFFVp)[20]. Therefore, we decided to isolate LLC clones expressing IL-12P2A under the control of either NFκBp or SFFVp. After cloning, we selected the clones based on the IL-12 expression by western-blotting (**Figure 1C**). We then confirmed that IL-12P2A was efficiently released by measuring its content in the culture supernatants (**Figure 1D**). Selected clones showed constitutive and LPS-induced expression of IL-12P2A under the control of SFFVp and NFκBp, respectively. Next we

analyzed the proliferation and viability of these clones in comparison to wild type LLC cells. In terms of cell viability, we found no differences among clones and wild type cells (**Table I**). When the proliferation was analyzed through indirect metabolic activity quantification, growth curves overlapped, except for the SFFVp-IL12P2A clone (**Figure 1 E,F**); this clone displayed a slight growth rate decrease, suggesting that constitutive IL-12P2A expression might affect to this clone proliferation without affecting to its viability.

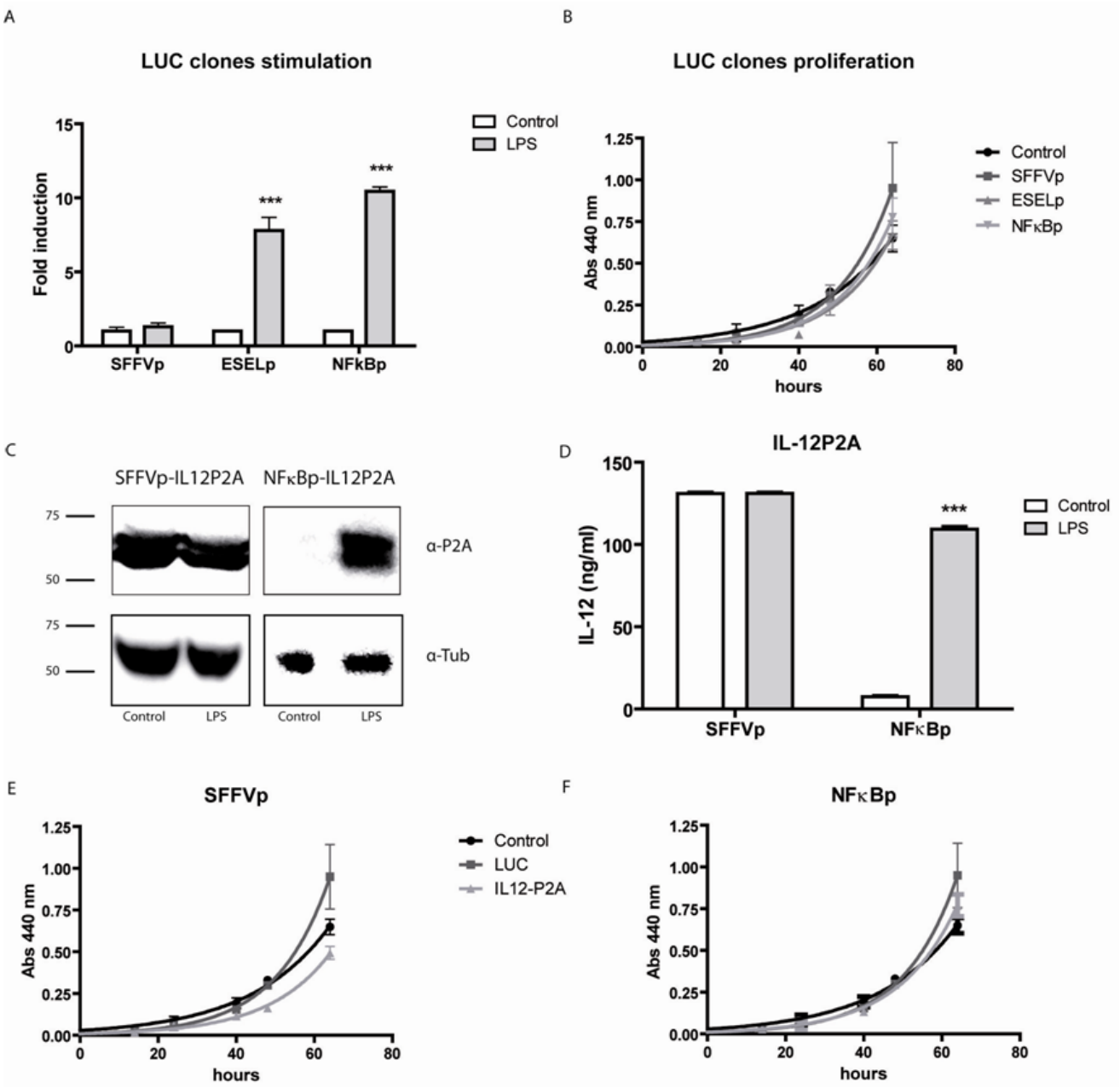

Figure 1

Characterization of luciferase- and IL-12P2A-expressing clones. (A,B) Isolated luciferase-expressing clones were tested for promoter inducibility and cell proliferation. (A) After serum deprivation, clones were incubated with LPS for 6 hours and luciferase activity was measured. Plot shows fold induction upon treatment. (B) Cell proliferation assays were performed through indirect metabolic activity quantification by incubation with WST-1 reagent followed by measure at 440 nm. Plot shows growth curves of the indicated clones. Non-transduced LLC were employed as a control. (C) IL-12P2A expressing clones were isolated, grown and, after serum deprivation, incubated with LPS for 6 hours in the presence of Brefeldin A (protein transport inhibitor). Cell extracts were analyzed by western blotting using either anti-P2A (top) or anti-α-tubulin (bottom) antibodies. (D) Supernatants from the IL-12P2A-expressing clones were collected and the IL-12 content measured by ELISA. (E and F) Cell proliferation assays were performed as in B. Plots show growth curves of the SFFVp (left) and NFκBp (right) containing clones. WST-1, water-soluble tetrazolium salt-1; ESELp, E-selectin promoter; NFκBp, 6xNFκBp sites; SFFVp, spleen focus-forming virus promoter; LPS, lipopolysaccharide. Plots show mean ± standard deviation. ***P< 0.001.

Table I
Clones viability percentage determination at 72h

Clone	Viability (% ± SD)
Control	93.50 ± 1.68
SFFVp-Luc	98.64 ± 0.59
NFκBp-Luc	98.51 ± 0.70
SFFVp-IL12P2A	97.28 ± 2.10
NFκBp-IL12P2A	96.74 ± 0.66

SD= standard deviation

Local IL-12P2A release by the inflammation-regulated expression system reduces tumor burden, increases mice survival and activates CD8+ T cells

LLC clones isolated and characterized were employed for *in vivo* experiments. These cells were ectopically implanted into syngeneic mice (C57/BL6; n=5). Luciferase activity and tumor burden were monitored periodically. Bioluminiscence analysis confirmed the transcriptional activity of these promoters after *in vivo* implantation (**Figure 2A**); as NFκBp and ESELp are inflammation-regulated promoters, the results reveal that tumor environment is pro-inflammatory soon after implantation in this mouse model. To assess the anti-tumor activity of IL-12P2A, tumor sizes were monitored and the percentage of survival calculated (**Figure 2 B,C**). Animals were implanted with IL-12 expressing clones under the control of either SFFVp or NFκBp. As