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Cytokine Gene Therapy of Autoimmune Disease

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Contributions of Authors

I would like to acknowledge the contribution of Drs. Yigang Chang and Keli Song in the studies described in Chapters III and IV of this thesis. In Chapter III, Dr. Keli Song constructed the IFN-γ plasmid DNA expression vector (pCI-IFN-γ), while Dr. Yigang Chang performed the experiment involving IFN-γ plasmid DNA (pCI-IFN-γ) therapy of NOD mice. Furthermore, Dr. Yigang Chang has constructed the pVR-IL-4-IgG1 expression vector, which I used in the studies described in Chapter IV. All other experiments described in Chapters II, III and IV were designed, performed and analyzed by the author of this thesis, under the supervision of Dr. Gérald J. Prud'homme.

Abstract

Proinflammatory cytokines by Th1 cells and macrophages are involved in the pathogenesis of several organ-specific autoimmune diseases. Clinically, cytokine therapy has the potential of influencing disease outcome by altering the balance between proinflammatory versus immunosuppressive cytokine profiles. Somatic cytokine gene therapy is an attractive alternative to cytokine immunotherapy, because it eliminates the need for frequent protein injections, and generates more constant cytokine levels *in vivo*, which may reduce toxicity and increase therapeutic efficacy. To study the immunoregulatory effects of TGF-β1 and IL-4 *in vivo*, we used a novel method of i.m. cytokine gene therapy in 2 experimental models of Th1 cell-mediated autoimmune diseases: murine diabetes and EAE. In our first model, i.m. TGF-β1 gene administration is effective at suppressing a DTH response, and at protecting NOD mice from autoimmune insulitis and diabetes. In this model, disease protection was associated with a decreased intrapancreatic IL-12 and IFNγ mRNA expression. In our second model, TGF-β1 and IL-4-IgG1 gene therapy resulted in anti-encephalitogenic effects in mice with MBP-induced EAE. TGF-β1 gene delivery had pronounced downregulatory effects on MBP-stimulated T cell proliferation and production of IFN-γ and TNF-α. IL-4-IgG1 gene delivery also suppressed these responses and enhanced endogenous secretion of IL-4. Cytokine gene therapy resulted in a decrease in the severity of CNS inflammatory lesions. With either treatment, CNS IL-12 and IFNγ mRNA expression was significantly diminished, while IL-4 and TGF-β1 mRNA levels were increased compared to control mice. In summary, i.m. delivery of cytokine plasmid vectors suppressed pathogenic Th1 responses, while enhancing the production of protective, regulatory cytokines in peripheral lymphoid and/or target tissues. Somatic cytokine gene therapy proved to be an effective therapeutic strategy of cytokine delivery in autoimmunity-prone mice.

Abrégé

Les cytokines proinflammatoires produites par les cellules Th1 et les macrophages contribuent à la pathogénèse de plusieurs maladies autoimmunes impliquant des organes spécifiques. En clinique, la thérapie aux cytokines peut influencer l'issue de ces maladies en modifiant l'équilibre entre les cytokines proinflammatoires et immunosuppressives. La thérapie génique des cytokines est une alternative intéressante à l'immunothérapie traditionelle, puisqu'elle évite les injections multiples, et génère des concentrations sanguines prolongées et constantes. La résultante est une toxicité très réduite et une plus grande efficacité thérapeutique. Afin d'examiner les effets immunorégulateurs de TGF-β1 et IL-4, nous avons utilisé une approche innovatrice de thérapie génique i.m. des cytokines dans 2 modèles animals d'autoimmunité: le diabète murin et l'encéphalomyélite allergique expérimentale (EAE). Dans notre premier modèle, l'administration du vecteur TGF-β1 peut supprimer une réaction d'hypersensibilité retardée, et protéger les souris NOD d'une insulite et du diabète. Cette protection est associé à une diminution de l'expression intrapancréatique de l'ARNm IL-12 et IFN-γ. Dans notre deuxième modèle, la thérapie génique aux vecteurs TGF-β1 et IL-4-IgG1 résulte dans des effets anti-encéphalitogéniques dans les souris atteintes de EAE. La thérapie au vecteur TGF-β1 a notamment supprimé la prolifération des cellules T, et la production de IFN-γ et TNF-α. Le traitement avec le vecteur IL-4-IgG1 a également supprimé ces réactions et a augmenté la production de IL-4. La thérapie génique des cytokines a diminué la sévérité des lésions du SNC. Chaque traitement a réduit de manière significative l'expression d'ARNm IL-12 et IFN-γ dans le SNC, et de plus, a augmenté l'expression de TGF-β1 et IL-4, comparé aux souris contrôles. En sommaire, la thérapie génique i.m. des cytokines a supprimé les réactions pathogéniques Th1, tout en augmentant la production des cytokines régulatrices. Donc, cette forme de thérapie génique est une stratégie efficace pour l'administration des cytokines dans les souris susceptibles aux maladies autoimmunes.

TABLE OF CONTENTS

PAGE

PAGE

PAGE

PAGE

CHAPTER II

CHAPTER III

PAGE

CHAPTER IV

PREVENTION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS BY INTRAMUSCULAR GENE THERAPY WITH CYTOKINE-ENCODING PLASMID VECTORS 188

PAGE

CHAPTER V

INDEX TO FIGURES

Chapter II: Factors Influencing Foreign Gene Expression Following Injection Of Plasmid DNA Into Mouse Skeletal Muscle

Chapter III : Transforming Growth Factor β1 (TGF-β1) Somatic Gene Therapy Prevents Autoimmune Disease In NOD Mice

PAGE

ABBREVIATIONS

APC : antigen presenting cell

β-GAL : beta galactosidase

BSA : bovine serum albumin

CAT : chloramphenicol acetyltransferase

CD : cluster of differentiation

CF : cystic fibrosis

CFA : complete Freund's adjuvant

CIA : collagen induced arthritis

CMV : cytomegalovirus

CNS : central nervous system

CPM : counts per minute

CTL : cytotoxic T lymphocyte

CTLA-4 : cytotoxic T lymphocyte associated-4

CYP : cyclophosphamide

DNA : deoxyribonucleic acid

DTH : delayed type hypersensitivity

EAE : experimental allergic/autoimmune encephalomyelitis

ELISA : enzyme-linked immunosorbent assay

GAD : glutamic acid decarboxylase

GMCSF : granulocyte-macrophage colony stimulating factor

HLA : human leukocyte antigen

HVJ : hemagglutinin virus of Japan

ICA : islet cell antigen

IDDM : insulin-dependent diabetes mellitus

IFN : interferon

Ig : immunoglobulin

IL : interleukin

iNOS : inducible nitric oxide synthase

IP : intraperitoneal

IV : intravenous

JAK : Janus kinase

LAK : lymphokine-activated killer

LCMV : lymphocytic choriomeningitis virus

LNC : lymph node cell

LPS : lipopolysaccharide

LUX : luciferase

mAb : monoclonal antibody

MHC : major histocompatibility complex

MBP : myelin basic protein

NK : natural killer

NO : nitric oxide

NOD : non-obese diabetic

OVA : ovalbumin

PBS : phosphate-buffered saline

PCR : polymerase chain reaction

RF : rectus femoris

RT : reverse transcription

SLE : systemic lupus erythematosus

SNC : système nerveux central

STAT : signal transducer and activator of transcription

TCR : T cell receptor

TGF : transforming growth factor

Th : T-helper

TA : tibialis anterior

TNF: tumor necrosis factor

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" Nature reveals her secrets not to those who rush by,

but to those who pass by with an observing eye

and a loving heart."

- Anonymous

To my Mother, Maria-Donata...

To my Father, Giovanni...

To my Brother, Antonio...

To my Wife, Nadine...

Your Love makes me live.

I dedicate my work to you, with love and affection.

+ Pax +

CHAPTER I

GENERAL INTRODUCTION

A. Gene transfer and therapy

A. 1 Experimental objective of gene therapy

Gene transfer into mammalian cells by physical, chemical and biological means has been an active area of research in the past 25 years. The possibility of manipulating certain cellular functions by expression of a newly transfected gene was the foundation for rapid development in the treatments of human disease by gene therapy. Gene therapy can be generally defined as the delivery of genetic information, in the form of functional nucleic acids (genes), into specific cells or tissues, by an efficient, safe and non-toxic manner, for the benefit of the organism (1). The successful application of this approach begins with the development of suitable carriers for the target specific delivery of genetic material followed by expression of DNA at that site. The nucleic acids may be DNA (cDNA), RNA (mRNA), sense or antisense, single, double or triple stranded. Different kinds of systems are being used to effectively transfer and express the foreign DNA in various types of mammalian cells. *In vitro*, genes can be introduced into cells by a variety of ways including microinjection, electroporation, calcium phosphate, DEAE dextran, and liposomes (2). For *in vivo* gene transfer, genetic information can be packaged into a variety of viral particles. Without viruses, it can be delivered together with either proteins, lipids, carbohydrate (eg. asiologlycoproteins), synthetic molecules (eg. polylysine conjugates), or administered

in purified (naked) form (3). The numerous potential benefits of gene therapy include the generation of immune responses against infectious microbes or cancer cells, production of therapeutic proteins, and correction of defective gene expression in a variety of genetic and acquired diseases (2,3).