a control, a clone expressing luciferase under each promoter was also employed. Results clearly show that IL-12P2A expression by each promoter significantly reduced tumor sizes and increased the percentage of survival. Notably 3 out of 5 animals bearing NFκBp-IL-12P2A cells showed complete or partial tumor regression and stabilization, surviving for at least 60 days, when they were euthanized. This observation is very important as neither complete nor partial regression was detected when SFFVp-IL-12P2A cells were implanted. Finally, we studied the activation state of T lymphocytes isolated from spleens of euthanized mice (**Figure 2D**). Flow cytometry analysis showed that implantation of IL-12P2A expressing cells activates both CD4+ and CD8+ T cell populations, suggesting an acquired immune system activation due to IL-12 expression. In the case of NFκBp, only the activation of CD8+ T cells was statistically significant.

Discussion

Recombinant IL-12 has shown relevant anti-tumor 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. An interesting alternative to conventional drug treatments can be gene therapy approaches. In this regard, a number of local gene therapy approaches have been undertaken in veterinary clinical oncology [26] demonstrating that IL-12 based gene therapy is an effective approach. Most recently initiated IL-12-based clinical trials are focused on local tumor treatment by gene therapy [27]. Clinical trials based on intratumoral IL-12 expression have proven that local production of IL-12 inside a tumor can stimulate infiltration by effector immune cells and that in some cases this is followed by tumor regression. Our lentivector-based inflammation-regulated expression systems would allow local expression of IL-12 controlled by the inflammatory state of the tumor microenvironment that might result in an enhanced anti-tumor activity with reduced toxicity.

Our *in vivo* results show that the transcriptional activity of ESELp and NFκBp is regulated during tumor growth. Interestingly, ESELp-driven expression increased at day 8 post-implantation, reached the highest level at day 11-13 and then dropped gradually. In the case of NFκBp-driven expression, it is characterized by high transgene expression levels (100 times higher than ESELp-driven expression) and did not show a common activation pattern after implantation. As NFκB pathway participates in both initiation and resolution of inflammation[28], its complex regulation during tumor growth might account for the different expression patterns observed.

Regarding IL-12P2A, its expression under the promoters tested reduces tumor growth causing improved survival rates. It is important to mention that the endpoint criterion for these experiments was the appearance of ulcers within the tumoral mass, and none of them suffered spontaneous death. Importantly, IL-12P2A expression under NFκBp showed a strong anti-tumoral effect, causing 1 partial and 2 complete tumor regressions. The NFκB pathway has a promoting role in most cancers [29] and here we show that it is possible to employ an NFκB-based promoter to drive transgene expression within tumor microenvironment. In particular, our experiments clearly show that NFκBp upregulation releases therapeutic levels of IL-12P2A. This expression system could be employed to control the local secretion of other immunotherapeutic agents with anti-tumor activity. In addition, it could be transfer to other vector platforms to be employed in inflammation-related diseases.