Current approaches of somatic gene therapy can be divided in two general categories (4,5): *ex vivo* gene transfer involves the removal of cells from an organism followed by gene transduction *in vitro* and reimplantation of the genetically modified cells into the appropriate tissue *in vivo*. In contrast, *in vivo* gene transfer involves the introduction of a recombinant gene into the appropriate cell type *in vivo* without the need to remove and culture cells from the recipient organism. Therefore, *in vivo* gene therapy circumvents many limitations associated with *ex vivo* gene transfer protocols.

A.2 Viral vectors

Although over a hundred human clinical trials in gene therapy have already been approved and applied, the technology is still in its infancy. The development of suitable expression vectors for *in vivo* gene transfer remains the most challenging and urgent task (4,5). In contrast to traditional protein prophylactic therapy, very large molecules, such as DNA, must be delivered to many or the majority of cells of a given tissue for any therapeutic benefit (6). Viruses are natural vehicles for gene transfer into mammalian cells. They have evolved specific mechanisms for efficient replication, condensation and packaging of their own genetic material. They have also developed

highly adaptive ways to attach, penetrate and maintain themselves inside mammalian cells (7). Therefore, viruses have played a pivotal role in the developmental phase of gene therapy. However, infectious viral life cycles are not compatible with the general aim of gene therapy and important modifications of viral structure and function are required (8).

There are several types of viral vectors currently used for gene therapy protocols and each having distinct advantages and disadvantages for different applications. The more common viral vectors include :

A.2.1 Retroviruses

These are the most common type of viral vector used in gene therapy protocols (4,5). Replication-deficient retroviral vectors can carry up to 9 kb of genetic information. These vectors generally contain the gene of interest in a viral capsule with all other coding viral sequences being entirely removed from its genome. Other viral sequences such as long terminal repeats (LTR) and packaging sequences (ψ) are required for genome replication, encapsulation, reverse transcription and integration (2). LTRs also serve as enhancer-promoter sequences which regulate the expression of viral genes (2). The packaging sequence (ψ) allows the viral RNA to be distinguished from other RNA species in the cell. By manipulating the retroviral genome, viral genes can be replaced with the gene of interest (transgene). Transcription of the transgene

may be under the control of the viral LTR's or enhancer-promoter elements engineered within the transgene (6). The viral proteins *gag*, *pol*, and *env* are also required but are provided by wild-type retroviruses in packaging helper cell lines (5). So, only the recombinant viral genomes are assembled to produce a retroviral vector. Minimal sequence overlap between vector and helper viruses help minimize the chance for generation of replication competent viruses (7,8).

The most important characteristic of retroviral vectors is integration of the provirus into the host cell genome resulting in the propagation of the transferred gene to all daughter cells of the infected cell (2-5). Retroviruses transfer this information into the genome of the target cell where it integrates and permanently modifies the genotype of the target cell. This is an advantage when treating hereditary genetic disorders such as Duchenne's muscular dystrophy, cystic fibrosis or adenosine deaminase deficiency (ADA) (5). Retroviruses have numerous potential risks, including toxicity resulting from permanent overexpression of the transgene or insertional mutagenesis resulting from the random disruption or inactivation of tumor suppressor genes and oncogenes, respectively (4). The use of retroviral vectors is also limited by their sensitivity to immune inactivation and by the necessity for target cells to divide in order to successfully integrate the proviral DNA into the host genome (8). Furthermore, there are production problems associated with low titers, stability and recombination/rearrangement with wild-type infectious retroviruses. Retroviral vectors have almost entirely been used in *ex vivo* gene therapy protocols because retrovirus

transduction efficiency is higher *in vitro* (7). Retroviral vectors have been extensively used in cancer therapy with the intention of increasing the antigenicity of tumor cells or activating host antitumor immune responses. The generation of tumor cell vaccines harboring genes encoding cytokines, MHC, costimulatory molecules or tumor derived antigens, have been the primary use for these vectors (reviewed in 4). Furthermore, retroviral vectors have also been used to establish long term correction in hematopoetic cells, as is the case with adenosine deaminase (ADA) deficiency (9,10).

A.2.2 Adenoviruses

Adenoviral vectors are widely used tools for gene therapy, being the second vehicle of choice for gene delivery after retroviral vectors (reviewed in 11). The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells causing benign respiratory tract infections in humans (11). The adenoviral genome contains over a dozen genes and they typically do not integrate into the host genome. Instead, they replicate as episomal structures within the nuclei of target cells (12). First generation adenoviral vectors which are replication defective can be produced by replacing the E1 gene (involved in viral replication) with the transgene (13). The recombinant vectors are then propagated in cell lines that express the E1 proteins (3-5).

Replication-defective adenoviral vectors can accommodate genes up to about 7.5 kb of DNA (5). These vectors enter cells by means of distinct cellular receptors (14): one for the adenovirus fiber and the other, $\alpha_v\beta_3$ (or $\alpha_v\beta_5$) surface integrins which serve as a receptor for the adenovirus penton. Adenovirus vectors are well suited for gene therapy trials because they can be produced in titers up to 10^{13} adenoviral particles/ml (15). These vectors are advantageous because they can infect both dividing and non-dividing cells, thus increasing the target cell range (11). The transferred gene remains episomal (extrachromosomal) therefore reducing the risk of modifying the host genome or of insertional mutagenesis (2-5,11,15).

One very serious drawback with adenoviral expression vectors is their inherent capacity to evoke potent humoral and cellular immune responses against the vector and virally infected cell. In the cellular response, potent cytotoxic CD8+ T lymphocytes destroy transfected cells and, in turn, limits the duration of transgene expression (16). In fact, expression lasts only 5-10 days post-infection. The anti-adenovirus humoral immune response also appears to limit the efficacy of repetitive administration of these vectors (17). Most of the human population probably have neutralizing antibodies to adenoviruses from previous infection with the naturally occurring virus (17). Thus, immunity to the adenovirus compromises gene transfer. Second generation adenoviral vectors, with a significantly reduced protein-encoding capacity, have also been developed to further eliminate any potential immune response directed against transfected target cells (18). Consequently, these vectors showed

longer-term expression, but a decline after 20-40 days was still observed (19). This idea has been extended further with the generation of "gutless" adenoviral vectors which have all of the viral genes deleted (with the exception of elements designating the beginning and end of the genome, and the packaging sequence). In a recent study, these "gutless" vectors expressed their transgenes for 84 days (20). Adenoviral gene transfer in nude mice, or into mice receiving immunosuppressive drugs, leads to sustained, high-level transgene expression (21).

A.2.3 Adeno-associated viruses

Adeno-associated viruses (AAV) are non-pathogenic viruses that are widespread in the human population (22). Gene therapists became interested in this virus because it is the only known mammalian virus which integrates in a site-specific fashion in the human genome, specifically on the short arm of chromosome 19 (23). Since AAVs do not produce disease, their integration site on chromosome 19 has been designated as a safe integration region. Paradoxically, most current vectors do not retain this integration specificity (24).

AAVs have 2 genes (*cap* and *rep*) which are positioned between both inverted terminal repeats (23). The AAV genome also has a packaging sequence. The *cap* gene encodes viral capsid proteins and the *rep* gene encodes a protein involved in viral

replication and integration. AAV also needs additional genes to replicate and these are usually provided by a helper virus, such as an adenovirus or herpes simplex virus (25).

This viral vector has the advantage of infecting a wide variety of cell types, both dividing and non-dividing (23, 25). Although production of high titers of virus is a feasible process, it is nevertheless a laborious and expensive endeavor. To date, AAV vectors can only accommodate about 4 kB of transgene DNA. Like adenoviral vectors, AAV vectors are immunogenic and thus potent humoral and cellular immune responses are generated against the vector and virally infected cell (26). This anti-AAV immune response limits the duration of transgene expression and the efficacy of repetitive administration of these vectors (26).

A.2.4 Other viral vector

Other viral systems used for gene therapy protocols include the herpes simplex virus, vaccinia virus (poxvirus) and the Sindbis virus (also called the hemagglutinin virus of Japan, HVJ) (3-5). In addition, hybrid systems have been developed where an adenoviral vector is used to carry a retroviral vector into a cell that is normally inaccessible to retroviral transduction (27). Generally, expression is short-lived with these systems. The use of these viral systems as gene transfer vehicles is still in its infancy.