The anti-tumor effects of IL-12 can be mediated by CD4+ and CD8+ T cells, depending on the tumor type and more importantly the delivery system employed[11,19,30,31]. In the case of implantation of IL-12-secreting cells, both T-cell populations are required to eliminate tumor cells[11]. To determine whether T-cell activation was taking place after implantation of IL-12P2A expressing cells, splenocytes from tumor-bearing mice were analyzed by flow cytometry.

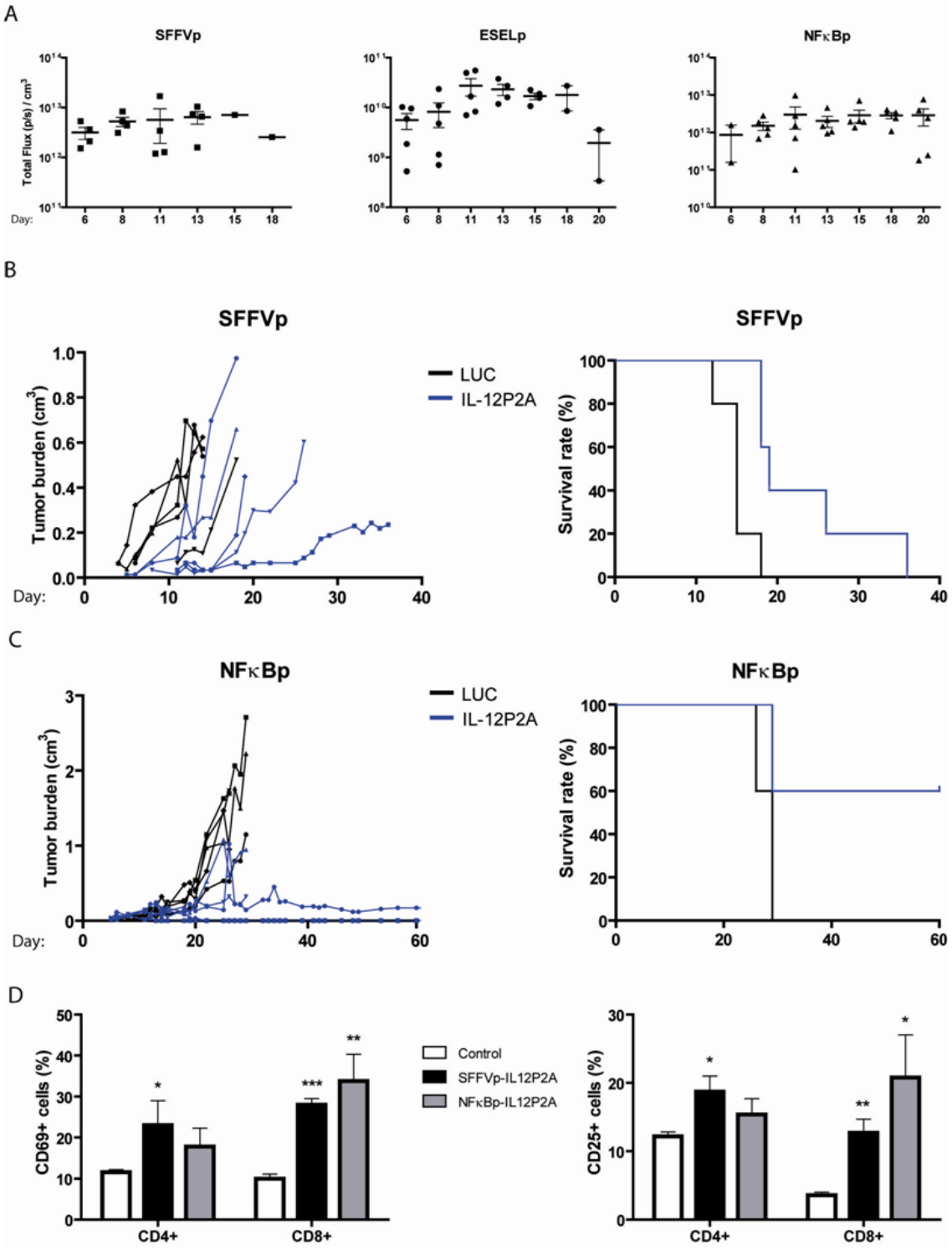

Figure 2

Implantation of IL-12P2A expressing cells in mice causes smaller tumors, longer survival and CD8⁺ T cells activation. (A) One LLC clone expressing luciferase under the control of either the constitutive viral promoter SFFVp (left) or the inflammation-inducible promoters ESELp (middle) and NFκBp (right) were implanted ectopically in C57/BL6 mice (n=5 per group). Bioluminiscence was monitored three times per week and is represented as luciferase activity relative to tumor volume (Total flux/cm³). (B-D) IL-12P2A (blue) and Luciferase-expressing clones (black) under the control of either SFFVp (B) or NFκBp (C) were individually and ectopically implanted in C57/BL6 mice (n=5 per group). Tumor diameters were measured and total tumor volumes (left, one line per animal) and survival rates (right) were calculated. (D) Splenocytes from the indicated implanted mice were isolated and the activation state of CD4⁺ and CD8⁺ T cell populations analyzed by flow cytometry. Splenocytes from animals implanted with luciferase-expressing cells were employed as controls. ESELp, E-selectin promoter; NFκBp, 6xNFκBp sites; SFFVp, spleen focus-forming virus promoter. *P < 0.05, **P< 0.01, and ***P< 0.001. Standard error of the mean is represented.

We found that $CD8^+$ T cell activation was statistically significant when IL-12P2A was expressed under either SFFVp or NFκBp. In the case of $CD4^+$ T cell activation, the results were statistically significant only when IL-12P2A was constitutively expressed (SFFVp). As the NFκBp-IL-12P2A group showed the highest survival rate, and in these animals only $CD8^+$ T cell population was significantly activated, these results highlight the crucial role of activated $CD8^+$ T cells for the IL-12-dependent anti-tumor effect in this solid tumor model. Previous results have also shown that $CD8^+$ T cells were the primary effectors in IL-12-mediated anti-tumor immunity[32,33]. Further analysis of samples collected from these experiments (blood, spleen and tumor) as well as future experiments will allow us to confirm these observations.

Once we have demonstrated the bioactivity of the muIL12opt-P2A in mice by employing IL-12P2A-producing clones, our efforts will be directed to isolate, characterize and test *in vivo* an ESELp IL-12-expressing cell clone. As we have showed, this promoter which is regulated by tumor-inflammation signals, produces the lowest levels of transgene expression, so potentially would be the best indicated to avoid side-effects. However is important to analyze whether these levels of IL-12 expression would be sufficient to produce anti-tumor effect. It will also be important to study whether long-term protection can be established by immune memory.

Published results have shown that delivery of IL-12 by cancer cells is an effective route for immune activation both in leukemia and in solid tumor mouse models [11,19]. We have shown that subcutaneous implantation of IL-12P2A-expressing LLC clones results in tumor growth impairment, being the inflammation-regulated system the most efficient one. These experiments have proven that our inflammation-regulated system is efficiently expressed in LLC solid tumors. Therefore, we will further test the anti-tumoral efficacy of our inflammation-regulated LV systems by local intratumoral administration in different solid tumor models. These inducible promoters might represent excellent systems for the inflammation-regulated expression of different anti-tumor agents. For future clinical applications, our expression systems can be transferred to vector platforms safe enough for human applications.

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5 Discussion

5.1 Summary

The inflammatory response is precisely controlled by the expression of cytokines whose local levels are directly related to the severity of the process. A major challenge in the treatment of chronic inflammatory diseases is the development of an expression system that is tightly regulated by the variable levels of these cytokines. We have described a long term lentiviral expression system based on the E-selectin promoter, which is locally induced by inflammatory stimuli in direct correlation with the intensity and duration of the inflammatory response. We have also generated lentivectors incorporating other previously described inflammation-inducible promoters, such as a hybrid human IL-6 promoter joined to the enhancer region of the human IL-1 promoter (IL1-IL6p) and a chimeric promoter based on NFκB-binding sites (NFκBp); the latter is potently stimulated, reaching expression levels similar or even superior to those obtained with a constitutive strong viral promoter such as SFFVp. These promoters have been useful for monitoring and understanding the inflammatory course of locally zymosan-induced inflammation, the altered inflammatory response of glia in ALS model derived from OM and the inflammatory context after LLC cells implantation in mice. The different expression patterns developed by these promoters have allowed us to investigate the effect of inflammation-induced cytokine expression. The results indicate their potential for chronic inflammatory diseases gene therapy treatments, as they are regulated by the severity of the process and for cancer gene therapy treatments, where the tumor inflammatory microenvironment drives the expression of the therapeutic molecule.