A.3 Non-Viral Methods of DNA Transfer

Viruses infecting mammalian cells have evolved specific mechanisms for cell attachment, penetration, survival and viral reproduction (1-3). However, viral vectors all induce an immunological response to some degree, may have safety risks, and their potential use is limited by difficulties in vector construction and large scale production, as mentioned above. These disadvantages have led to a research shift to explore alternative non-viral approaches to gene therapy. The ultimate goal is to imitate beneficial characteristics of viruses while avoiding their inherent limitations.

Non-viral methods of DNA transfer possess no antigenic proteins, have no infectious or mutagenic capability and large scale production is feasible. There are three main methods of non-viral DNA transfer, namely, cationic liposomes, molecular conjugates and naked plasmid DNA.

A.3.1 *Liposomes*

This form of gene transfer relies on the use of liposomes as vehicles for gene delivery. Liposomes are lipid monolayers or bilayers entrapping a fraction of aqueous fiuid. This approach relies upon the electrical charge properties of DNA (negative due to the phosphate backbone of the double helix), cationic lipids (positive), and

cell surfaces (net negative due to sialic acid residues) (28). DNA (plasmid) spontaneously associates with the outside surface of liposome and the resulting complex can adsorb to the cell membrane and deliver the nucleic acid directly to the cytoplasm, bypassing the lysosomal degradation pathway (29). The overall ratio of lipid to DNA, as well as the total concentration has to be evaluated in order to minimize toxicity (30). In vitro, up to 90% of certain cell lines may be transfected. By including a small amount of an anionic lipid in an otherwise cationic liposome, the DNA can be incorporated into the internal surface of the liposome, thus protecting it from enzymatic degradation. (31).

Attachment of ligands on the surface of liposomes is the most efficient way for their delivery to target tissue sites. For example, targeting proteins such as anti-MHC antibodies, transferrin, polylysine, or HVJ proteins have been included in liposomes (32-35). Some of these targeting proteins, such as the HVJ additionally allows the plasmid DNA to escape from the endosome into the cytoplasm, thus avoiding degradation (35). The inclusion of a DNA binding protein enhances transcription by bringing the plasmid into the nucleus (36). Further improvements include incorporating the Epstein-Barr virus EBNA1 gene in the plasmid to maintain the plasmid as an episomal element (37).

In theory, liposome/plasmid DNA complexes have several benefits as gene delivery vehicles. They can be used to transfer transcriptional units of nearly unlimited sizes, cannot replicate or recombine to generate infectious particles, and

provoke few inflammatory responses (5,30). They can transfer genes in a wide variety of dividing and non-dividing cell types, although transfection efficiency is significantly greater in dividing cells (38). The disadvantages of liposomes is that they are rather inefficient, requiring thousands of plasmids be presented to the target cell in order for successful gene transfer to ensue. Transgene expression tends to be short-lived (38).

Liposome/plasmid complexes have been applied in human clinical trial for cystic fibrosis (CF) (39). In this study, spray administration of DC-Chol/DOPE and CFTR cDNA plasmid complexes resulted in biological effects which became detectable by 24 hours and showed a maximum response by 3-4 days. Responses disappeared by 7 days post-treatment, with extensive variation among individual patients. Three other phase I gene therapy trials for CF using the cationic lipids DC-Chol/DOPE (40), DIMRIE/DOPE (41), and DOTMA (42) are currently in progress. Also, i.v. injection of DNA/liposome complexes has met with limited success (43). In this case, the cells of several organs are transfected (particularly endothelial cells), but the persistence of gene expression is usually much less than after i.m. delivery (see below for detail).

A.3.2. *Molecular conjugates*

Molecular conjugates consist of protein or synthetic ligands to which DNA has been attached. In this method, DNA is conjugated with a cell-specific carrier

molecule which is the ligand for a cell surface receptor (44). Delivery to the target cells can be improved by using similar techniques to those used for liposomes. Targeting proteins include asialoglycoprotein (45), transferrin (46), polylysine conjugates (47), and polymeric IgA (48). Advantages of these conjugates include low immunogenicity and accomodation of large DNA fragments. Transgene expression tends to be transient and is limited by endosome/lysosomal degradation (27,44).

A.3.3 *Naked plasmid DNA*

In recent years, there has been a great deal of interest in the use of plasmid DNA encoding antigens or therapeutic proteins for immunization and *in vivo* systemic delivery purposes, respectively. This interest began with an observation that direct injection into mouse skeletal muscle of "naked" plasmid DNA, or RNA, encoding either CAT, β-gal and luciferase reporter genes resulted in functional gene expression by individual muscle fibers (49). Reporter gene expression was long-term since it could be detected for at least 19 months following a single injection of DNA. It has also been reported that similar results could be obtained following DNA vaccination of Rhesus monkeys, providing direct evidence that this phenomenon was not limited to rodents (50). These results were astonishing because higher levels of gene expression

could be achieved using plasmid DNA alone compared to direct injection of DNA complexed with cationic lipids, hence the use of the term "naked".

Naked plasmid DNA can also be attached to gold particles and bombarded into tissue (51). This form of plasmid DNA administration in tissue is referred to as gene bombardment or "gene gun" ballistics. Both direct DNA injection and gene gun delivery result in prolonged low level expression *in vivo* (52). Plasmids are more readily taken up by adult muscle fibers than retroviral and adenoviral vectors (53). This is of importance, especially in monogenic diseases like Duchenne's muscular dystrophy where achieving the corrected phenotype before the muscle fibers undergo degeneration might assist in preventing further muscle damage. The simplicity of these methods and sustained expression has led to the development of numerous DNA vaccines (see section A.3.3.2). There is no reported DNA length restriction and no immunological problems have been associated with them (54).

The process of plasmid DNA entry into individual skeletal muscle fibers is not completely understood, although both active and passive mechanisms have been suggested to be involved. Several uptake mechanisms have been postulated, including plasma membrane disruption, receptor-mediated endocytosis, and potocytosis (reviewed in 55). There are many barriers for plasmid DNA molecules once they are injected in muscle tissue. They must cross the ECM, plasma membrane, sarcolemma of individual muscle fibers, and then find their way to the nucleus. Although it is currently unclear on how plasmid DNA molecules reach the nucleus, it

is generally believed that T-tubules play a role in plasmid DNA uptake and trafficking (56). In addition, many factors such as muscle fiber type, muscle size, vascularization, age and sex appear to influence plasmid DNA uptake and expression (57, Chapter II). Direct injection of naked plasmid DNA has also been performed in other tissues including skin, liver, tongue, thyroid, tumors, and lung (58). Expression levels in these tissues were considerably lower than skeletal muscle. The reason why skeletal muscle is so readily transfected by plasmid DNA is unclear. It may reflect uptake mechanisms mediated by structures unique to or prevalent in skeletal muscle, such as the T-tubule system or caveolae, respectively (59).

A.3.3.1 Naked Plasmid DNA Expression Vector Design and Construction

The essential components of naked plasmid DNA expression vectors designed for vaccination or therapeutic protein delivery are the same as for conventional eukaryotic expression vectors (reviewed in 60): namely, a coding DNA sequence, an upstream promoter/enhancer (P/E) and a downstream polyadenylation signal (poly A) /mRNA termination sequence (which may be isolated from a range of mammalian and viral sources); and, a bacterially derived plasmid backbone containing both an origin of replication (ORI) for *E.coli* and an antibiotic resistance selectable marker. Many plasmid expression vectors also contain intronic sequences which have been shown to greatly increase gene expression within transfected cells. Obviously, there are many

incremental changes possible within the above-mentioned framework. The promoter/enhancers that have been most widely used for DNA vaccination and systemic protein delivery are the cytomegalovirus immediate/early promoter-enhancer (CMV-I/E) and the Rous sarcoma virus long terminal repeat (RSV-LTR). Compared to most other promoters, these promoters are excellent for mediating the highest levels of protein expression by *in vitro* transfection of cell lines. While it is rather difficult to establish functional correlations between the abilities of a plasmid to express heterologous proteins *in vitro* and *in vivo*, it appears that plasmids providing better expression *in vitro* achieve superior results *in vivo* (61).