5.2 Inflammation-regulated lentiviral system for chronic inflammatory diseases

Studies in animal models have shown that gene therapy is an alternative for the local treatment of chronic inflammatory diseases. One of the critical factors in gene transfer is the type of vector employed. Non-viral vectors commonly yield low gene transfer efficiency[243,244]. Among the viral vectors, adenoviruses are the most widely used, but they are poor candidates for the treatment of chronic inflammatory diseases because of the immune response associated with their application and the rapid loss of transgene expression due to lack of persistence of the viral genomes[132,245]. Adeno-associated viruses (AAV) have emerged as a very promising alternative, since although AAV vectors have limited cargo capacity, they stably transduce host cells and show low immunogenicity. However, recent studies have reported an inflammatory response after AAV application[133-

[135]. In addition, technical restrictions limit the scalability of AAV vectors, making it difficult to produce adequate viral titers[246-248]. As an alternative, lentivirus-derived expression systems have been employed in animal models of neuroinflammation[139]. LVs not only infect dividing and quiescent cells, but they also provide long term expression and show low immunogenicity. In addition, the biosafety profile of lentiviral vectors has been improved significantly by minimizing the regions of homology between vector and helper sequences (split configuration), and by using heterologous promoters[249]. Furthermore, the use of vesicular stomatitis virus glycoprotein (VSV-G) confers efficient transduction in a wide range of cell types from many species, and allows high titers of the lentiviral particles for clinical applications[250,251]. Our study suggests that LVs may also be a valuable alternative in the treatment of chronic inflammatory diseases.

Several inflammation-inducible systems have been described recently, all of which are based on chimeric promoters. The precise *in vivo* regulation of these tailored promoters is still unknown. Our expression system is based on the proximal promoter region that controls the expression of the E-selectin gene. This gene is particularly attractive as it is induced early and transiently upon inflammation and its promoter region contains the binding sites for transcription factors activated by the early-induced pro-inflammatory cytokines TNF-α and IL-1. Compared to other described expression systems, the ESELp-based system shows the highest transcriptional activity in endothelial cells. In addition, our expression system is highly stimulated in endothelial cells by the early-induced pro-inflammatory cytokines TNF-α and IL-1. These results are important as activated endothelium plays an important role in inflammation initiation. We tested our expression system in an experimental model of chronic inflammation by administering repeated local injections of zymosan. ESELp-driven transgene expression is rapidly induced after zymosan administration, coinciding with the peak of inflammation 7 days after the first treatment, and is maintained until inflammation recedes. Compared to other inflammation-inducible systems, the ESELp-based is characterized by a low basal activity that after zymosan administration increased to levels similar to those observed with a strong viral promoter. The other expression systems analyzed showed either higher basal activity (NFκB-based system) or very low inducibility after inflammation (IL1-IL6p-based system). Our results show that ESELp-dependent transgene expression increases several fold, correlating with the severity of inflammation in the animal system tested.

Since chronic inflammatory diseases are characterized by flare-ups and remission phases, it was important to test whether the promoter was silenced *in vivo* and whether transgene expression could be re-induced after a second zymosan boost. Transgene expression again correlated with inflammatory status after a second zymosan boost, showing no evidence

of promoter silencing. We therefore consider the ESELp a valuable tool for the development of gene expression systems for the treatment of chronic inflammatory diseases. The use of ESELp-based gene delivery systems to selectively express anti-inflammatory agents in arthritis-affected joints might eliminate some of the problems of tolerability and compliance associated with systemic drug therapies.

One important issue in locally applied gene therapy is to study the putative migration of transduced cells *in vivo*. In this regard, it has been demonstrated that LV injection into the mouse footpad transduces DCs which are able to migrate to the draining lymph nodes and spleen[252]. However, we only detected residual luciferase activity in the popliteal lymph nodes draining the infected paw, suggesting that transduced DCs remain in the local inflammatory focus[160] (Garaulet G *et al.* Figure 3 f,g). This is in agreement with previously published results showing that IL-10 transgene expression modulates DC maturation[253,254]. The authors observed that DCs transduced with adenoviral vectors expressing IL-10 maintained an immature state characterized by low MHC class II, CD86, and IL-12 expression. The immaturity might affect to their migratory ability which would support our *in vivo* results. Further experiments should be performed to further characterize the impact of IL-10 expression on DC migration *in vivo*.

The occurrence of unpredictable relapses complicates the treatment of chronic inflammatory diseases. Rheumatoid arthritis, the most frequent inflammatory rheumatic disorder, is a paradigm of chronic inflammatory diseases characterized by an imbalance of pro- and anti-inflammatory molecules. Although systemic administration of anti-inflammatory agents is beneficial to patients with chronic RA, these treatments are limited by loss of efficiency and relapse after treatment cessation. There are also significant side effects associated with a prolonged systemic imbalance of the natural inflammatory response[255,256]. Viral vectors are promising candidates for gene therapy for local treatment of RA, and several clinical trials are underway[257]. However, there is still a need to develop new therapeutic approaches that provide prolonged remission from disease with limited side effects by targeting anti-inflammatory mediators to the diseased joints. The use of disease-regulated promoters to drive transgene expression might provide therapeutic levels of the anti-inflammatory agent exclusively during flare ups. In addition, administration of virus directly into arthritic joints should avoid the side effects associated with systemic administration and increase the site-specific effects of the therapeutic agent.

5.3 Therapeutic effect of murine IL-10 released by inflammation-inducible system

It has been shown that local administration of recombinant IL-10 effectively reduces pro-inflammatory cytokine activity in several animal models of human diseases; however, constant high levels of anti-inflammatory molecules might increase the risk of infection, therefore prolonged administration of IL-10 is limited by associated side effects. Our study demonstrates that activation of the LV-ESELp lentiviral expression system is regulated by the local level of inflammation. Moreover, the LV-ESELp system drives inflammation-regulated IL-10 expression at levels sufficient to reduce acute inflammation induced by zymosan with no effect in the IL-10 serum levels. Although the LV-ESELp system releases considerably lower local concentrations of IL-10 than the constitutive expression vector LV-SFFVp, it is noteworthy that regulated IL-10 expression is as effective as constant expression in reducing inflammation *in vivo*. Furthermore, the inducible system is switched off during remission of the inflammation, thus avoiding the risks associated with a sustained release of IL-10. Altogether, these results suggest that local administration of LV-ESELp-IL10 should not increase animal susceptibility to opportunistic infections. As expected, the levels of inflammation and transgene induction are lower after the second bout than observed at disease onset. This endogenously-regulated system for the local expression of anti-inflammatory molecules provides a potential new approach for the local treatment of chronic inflammatory diseases.

5.4 Inflammatory response in ALS cell model

Besides the classical chronic inflammatory diseases, many data support the implication of neuroinflammation in neurodegenerative pathologies. Multiple lines of evidence have shown the immune system, including astrocytes and microglia, to be deleterious for motor neurons in ALS. Reactive astrocytes and microglia may release pro-inflammatory factors such as cytokines and chemokines which are harmful for the neighbouring cells[258]. However, their role as the primary cause of the disease remains undetermined. Our data indicate that there is no increased innate immune response of glia in ALS: using the established model of SOD1 overexpression in human astrocytes we did not observe increased pro-inflammatory response after LPS treatment; this result is similar when OM cells from healthy donors and those from ALS patients are compared. However, co-culture with motor neurons increases glial sensitivity to pro-inflammatory stimuli in ALS: we observed augmented NFκB-dependent reporter activity both in the SOD1-overexpressing

astrocyte model as well as in OM cells from ALS patients. Consistent with this, NF-κB activation has previously been observed in spinal cord astrocytes in ALS patients as well as in TDP-43 animal models where it was also demonstrated that the inhibition of NF-κB with Withaferin A reduced denervation in neuromuscular junctions[259,260]. These results indicate that alterations in the innate immune response of glia in ALS might be a consequence of their interaction with damaged neurons rather than the cause of initial neuronal damage. Nevertheless, once sensitized, the modified pro-inflammatory response of glia in ALS could further worsen the state of neighboring neurons. In agreement with this concept, previous work using a SOD1 transgenic mouse model demonstrated that microglia and T cells initially slow disease progression; but at later stages after accumulation of SOD1 protein, it contributes to acceleration of the disease[261]. Moreover, it has been shown in both ALS patients as well as in mouse models that activation of microglia and astrocytes takes place only after distal axon degeneration[262].

Thus, our inflammation-inducible LVs have been important to determine the activation of inflammatory responses in patient-derived OM when were co-cultured with human spinal cord neurons. This strategy reproduces, among others, this feature of the non-neuronal contribution to ALS and therefore shows the potential of OM cells as new cell model for ALS.

5.5 Efficient expression of bioactive murine IL-12 as a self-processing P2A polypeptide

The production of IL-12 requires the coordinated expression of p35 and p40 subunits to form the functional heterodimeric interleukin. The biosynthesis of IL-12 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[263]. In the present work, we have used an expression-optimized IL-12 plasmid (muIL12opt) described by Dr. Felber and coworkers[263], which encodes significantly higher levels of bioactive IL-12 compared to the wild-type IL-12 sequences. We have generated a P2A-based IL-12 construct encoding both IL-12 subunits in a single coding sequence in order to achieve concomitant, stoichiometric production of both IL-12 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) [264]; 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[265] of the subunits. This is especially important in the case of IL-12, 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 IL-12 antagonists[232]. This makes the P2A strategy more suitable for the expression of heterocomplexes such as IL-12 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[266]. 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 (Lorenzo C *et al.* Figure 1c). The cellular processing of IL-12P2A was apparently successful in all the tumor cell lines tested: HEK-293, LLC and B16-F10, as transduced cells produced and released functional heterodimeric IL-12 (p70), measured by ELISA (Lorenzo C *et al.* Figure 4). IL-12P2A bioactivity was further demonstrated in lymphocyte proliferation experiments (Lorenzo C *et al.* Figure 5). Thus, our results confirm that the IL-12 polypeptides are processed normally and secreted from the cell and that the P2A sequence employed does not interfere with heterodimeric IL-12 folding, secretion and activity.