A.3.3.2 DNA Vaccination

The potential significance of the above-mentioned results for DNA vaccine development became evident when Ulmer and colleagues (62) demonstrated that immunization of mice with a plasmid encoding a full length influenza nucleoprotein (NP) gene conferred protection to plasmid-treated mice from lethal challenge with the influenza virus. This therapy was referred to as DNA vaccination (60,62). Protection in this model was probably mediated by antigen-specific T cell immunity and vaccination produced potent cytotoxic T lymphocyte (CTL) responses directed against an NP peptide epitope. DNA vaccination of mice using plasmids encoding the hemagglutinin (HA) gene resulted in strong neutralizing antibodies against influenza and complete

protection from homologous viral challenge (62,63). Furthermore, other reports have demonstrated the partial protection from influenza challenge with HA DNA-vaccinated chickens, although neutralizing antibodies were not detected (64), and HIV gp160-specific antibody and T cell responses in mice using a gp160/rev-expressing plasmid (65).

DNA vaccines have been used to elicit specific humoral and cellular immune responses against a variety of antigens in several animal species. Some examples include hepatitis B surface antigen in mice (66,67), herpes simplex virus-1 glycoprotein B in mice (68), bovine herpesvirus 1 glycoprotein IV in cattle (69), rabies virus glycoprotein in mice (70), malaria circumsporozoite protein mice (71), *Leishmania* gp63 in mice (72), lymphocytic choriomeningitis virus (LCMV) NP in mice (73), carcinoembryonic antigen in mice (74), and MHC class I antigens in rats (75).

DNA vaccines have demonstrated a surprising ability to initiate both humoral and cellular protective immunity. This protective immunity is both potent and long-lived in certain animal models (62,63,66,67). Generally, both types of immune responses have been obtained only with live, attenuated viral vaccines or live, recombinant vaccinia vectors rather than killed organisms or recombinant subunit vaccines which produce humoral immunity predominantly (60). Whether a vaccine can elicit strong CTL activities appears to correlate with its ability to produce protein synthesis *in vivo* following vaccination. *In vivo* synthesis of foreign protein allows

processing and presentation of vaccine-derived epitopes in association with MHC class I molecules, which is required for the generation of CD8+ CTL responses. Furthermore, because DNA vaccine-derived antigens are synthesized *in vivo*, native proteins structure and conformation, including the appropriate post-transcriptional modifications, should be obtained. Thus, it could be expected that more potent neutralizing antibody responses would be generated using DNA vaccines than may be obtained when using vaccines based on chemically modified proteins (60).

A.3.3.3 Systemic delivery of therapeutic proteins by direct i.m. DNA injection

Clinically, protein therapy is applied in a variety of metabolic, neoplastic, inflammatory and autoimmune disorders (76). Conventional protein therapy, including cytokine immunotherapy, has been limited by the need to administer large doses of recombinant protein, often in boluses, with temporarily high systemic levels. These elevated levels can often result in untoward systemic toxicity (77). Moreover, most proteins have a short $T_{1/2}$, thus reducing bioavailability and resulting in poor therapeutic efficiency (78). Isolation and production of highly purified and stable therapeutic proteins are particularly difficult, laborious and expensive processes. Somatic gene therapy has the potential of overcoming these drawbacks, by eliminating the need for frequent protein injections and by allowing long-term constant delivery of

proteins at therapeutic levels. The ease of preparation and relatively low production cost make naked plasmid DNA an excellent drug delivery system (79).

Skeletal muscle represents an attractive target for somatic gene therapy. Since the advent of i.m. gene transfer, much of the focus was directed towards the use of skeletal muscles for DNA vaccination purposes (60). Originally, little was known about i.m. gene transfer and expression of therapeutic proteins. Today, we know that i.m. gene delivery also provides numerous advantages for the systemic delivery of therapeutic biomolecules. Skeletal muscles are comprised of elongated, multi-nucleated cells which are easily accessible for i.m. plasmid DNA injection. The larger muscle mass and non-dividing (post-mitotic) nature of these cells provide advantageous features for plasmid DNA uptake, persistence and long-term expression of therapeutic proteins (49,80,81). Skeletal muscles are also highly vascularized, so protein production from plasmid DNA expression vectors can readily enter the circulation. Therefore, skeletal muscles can be used as *in vivo* biological factories for stable and long-term synthesis of therapeutic molecules (82).

Direct i.m. injection of naked plasmid DNA expression vectors has been applied for the systemic delivery of therapeutic proteins. In a recent study, investigators tested the *in vivo* expression of IL-5 which is involved in the growth and differentiation of B cells and eosinophils (83). I.m. administration of an IL-5 expression vector to mice rapidly increased the number of eosinophils in peripheral blood and elevations in serum IL-5 levels ranged between 10-63 pg/ml. The increase

in eosinophils persisted for 5 weeks (16 weeks in some experimental mice) after plasmid DNA injection, although serum levels were low. The authors argued that low level expression of IL-5 might be sufficient to induce long-term systemic eosinophilia.

In another study, systemic immunological effects were observed following i.m. injection of IL-2 (pIL-2), IL-4 (pIL-4) and TGF-β1 (pTGF-β1) plasmid DNA vectors (79). IL-2 plasmid therapy had enhanced humoral and cellular immune responses to human transferrin, an immunogenic protein, injected at a distal location from the site of DNA injection. These IL-2 effects were abolished with the co-injection of a pTGF-β1. TGF-β1 plasmid treatment markedly reduced the anti-human transferrin antibody response and caused a 8-fold increase in TGF-β1 activity. Intramuscular injection with pIL-4 selectively increased IgG1 levels but did not alter cellular immune responses to human transferrin. Furthermore, in lupus prone MRL/lpr/lpr mice, injection of pIL-2 or pTGF-β1 increased or decreased autoantibodies to chromatin, respectively. Interestingly, Raz and co-workers also evaluated the effects of pTGF-β1 and pIL-2 therapy on disease activity in MRL/lpr/lpr mice (84). In this study, pTGF-β1 treatment had beneficial effects in murine SLE with a prolonged survival, decreased anti-chromatin and rheumatoid factor antibodies and a 50% decrease in total IgG production. Also, renal function was improved and kidney inflammation was significantly reduced. In contrast, pIL-2 i.m. injections had harmful effects on disease progression with a decreased survival, enhanced total synthesis and autoantibody

production. Another group of investigators have used TGF-β1 i.m. gene therapy to improve experimental colitis in rats (85).

In our laboratory, we successfully modulated inflammatory responses in autoimmune diabetes and EAE, two models of organ-specific autoimmunity, by i.m. administration of naked plasmid DNA expression vectors encoding TGF-β1 or IL-4-IgG1 (86), as discussed in Chapter III and Chapter IV, respectively. Wahl and co-workers have recently documented therapeutic effects of i.m. TGF-β1 gene therapy in a model of streptococcal cell wall-induced arthritis (87). Systemic delivery of mouse IL-10 by i.m. injection of plasmid DNA also prevented autoimmune diabetes in NOD mice (88). In all of the above-mentioned studies, cytokine plasmid treatments significantly elevated serum/plasma levels of each respective cytokine, compared to control plasmid-treated mice. These levels were sustained and accompanied with long-term therapeutic benefits.

Tripathy and co-workers recently described the construction and characterization of a plasmid DNA expression vector which drives high-level production of murine erythropoietin (EpO) *in vivo* (89). A single i.m. injection of the EpO plasmid vector produced physiologically significant serum levels of EpO, compared to control plasmid treated mice, and resulted in significant elevations in hematocrits that were stable for at least 90 days. Secretion of circulating proteins following direct i.m. injection of plasmid DNA has also been demonstrated for human alpha-1-antitrypsin and rat Kallikrein-binding protein (82,90). In both studies, secreted

proteins were detected in the circulation at the end of the first week after injection, with subsequent disappearance of circulating protein due to a protein-directed antibody. Draghia-Akli and co-workers have described the characterization of a growth-promoting myogenic expression plasmid vector that drives high-level hGHRH production in skeletal muscle (91). Upon i.m. injection in immunocompetent adult mice, hGHRH was secreted into the circulation. Furthermore, a single administration of the plasmid vector subsequently elevated serum GH levels 3-4 fold for up to 2 weeks, enhanced liver IGF-1 gene expression and increased body weight approximately 10% as compared to control mice. Another group of investigators also reported the construction of a muscle-specific plasmid expression vector which provides long-term secretion of biologically active human growth hormone (hGH) into the systemic circulation following direct plasmid injection (92). A single treatment of this plasmid vector resulted in sustained serum levels of biologically active hGH. The expression and biological effects of hGH persisted for 21 days post-injection. As described above, the loss of hGH effect over time was coincidental with the appearance of anti-hGH antibodies.

The above-described studies provide strong evidence that direct i.m. injection of plasmid DNA expression vectors is an effective approach to *in vivo* delivery of cytokines and growth factors for therapeutic purposes.

B. Immunological tolerance and autoimmunity

The mammalian immune system has evolved several mechanisms to ensure self-nonself discrimination. These mechanisms allow tolerance to self-antigens to be achieved and reactivity to foreign antigens to be maintained, thus preventing any potential autoimmune pathology. Immunological tolerance is, therefore, an acquired physiological state in which the immune system does not harm the organism that harbors it (reviewed in 93).

T cells have developed multiple mechanisms for the establishment and maintenance of immune tolerance in order to avoid reactivity to self antigens. The fate of potentially self-reactive thymocytes appears to be primarily dictated by the site, nature and the level of self-antigen expression, MHC expression, and as well as by the available TCR repertoire (reviewed in 93). There are 3 general mechanisms of T cell tolerance identified to date: clonal deletion, anergy, and immunoregulation (immune deviation) (reviewed in 94 and 95).

B.1 Clonal deletion

Clonal deletion of T cells strongly reactive with self-antigens is a major mechanism contributing to central tolerance. During T cell development, only a few of the many thymocytes which are produced go on to become mature and functional T cells. The production of mature T cells in the thymus involves both positive and

negative selection directed toward a plethora of self antigens associated with either MHC class I or II molecules (96). Thymocytes must be postively selected by interaction of their TCR and MHC/antigen peptide complexes expressed on cortical epithelial cells of the thymus. Most developing thymocytes have a negligible affinity for MHC/peptide complexes on epithelial cells and die within a few days by neglect. A small fraction of thymocytes do have a significant affinity for peptides on self MHC molecules. These cells receive a low level signal, mediated by their αβ TCR and CD4/CD8 co-receptors, and are salvaged from neglect. This in turn induces the maturation of thymocytes into single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ cells. Thymocytes experiencing strong reactivity for self-antigen/MHC complexes expressed on cortico-medullary bone marrow derived APC (dendritic cells) undergo clonal deletion via apoptosis. (97). Negative selection ordinarily takes place at the $CD4^+CD8^+$ (double-positive, DP) stage of thymocyte development, requiring the participation of CD4 and MHC II molecules (98). Although these CD4+CD8+ thymocytes represent the cell type in which most repertoire selection events occur, the differentiation of their immediate precursors, $CD4^-CD8^{low}$ cells, can be aborted by TCR engagement. This provides a way for eliminating early thymocytes expressing autoreactive TCRs (99).

Intrathymic clonal deletion of T cells strongly reactive with self-antigens is a major mechanism contributing to central tolerance. Kappler and Marrack were the first to describe clonal deletion in normal mice expressing endogenously encoded viral superantigens (100). In this study, Vβ17a-bearing T cells which were reactive for the

superantigen were subsequently deleted and were not present in the periphery of these mice. This mechanism was then solidified in transgenic mice in which genes that encode a TCR for known antigens had been introduced into the germline, under conditions where the pertinent antigen could be expressed in the thymus (101). In this model, high-affinity interaction results in the physical elimination (by apoptosis) of self-reactive T cells in the thymus, with a subsequent absence of T cells with the transgenic TCR in the periphery. Conversely, low to moderate affinity binding thymocytes are permitted to continue their developmental program. Clonal deletion can also occur in the bone marrow for the induction of B cell tolerance (102).

In instances where an a self-antigen is not expressed intrathymically, it can be assumed that peripheral mechanisms of tolerance operate to preclude harmful autoreactivity of self-reactive T cells (103). In addition, these mechanisms must also operate against self-reactive T cells that escaped intrathymic clonal deletion. Peripheral clonal deletion is a tolerance mechanism which consists in the physical elimination of self-reactive T cells in peripheral tissues. Peripheral deletion of self-reactive T cells was first described for superantigen-reactive T cells (104). Also, conventional self-antigens that are expressed in the periphery can mediate tolerance by clonal deletion, in analogy to the activation-induced programmed cell death by clonal exhaustion that has been reported for foreign antigens (105).

B.2 T cell Anergy

Distinct cell-surface molecules deliver 2 different types of signals, both required for complete T cell activation during the development and regulation of immune responses. This is referred to as the 2 signal model of T cell activation (reviewed in 106). Signal 1 is delivered by the TCR upon appropriate antigen/MHC recognition, binding and engagement. Signal 2 is a co-stimulatory signal delivered by multiple cell surface receptor/ligand interactions which may include co-stimulatory molecules (CD28, CTLA-4, B7.1 and B7.2), cytokines or other signals from the APC surface. In this model, an absence of either one of these signals would result in a lack of T cell activation and proliferation. T cell unresponsiveness was originally induced in CD4+ Th clones as a consequence of their stimulation with antigen/MHC complexes in the absence of co-stimulatory signals provided by APC (107). In this study, the capacity of unresponsive T cells to produce IL-2 or to proliferate was found to be blocked, regardless of the magnitude of antigen presentation and costimulation provided by APC during a subsequent antigenic challenge. Therefore, T cell anergy refers to a state of proliferative hyporesponsiveness, resulting from the inability of T cells to enter cell cycle and produce IL-2 upon antigen challenge (106). Anergy of regulatory T cells (Th2 cells, see below), in the thymus or periphery, may be harmful to the organism and cause autoimmune disease.

B.3 T cell Immunoregulation and Immune deviation

The concept of "immune deviation" was originally defined in the 1960's by Asherson and Stone to describe the antigen-specific prevention of a DTH reaction which was associated with the induction of a T cell dependent antibody response (108). Specifically, guinea pigs were immunized with protein in alum (instead of CFA) and a dramatic decrease in skin reactions was observed 24 hours later. This was also associated with a loss of antigen-specific IgG2a and an increase of IgG1 antibodies. This notion of an antagonism between humoral and cell mediated immunity was further extended by Parish and Liew (109), and Bretscher (110) who proposed that this cross-regulation might be controlled by 2 antagonistic T cell populations. Therefore, T cells in the process of immune deviation respond strongly to a particular antigen, but in a way that differs from the standard T cell response. This phenomenon is generally explained by the distinct actions of Th1 and Th2 cell subsets, as discussed below.

The well documented observation that autoreactive T cells can be found in normal individuals strongly suggests that negative selection in the thymus is not a fail-safe mechanism (111). As described above, it also suggests that other mechanisms exist in the periphery to actively maintain tolerance to self-antigens or, at least, functionally inhibit the autoaggressive potential of these cells. Furthermore, the loss of tolerance to self-antigens that are often encountered in the thymus, can frequently lead to clinical manifestations of autoimmune disease (112). In addition to anergy, T cell

immunoregulation, by means of immune deviation, appears to be an important mechanism for the induction and maintenance of peripheral T cell tolerance (113,114). Unlike clonal deletion and anergy, immune deviation can be self-perpetuating because cytokine responses of fully differentiated T cells can influence the differentiaton of newly generated, self-antigen-specific T cells (115).

C. Cytokines and immunoregulation

C.1 Role of cytokines in T cell immunoregulation

Since its first description, immune deviation was not understood until the characterization of CD4+ T cell subsets. CD4+ Th cells comprise heterogeneously and functionally distinct subsets which play important roles in the regulation of immune responses by producing various cytokines following activation (116). Although there are no clear phenotypic differences between these subsets, they are nevertheless distinguished by their differential cytokine expression profile (117). Although these subsets were first identified by *in vitro* analysis of murine T cell clones, strong evidence now exists for similar T cell subsets in mice, rats and humans. In mice, CD4+ T cells can be subdivided into at least 3 major subsets (118): Th1 cells, which secrete IL-2, IL-3, TNF-α, TNF-β, IFN-γ and GM-CSF; Th2 cells which selectively secrete IL-3, IL-4, IL-5, IL-10, IL-13 and GM-CSF; and Th0, which are not restricted in their cytokine profile and produce a mixture of Th1 and Th2 cytokines.

Recently, it has been proposed that there are additional subsets of CD4+ regulatory T cells (reviewed in 119). Th3 cells which are generated during oral administration of autoantigens, produce little or no IL-4 and IL-10, but they differ from conventional Th2 cells in their capacity to produce large amounts of TGF-β1 (120-122). Other regulatory CD4+ T cell subsets include T cell regulatory (Tr1) and $CD45RB^{low}$ T cells which express large amounts of immunosuppressive cytokines IL-

10 and TGF-β1, respectively (123,124). Whether these cells represent distinct T cell lineages or different differentiated states of common CD4+ Th precursors is still unclear. Nevertheless, although these regulatory Th cell populations, generated under different *in vitro* or *in vivo* conditions, have unique cytokine expression profiles, they apparently have the common capacity to regulate pathogenic immune responses primarily via TGF-β1, and to a lesser extent IL-10 (119). The functional relevance of these cells will be discussed below.