5.6 IL-12 expression driven by inflammation-inducible promoters in tumor cell lines

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 anti-tumor response] and gene therapy. Up to 58 clinical trials based on IL-12 therapy have been started or

completed to date[113]. Recombinant IL-12 has shown relevant anti-tumor 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 anti-tumor activity and reduced toxicity. In this regard, a number of local gene therapy approaches have been undertaken in veterinary clinical oncology[267]. By employing viral and non-viral gene delivery methods local anti-tumor 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 IL12-based clinical trials are focused on local tumor treatment by gene therapy[113]. These strategies are trying not only to minimize the IL12-dependent toxic effects but also to induce specific anti-tumor mechanisms by overcoming the strong immunosuppressive tumor microenvironment[268]. In this regard it is important to mention that the inflammation-regulated promoters ESELp and NFκBp are still induced in the presence of IL-10 [[160] 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 infiltration by effector immune cells and that in some cases this is followed by tumor regression. Therefore IL-12 is still considered as an anti-cancer cytokine and its importance in cancer immunotherapy keeps growing.

Systemic expression of IL-12 has proved to cause significant toxicity and negative side effects[113]. Thus, a major challenge in the treatment of cancer using IL-12 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 NFκBp) 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 IL-12 production among the different cell lines and promoters (Lorenzo *et al.* Figure 4). Among them, the IL-12 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 anti-tumor immunity. This expression system would fulfil the requirements of an inflammation-induced system as it produces very low IL-12 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 IL-12 production of the NFκBp-based system was very low (39 pg/ml) while it was significantly increased after pro-inflammatory stimulation (666 pg/ml); therefore, the NFκB-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 IL-12 expression in the tumor environment but also to achieve local IL-12 release controlled by the inflammation state of the tumor, hopefully avoiding toxic side effects associated with systemic IL-12 administration.

5.7 Inflammation-regulated promoters in a syngeneic tumor model

Although gene therapy approaches exploiting the anti-tumoral 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[109,269,270]. 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 fulfil these two features, long term and inducible transgene expression. Recent preclinical studies in solid tumor models have shown that the level of IL-12 expression is essential for tumor clearance and protection[145]. For this, the authors isolated and characterized IL-12-producing tumor cell clones. Once the bioactivity of IL-12P2A has been confirmed in cell culture-based assays and the inducibility of the promoters has been tested *in vivo* [162], we have tested the bioactivity of the IL-12P2A in mice by employing IL-12-producing clones from transduced LLC cells. ESELp and NFκBp showed early activation and tumor-regulation of transgene expression in contrast to the non-inducible SFFVp expression. Interestingly, modulation of ESELp-driven expression begins with an increase starting at day 8 post-implant reaching a maximum around 11-13 days and followed by a constant fall. In contrast NFκBp-driven expression is characterized by high transgene levels (100 times higher than ESELp-driven expression) and did not show a common activation pattern after implantation. As NFκB pathway participates in both initiation and resolution of inflammation[271], its complex regulation during tumor growth might account for the different expression patterns observed.

Regarding to IL-12P2A, its expression under the promoters tested reduced tumor cell growth *in vivo*; as a result the survival rate of the animals bearing IL-12P2A-expressing cells was significantly improved. It is important to indicate that the endpoint criterion was the detection of ulcerated areas within the tumor mass; none of them suffered

spontaneous death. Importantly, the results obtained with the NFκBp-based inflammation-inducible system were quite impressive as there were 1 partial and 2 complete tumor regressions; these animals remained stable till the end of the experiment, up to 60 days post-implantation. The NFκB pathway has been recognized to play a promoting role in most cancers [31] and here we show that it is possible to make use of this NFκB-induced expression to drive transgene expression within tumor microenvironment and obtain a therapeutic effect by expressing IL-12. To determine whether T-cell activation was taking place after implantation of IL-12P2A expressing cells, splenocytes from tumor-bearing mice were analyzed by flow cytometry. We found that $CD8^+$ T cell activation was statistically significant when IL-12P2A was expressed under either SFFVp or NFκBp. In the case of $CD4^+$ T cell activation, the results were statistically significant only when IL-12P2A was constitutively expressed (SFFVp). As the NFκBp-IL-12P2A group showed the highest survival rate, and in these animals only $CD8^+$ T cell population was significantly activated, these results highlight the crucial role of activated $CD8^+$ T cells for the IL-12-dependent anti-tumor effect in this solid tumor model. This is in accordance with previous published results showing that $CD8^+$ T cells were the primary effectors in IL-12-mediated anti-tumor immunity[272,273]. T cell activation in IL-12-expressing groups was confirmed by an increase in the percentage of $CD69^+$ (one of the earliest cluster of differentiation expressed in activated T cells), and $CD25^+$ (the α-chain of IL-2 receptor) cells, both surface proteins described as T cell activation markers [274,275]. These results suggest that intratumor IL-12 expression triggers T cell responses that would be beneficial by hampering tumor progression.