Cytokine responses that resemble Th1 or Th2 responses, but not necessarily made by CD4+ T cells, are referred to as type 1 or type 2, respectively (115). Type 1 responses generally promote inflammatory and cellular responses (DTH, CTL, macrophage activation, complement-fixing IgG2a antibody), while type 2 responses are associated with non-complement fixing antibody (IgG1) and allergic responses (eosinophilia and IgE production). Although individual T cells can secrete other combinations of cytokines, CD4+ T cell responses in disease states often are predominantly Th1- or Th2-like in nature (125).

CD4+ T cells typically go through a Th0 precursor stage of differentiation before terminally differentiating into either Th1 or Th2 (116-118). Although cytokine responses can remain mixed, the same CD4+ T cells can be induced to secrete either type 1 or type 2 cytokines, depending on the environment in which they are activated. *In vivo* induction of Th1 or Th2 cells has critical immunological consequences, either pathogenic or protective, as evidenced by studies in the *Leishmania* model (126) . In

this model, strains of mice that are genetically prone to generate a Th1 response against *L. major* (C57BL/6 mice) are resistant to infection, while mice generating a Th2 response (BALB/c mice) are susceptible to infection by the parasite.

In recent years, several factors capable of influencing T cell differentiation have been identified. Some of these factors include :

A. Cytokine microenviroment

The development of Th1 and Th2 cells is primarily influenced by the cytokine milieu during the initial phase (T cell activation) of the immune response. The cytokines produced by each T cell subset are inhibitory and cross-regulatory for the opposing subset, and this drives CD4+ T cells towards either the Th1 or Th2 developmental pathway (reviewed extensively in 116-118). For example, both IL-12 and IFN-γ induce/increase the synthesis of IFN-γ and generally inhibit the production of Th2 cytokines. (127,128). IL-12, and to a lesser extent IFN-γ, are potent inducers of Th1 cell development and related cytokine production (127). In addition, IFN-γ itself inhibits Th2 cells thus positively contributing to the inflammatory effect of IL-12 (127,128). Conversely, Th2 cells produce IL-4 which stimulates CD4+ T cells to secrete IL-4 and other Th2 cytokines, and promotes Th2 responses (129). IL-4 also inhibits the synthesis of Th1 cytokines, particularly IFN-γ, and suppresses the development of Th1 cells through downregulation of IL-12 production by activated macrophages or IFN-γ synthesis by NK cells (129). Interestingly, if IL-4 is critical for

the priming of naïve T cells and subsequent Th2 cell development, IL-4 may initially be provided by basophils or NK1+ T cells which expresses this cytokine without prior encounter with antigen or IL-4 (130). Furthermore, the production by Th2 cells of other potent macrophage deactivating cytokines like IL-10 and IL-13 provides strong foundation for their anti-inflammatory effects *in vivo* (131). TGF-β1 primarily suppresses the development of Th1 cells and appears to promote the generation of either Th1- or Th2-like response in different situations (see below).

B. Co-stimulatory molecules

Another factor suggested to influence T cell subset development are membrane-bound co-stimulators, such as B7-1 and B7-2, which are signals provided by APC, and together with antigen have been shown to enhance specific T cell responses via their interaction with CD28 (133). It has been shown that in some experimental systems that co-stimulators may differentially regulate Th1 and Th2 differentiation such that B7-1 ligation favors Th1 development while B7-2 ligation promotes Th2 differentiation (134). In another model, CD28 co-stimulation induces IL-4 production, reinforces Th2 cell development and protect NOD mice from a Th1 mediated insulitis and autoimmune diabetes (135, see below).

C. Nature of APC

The relationship between type of APC and polarized cytokine production is not well understood. A possible link between these two parameters was provided by De Becker and co-workers (136). They immunized mice with antigen-pulsed splenic dendritic cells or activated B cells and reported to generate both IgG1 (Th2) and IgG2a (Th1) responses. Conversely, when they immunized with antigen-pulsed peritoneal macrophages, only IgG1 responses were generated. This study provided evidence that antigen presentation by dendritic cells or B cells might be more likely to support IFN-γ production which, in turn, enhances IgG2a responses, than would antigen presentation by peritoneal macrophages (115,116,136). Similar findings were observed in a model of EAE where autoantigen was coupled to splenic B cells and disease was prevented by a Th2 mediated mechanism (137).

D. Site of antigen presentation

It is reasoned that various tissues may have differential abilities to initiate and support specific T cell responses. In a recent study, it was found that inhaled OVA has a greater tendency than parenterally injected OVA to stimulate Th2 responses (138). When administered orally, antigens that stimulate a strong antibody response can induce tolerance which is associated with T cell production of TGF-β1 (139). These findings may reflect unique populations of T cells or APC in the lung or gut, differential cytokine production at these sites, or other uncharacterized factors (115).

E. Other factors

Additional factors which may affect T cell differentiation include the nature and dose of antigen, MHC class II haplotype, non-MHC genes and overall affinity between MHC, antigen and TCR molecules (116).

In light of the aforementioned factors which influence CD4+ T cells differentiation, the induction of disease-protective CD4+ Th cell subsets should be a rational approach for antigen-specific therapies of organ-specific autoimmune diseases such as EAE and autoimmune diabetes. Since priming of CD4+ T cells with antigen and cytokines plays a dominant role in the induction of protective T cell subsets, cytokine-induced immune deviation might be a therapeutic approach capable of selectively modulating the immune response of autoantigen-specific T cells *in vivo*, even in the absence of information related to the identity of the autoantigen and TCR fine specificity.

C.2 Transforming growth factor beta 1 (TGF-β1)

For our *in vivo* cytokine gene transfer studies, we focused on TGF-β1. We chose TGF-β1 as our candidate cytokine for 2 main reasons:

1) TGF-β1 has many well-defined immunomodulatory functions which can easily be assessed *in vivo* (140). Moreover, the immunoregulatory properties of TGF-β1 have been demonstrated in several models of autoimmunity, as described later.

2) In order to achieve significant suppression of autoimmune responses, microgram doses of purified recombinant TGF-β1 are required. Since TGF-β1 isolation and purification are particularly difficult and very expensive processes, TGF-β1 gene therapy could alleviate these burdens by providing relatively constant TGF-β1 delivery *in vivo*.

C.2.1. Biochemistry and molecular biology

C.2.1.1 General Features :

TGF-β is a family of closely related multifunctional growth factors involved with a variety of different biological processes including embryonic development, tumorigenesis, fibrosis, wound healing, hematopoiesis, and immunoregulation (reviewed in 141). The original characterization of TGF-β was made in the field of tumorigenesis. It was found that certain tumors produced soluble mediators, called

transforming growth factor, which would allow normal fibroblasts to growth and survive in soft agar, a trait of transformed cells (142). The originally described form of TGF-β, now known as TGF-β1, is only one member of a family of regulatory proteins distantly related to TGF-β1 with a 30-40% sequence identity. These include the activins, inhibins, and bone morphogenetic proteins (BMPs), and a number of more closely related proteins (70-80% sequence identity) designated TGF-β2, TGF-β3, TGF-β4, and TGF-β5 (143). Three TGFβ isoforms, termed TGFβ1, TGFβ2 and TGFβ3, have been described in mammals, each encoded by distinct genes, and expressed in unique patterns *in vivo* (144). The conservation of the amino acid sequence of TGF-β1, TGF-β2, or TGF-β3 in different mammalian species is close to 100%, thus suggesting critical functions for these proteins throughout mammalian evolution (145).

TGF-β1 has been found in the highest concentration in human platelets and mammalian bone, but it is produced by many cells in smaller amounts (140). Antigen-specific T cells and activated macrophages produce both active and latent TGF-β1 (146). TGF-β2 is produced by many cell types and has been found in the highest concentration in porcine (but not human) platelets and mammalian bones. TGF-β3 has been detected in human, porcine, and avian sources, mainly in cells of mesenchymal origin, suggesting a different role for this protein than for TGF-β1 or β2 (140,141). TGF-β4 has been detected so far only in chick embryo chondrocytes and its

distribution in other types of cells has not yet been characterized (140,141). TGF-β5 has been detected in *Xenopus* embryos and adult tissues (140,141).

TGF-β1 is the best characterized protein of the TGF-β family of proteins. Murine TGF-β1 is synthesized as a 391 amino acid precursor polypeptide containing a hydrophobic signal sequence, pro-region and mature peptide (147). The biologically active factor which is generated by proteolytic processing, is a disulphide-linked homodimer, with each monomer representing the carboxy-terminal 112 amino acids of the precursor. The active TGF-β1 25kD homodimer is non-covalently associated with a precursor molecule (latency associated peptide, LAP) to form a 75 kD complex (147). In some cell types, such as platelets, TGF-β1 exists as a large latent complex, where the latent TGF-β1 binding protein (LTBP) is covalently linked to the LAP molecule (148). LTBP is important for the secretion of TGF-β1 by platelets. LTBP has been shown to associate with LAP during secretion from the cell, and if LTBP does not complex with LAP, TGF-β1 is secreted very slowly (148).

C.2.1.2 TGF-β1 receptor signaling

The biological functions of TGF-β1 are mediated by binding to cell surface receptors that, at present are incompletely characterized. Cross-linking studies with iodinated TGF-β1 show that most cells have three size classes of binding receptors. These are Type I (53 kDa), Type II (70-85 kDa), and Type III (250-350 kDa) (149). The Type III receptor has been identified as a chondroitin/heparin sulfate and beta-

glycan receptor that exists in both membrane-bound and soluble forms (150). Recent studies involved with the production of TGF-β-resistant mutants and restoration of sensitivity by complementation, has shown that Type I and Type II receptors are involved with mediation of biological responses to TGF-β1 (151,152). TGF-β1 binds to the Type II receptor, with subsequent recruitment of the Type I receptor (153). This Type I/II receptor complex then signals through an intracellular serine/threonine kinase domain on the Type II receptor, which phophorylates downstream substrates (154). These downstream effector molecules include the Smad cytoplasmic proteins which translocate to the nucleus following phosphorylation (155). The Type III (beta-glycan) receptor is apparently not capable of signal transduction (152).

All three major components of the TGF-β1 receptor complex have now been cloned, and studies are beginning on the expression of each of these components and on their interactions in reconstituted complexes (Type I, Type II, Type III). From these studies, a model for the interaction of TGF-β1 with its receptors is emerging. In this model, for cells expressing Type III receptor on their surfaces, TGF-β1 binds to betaglycan and is presented to the Type II receptor with the subsequent formation of a high-affinity complex consisting of TGF-β1 and the Type II/III receptors (156). The betaglycan is then displaced from this complex by the Type I receptor, and a high-affinity complex of TGF-β1, Type I/II receptors initiates signal transduction (156). Under these conditions, cells are equally responsive to TGF-β1 or TGF-β2. Alternatively, for cells lacking Type III receptor on their surfaces, a high-affinity,

signal-transducing complex of TGF-β1, Type I/II receptors form directly (153). Under these conditions, cells are much more responsive to TGF-β1 than to TGF-β2. Cellular responsiveness to TGF-β2 appears to be dependent on the presence of beta-glycan and the formation of a subset of receptors with high-affinity for TGF-β2 (157). This model provides explanations for equal activities of the TGF-β isoforms despite differential affinities for the Type I and Type II receptors and for the observed differential responses of some cells to TGF-β1 or TGF-β2.

X-ray crystallography and NMR analysis have been used to study the molecular structure of TGF-β1. These studies have revealed some unusual features of the structures of these isoforms of TGF-β and have suggested regions of these molecules that might contribute to the specificities in receptor binding previously observed (158). Recently, a 60 kDa type IV and 400 kDa Type V receptor have been identified on pituitary and transformed cells, respectively (159).

There are a few reports of other TGF-β-binding surface components (160). Latent or activated TGF-β binds to a variety of interstitial matrix components, including decorin and endoglobin (161,162). Circulating TGF-β binds to α-macroglobulin to form a high molecular weight inactive latent complex unable to bind cellular receptors, and is which is cleared within 3 minutes (163). Therefore, active TGF-β1 is rapidly eliminated from plasma and probably exerts only transient local effects. In addition, thrombospondin, fibronectin and type IV collagen also bind TGF-β1 (164-166). Thus, the cytokine is sequestered in the extracellular matrix, which may

serve as a reservoir, though these bound forms are usually inactive. The full significance of TGF-β1 interactions with the above-described proteins remains to be determined.

The high degree of sequence homology, along with the numerous redundant functions observed *in vitro*, have led to the possibility that TGF-β1, β2 and β3 may be functionally redundant *in vivo* (143). However, this might not be the case since gene ablation of each isoform produces a distinct phenotype (167,168). Although it is possible that each TGF-β isoform substitutes for another in a context-specific fashion, the occurrence of complete redundancy is unlikely due to the presence of distinct genes encoding for each, and by the unique pattern of expression *in vivo* (144). As described below, this is further confirmed by studies in TGF-β1 knockout mice.

C.2.1.3 Activation of latent TGF-β1

As described above, the three mammalian TGF-β isoforms are each initially synthesized as part of a larger precursor molecule containing the mature form of TGF-β. The two portions of the precursor are secreted as a biologically inactive, noncovalently-bound complex consisting of dimers of both LAP and mature TGF-β1 (169). For secretion to occur, TGF-β1 must be in its inactive latent form (170). Since latent TGF-β1 is not biologically functional, it must therefore be activated following secretion (141). Mature TGF-β1 can be released from the complex in an active form *in vitro* by a variety of treatments, most commonly acidification (171). Although several

mechanisms of activation have been proposed, the precise process of activation *in vivo* is not completely understood. Studies have suggested roles for extreme pH, free radicals, transglutaminase or platelet-derived thrombospondin in the mechanism of activation of latent TGF-β1 (reviewed in 155).

Strong experimental evidence support the notion that proteolytic cleavage of the LAP can cause the release of active TGF-β1 from the latent complex (172). It is known that LAP is glycosylated and that some of the carbohydrate chains contain mannose-6-phosphate (man-6-P). It has also been observed that latent TGF-β1 binds to the cation-dependent man-6-P/IGF-II receptor on the surface of many cells (173). Inhibition of *in vivo* activation of latent TGF-β1 by exogenous man-6-P, or antibodies against the man-6-P/IGF-II receptor, suggests that binding of the latent complex to this receptor is involved somehow in activation (173). In addition, TGF-β1 activation may also involve other molecules such as plasmin, cathepsin or other proteases, and may be mediated by macrophages in inflammatory sites (172,174). Whatever the physiological mechanism for activation may be, it represents an important control step for the regulation and localization of the effects of TGF-β1.

C.2.2 Immunoregulatory functions

In the immune system, the actions of TGF-β1 are highly pleiotropic (reviewed extensively in 140 and 175). While specific receptors for this protein have been found on almost all mammalian cells examined, the nature of the response depends on a variety of conditions, including the cell type, growth conditions, the state of cell differentiation, and the presence of other growth factors or cytokines (140,175). The effects of TGF-β1 on proliferation, differentiation and function may be stimulatory or inhibitory, depending on these conditions. Therefore, TGF-β1 functions as a complex regulatory molecule whose biological roles are "context-dependent" (175).

TGF-β1's anti-proliferative nature suggests that this molecule has profound effects on the cell cycle (reviewed in 176). Inhibition of proliferation is a complex process which involves an obstruction of the phosphorylation of the retinoblastoma tumor suppressor protein (177). Depending on the cell type, TGF-β1 can downregulate the activity of several G1 cyclins and associated cyclin-dependent kinases (177) . Recent findings suggest that these inhibitory events are initiated by TGF-β1-induced expression of $p15^{Ink4B}$ and $p21^{Cip1}$ cyclin-dependent kinase inhibitors (176,177). Generally, TGF-β1 is stimulatory for cells of mesenchymal origin, and inhibitory for cells of epithelial or hematopoetic origin (176). TGF-β1 also has tumor suppressor functions, since the loss of expression of the TGF-β1 Type II receptor correlates with

susceptibility of malignant transformations in both hematopoetic and epithelial cells (178).

TGF-β1 has important downregulatory functions on the growth, differentiation and activities of a number of different cell types involved in specific and non-specific immunity (reviewed in 175). Its unique capacity to stimulate its own production by an autoinductive mechanism and almost exclusive production of the β1 isoform by immune cells, such as activated T cells and macrophages, suggests critical roles in hematopoiesis and immune function (175,179,180). Generally, TGF-β1 is considered to be the most immunosuppressive of all cytokines, and potent inhibitory effects have been reported on B and CD4+ T lymphocyte proliferation, cytotoxic CD8+ T lymphocytes, NK cells, LAK cells, and macrophages (reviewed extensively in 140, 141, and 175). In T cells, it suppresses Th1 cytokine production such as IFNγ, TNF-α and IL-2 (181). Moreover, TGF-β1 blocks Jak-Stat signaling in T cells by preventing tyrosine phosphorylation and activation of Jak-1, Stat-5 and Tyk-2 (182). It inhibits IL-2R (182) and IL-12R (183) expression, and can induce apoptosis in T cells (182). In macrophages, it inhibits inducible iNOS activity (184), antagonizes the activities of TNF-α and IFN-γ (185), suppresses the production of NO and superoxide (186,187), and alters costimulatory molecule expression (183,188). In addition, TGF-β1 has downregulatory effects on MHC class I and II antigen expression on a variety of cell types including B cells and macrophages (189). TGF-β1 is an inhibitor of the secretion of IgG and IgM by B lymphocytes (175,181). TGF-β1 can also alter the expression of

various adhesion molecules, such as E-selectin, and thus interferes with the adhesion of neutrophils and lymphocytes to the vascular endothelial cells (190,191). In this regard, TGF-β1 is an "anti-cytokine" and may trigger the suppression of immune responses (175).