Systemic IL-12 administration causes significant toxicity and side effects [113]. Samples and organs collected from this last *in vivo* experiment will allow to address this important question (i.e., toxicity and side effects) when our IL-12P2A expression systems were employed. The analysis of blood samples weekly collected will indicate the IL-12 bloodstream levels, and will show whether the use of the inflammation-regulated promoter has any impact on IL-12 systemic levels. In addition tumor cell composition will be determined by looking for tumor infiltration of effector immune cells (macrophages, NK and T cells). Once we have demonstrated the bioactivity of IL-12P2A in mice by employing IL-12-producing LLC clones, our efforts will be directed to isolate, characterize and test *in vivo* LLC cell clones expressing IL-12P2A under ESELp. As we have shown that this promoter, which is regulated by tumor-inflammation, produces the lowest basal levels of transgene expression and a high fold induction upon stimulation, it would potentially be the best indicated to avoid side effects. However it is important to analyze whether these levels of IL-12 expression are therapeutic and have an anti-tumor effect. It will also be

important to study whether long term protection can be established by immune memory. An important characteristic of IL-12 is that it synergizes with several other cytokines[276]. 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[113]. Nevertheless, despite these results, IL-12 remains as a recognized anti-cancer agent with a great potential for synergistic combinations with other immunotherapies and/or conventional cancer treatments[277,278].

We have shown that subcutaneous implantation of IL-12P2A-expressing LLC clones results in tumor growth impairment, being the inflammation-regulated system the most efficient one. These experiments will constitute a proof-of-concept to study the anti-tumoral efficacy of our inducible LV systems after local intratumoral administration. For future clinical applications, our expression systems can be transferred to vector platforms safe enough for human applications. Importantly, it has been recently shown that the virus mimicking effect of viral vectors would likely trigger the IFN-response needed for efficient IL-12 dependent immunotherapeutic effects, improving its anti-tumor properties [147]. These inducible promoters might represent excellent systems for the inflammation-regulated expression of different anti-tumor agents for future clinical applications.

6 Conclusions/Conclusiones

The conclusions drawn from this work can be summarized as follows:

1. Lentiviral systems based on inflammation-inducible promoters respond to pro-inflammatory stimuli *in vitro* in different cell types such as immune, endothelial, glia and tumor cells.

2. Local administration of the inflammation-regulated lentiviral system based on the human E-selectin promoter releases therapeutic levels of murine IL-10 which attenuate acute inflammation induced by Zymosan injection. Promoter activation correlates with the inflammatory state within the affected area.

3. Murine IL-12 expressed as a self-processing polypeptide based on the P2A peptide sequence (IL-12P2A) efficiently induces murine lymphoblast proliferation and IFN-γ release *ex vivo*.

4. Subcutaneous implant of LLC cells transduced with the inflammation-regulated lentiviral systems in mice stimulates transcription from their promoters. These results suggest the presence of an early pro-inflammatory response within the tumor microenvironment.

5. In the syngeneic model, the survival rates improve when tumor cells expressing IL-12P2A are implanted. In the case of the inflammation-regulated inducible system based on the NFκB promoter, up to 60% of total or partial tumor regression was observed.

6. Subcutaneous implant of LLC cells expressing IL-12P2A in mice increases the percentage of CD69 and CD25 positive cells within the $CD8^+$ T cell population. These data suggest the activation of cytotoxic T lymphocytes that might contribute, at least in part, to the decrease of tumor growth rate in these animals.

Las conclusiones extraídas de este trabajo son las siguientes:

1. Los sistemas lentivirales basados en promotores inducibles por inflamación responden *in vitro* a estímulos pro-inflamatorios en diferentes tipos celulares incluyendo células del sistema inmune, endoteliales, de la glía y tumorales.

2. La administración local del sistema lentiviral regulado por inflamación basado en el promotor de la selectina E humana secreta niveles terapéuticos de IL-10 murina que disminuyen la inflamación aguda inducida por la inyección de Zymosan. La activación del promotor es proporcional al estadio inflamatorio de la zona afectada.

3. La IL-12 murina expresada como un polipéptido auto-procesable empleando la secuencia del péptido P2A (IL-12P2A) induce en células aisladas de bazo de ratón tanto su proliferación como la producción de IFN-γ.

4. El implante subcutáneo de células LLC transducidas con los sistemas lentivirales regulados por inflamación estimula la actividad transcripcional de sus promotores. Estos resultados sugieren la presencia temprana de un ambiente tumoral pro-inflamatorio post-implante.

5. En el modelo de tumor singénico empleado, la tasa de supervivencia mejora cuando se implantan células tumorales que expresan IL-12P2A, llegando a observar una regresión total o parcial de la masa tumoral en un 60% de los animales cuando se emplea el sistema lentiviral inducible por inflamación basado en el promotor NFκB.

6. El implante subcutáneo de células LLC transducidas que expresan IL-12P2A aumenta el porcentaje de linfocitos T $CD8^+$ que expresan los marcadores de activación CD69 y CD25. Estos datos sugieren la activación de una respuesta inmune de linfocitos T citotóxicos que podría contribuir, al menos en parte, a la disminución de la tasa de crecimiento tumoral en estos animales.

7 Bibliography

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