Although TGF-β1 is largely a negative regulator of inflammatory responses, it may have some stimulatory effects as well. TGF-β1 is a potent chemoattractant for macrophages/monocytes, neutrophils and lymphocytes during the recruitment or activation phase of the immune response (192-194). TGF-β1 can also enhance the growth of naïve T cells (195), and influence the differentiation of dendritic cells (196). In addition, in cooperation with IL-2 and IL-5, it upregulates IgA secretion by activated B lymphocytes (181). The biphasic nature of TGF-β1, functioning as a chemoattractant and inducer of cytokine production in the early stages of the inflammatory response, while suppressing these events during the resolution of an immune response, is a distinct characteristic of this cytokine (140,175).

The potent immunosuppressive effects of TGF-β1 is most clearly demonstrated by the study of Shull and co-workers (197) who developed TGF-β1 knock-out mice. Animals homozygous for the mutated TGF-β1 allele (TGF-β1 -/-) die, within 3-4 weeks, with a multi-organ inflammatory syndrome involving the heart, lungs, liver, stomach, pancreas, brain, eyes and other tissues. In these mice, the inflammatory infiltrate is perivascular in nature and varies from primarily neutrophilic (stomach) to primarily lymphocytic (brain), with various mixtures in some organs. Additional

evidence for increased leukocyte/endothelial cell interaction in the phenotype of TGF-β1 -/- mice came from studies where either synthetic fibronectin peptides (198) or anti-LFA antibody treatment (199) reduced leukocytic infiltration and tissue damage, while significantly prolonging the survival of these mice. The absence of functional TGF-β1 is also associated with increased mRNA expression of several pro-inflammatory cytokines (197), including IL-2, IFNγ, TNFα, MIP-1α, and IL-1β. Furthermore, the TGF-β1 null mutation is associated with increased numbers of activated and proliferating cells in lymphoid organs, as well as an increased production MHC class I and II antigens (197). These findings provide strong evidence that TGF-β1 suppresses many aspects of inflammatory responses.

Studies from the TGF-β1 knockout mice have intimated critical roles for TGF-β1 in autoimmunity and immune tolerance. TGF-β1 is known to influence thymocyte differentiation by inhibiting the progression and differentiation of $CD4^{-}CD8^{low}$ precursor thymocytes into CD4+CD8+ double-positive thymocytes (94,95,200). TGF-β1 may control the rate at which CD4+CD8+ thymocytes are generated, and thus limit subsequent TCR selection events on cortical thymic epithelia. It is believed that in the absence of TGF-β1, double-positive thymocytes are produced too rapidly for autoreactive T cells to be efficiently deleted in the thymus (175). Thus, autoreactive T cells escape selection events and enter peripheral tissues, where they may be activated and cause disease.

The aberrant expression of MHC class I and II molecules in peripheral tissues of TGF-β1 -/- mice may also contribute to the autoimmune phenotype of these mice (197). TGF-β1 is a potent negative regulator of MHC class II antigen expression and this is consistent with its overexpression in these mice (189,197). Therefore, in the absence of TGF-β1, MHC class II antigens are permitted to be expressed on non-professional APCs such that inappropriate presentation of autoantigens may occur. In fact, increased expression of MHC class II antigens is often observed in a variety of animal models of autoimmune disease, and susceptibility to these diseases is frequently associated with particular MHC class II haplotypes (125,201).

The immunoregulatory influence of TGF-β1 has been studied in many *in vivo* models of autoimmunity including EAE (202-204), IDDM in NOD mice (86,205,206), SLE (84), and CIA (204). In some of these models, systemically administered purified TGF-β1 proved to be therapeutically effective at suppressing autoimmune responses and reduce the severity of histopathological lesions (203,204). TGF-β1 expression correlates with disease recovery/remission, while its production appears to be absent during active phases of inflammation (119,125,207,208). Evidence for an endogenous regulatory role of TGF-β1 came from studies where systemic delivery of anti-TGF-β1 mAbs reduced the severity of autoimmune disease expression (202). Furthermore, recent experiments have established a role for TGF-β1 in the induction of immune tolerance to target antigens. For example, T cells isolated from MBP-immunized mice orally tolerized to EAE and non-diabetic NOD mice have been shown to secrete TGF-

β1 (120-122,205,206,209). Interestingly, CD45RB^{high} CD4+ T cells (Th1-like cell population) transferred into SCID mice induces chronic inflammatory bowel disease (124). This disease was dependent on IFN-γ and TNF-α, and could be inhibited by co-transfer with CD45RB^{low} CD4+ T cells (Th2-like TGF-β1 producing T cell population) (124). It is interesting that TGF-β1 has a profound effect on the *in vitro* development of antigen-activated Th cells, leading to the development of the CD45RB ^{low} phenotype (124). In a model of parasitic infection, TGF-β1 administration inhibited IL-12 and IFN-γ production (Th1 response), thus rendering susceptible mice resistant to *T. gondii* (210). The inflammatory wasting syndrome that develops 3 weeks postnatally in TGF-β1-/- mice is ameliorated in TGF-β1-/-/SCID mice and in TGF-β1-/-/MHC class II-/- double knockout mice (211). Thus, CD4+ T cells appear to mediate much of the disease manifested in TGF-β1-/- mice. Supporting this notion, administration of anti-CD4 mAb improves survival in these mice (211). Although IL-4-driven Th2 responses can also be suppressed, it is generally believed that in the absence of functional endogenous TGF-β1, the equilibrium between Th1/Th2 cells may be disturbed, and Th1 responses may be allowed to expand uncontrollably (175,211). Thus, autoreactive T cell, which have escaped central selection mechanisms may be suppressed in peripheral tissues by means of TGF-β1.

It was initially demonstrated that TGF-β1 could play a role in Th1 cell development, but its mechanism of action remained unclear, particularly since this factor could also inhibit IFN-γ producing T cell clones (212). The ultimate effect of

TGF-β1 in these cultures depended on the genetic background of the mice and the amount of IL-2 used in the priming conditions (212). In other systems, TGF-β1, as well as IL-12 and IFN-α, produced by macrophages and B cells, particularly in reponse to intracellular bacteria, have been shown to play an important role in the induction of Th1 cells (213). It is becoming more clear that TGF-β1 may promote Th2 differentiation. Systemic administration of TGF-β1 in mice infected with *L. amazonis* led to increased production of IL-4 and decreased systhesis of IFN-γ (214). Previous studies demonstrated that TGF-β (β1 or β2) can modulate macrophage activity in a way that favors Th2 over Th1 differentiation by blocking IL-12 production and CD40 expression (215). TGF-β1 may also influence Th2 development by the attenuation of IL-12Rβ2 expression (183) and IL-12 signal transduction (216), which ultimately reduces IL-12 responsiveness in T cells (188). This results in decreased T cell proliferation and IL-12-induced IFN-γ production, increased T cell apoptosis, and suppressed Th1-mediated responses (183,188). Another group of investigators have recently shown that TGF-β1 alters APC function in NOD mice and consequently polarizes islet-antigen responses toward a Th2 phenotype (217).

A considerable effort is being deployed to identifiy the cytokine factors which appear to regulate endogenous TGF-β1 production *in vivo*. Seder and co-workers found that TGF-β1 producing T cells differentiate under priming conditions favoring induction of Th2 (IL-4) and inhibition of Th1 responses (IL-12 and IFN-γ) (218). This study was corroborated by the fact that primed CD4+ T cells from IL-4 -/- mice

produced less TGF-β1, while cells from IFN-γ -/- mice produced more TGF-β1, when compared to wild-type cells (124). In a model of oral tolerance, high doses of antigen, in contrast to low doses which promotes TGF-β1 production, leads to the elaboration of IFN-γ and IL-12 by Peyer's patch (PP) T cells. In this model, antibodies to IL-12 appears to augment TGF-β1 secretion and T cell apoptosis (219). This suggests that IL-12 and TGF-β1 are counter-regulatory cytokines involved in peripheral tolerance. Consequently, IL-12 inhibits the development of TGF-β1 secreting cells, and *vice versa* (218). Therefore, in the presence of TGF-β1, autoreactive Th1 cells are less prone to get activated, proliferate, and survive, thus allowing regulatory T cells (Th2 or Th3) to actively maintain tolerance. The above-described studies have important implications for the therapy of Th1-mediated autoimmune diseases, since inhibition of Th1 cell responses may not only interfere with the disease process, but also indirectly suppress disease by means of TGF-β1 production.

In recent years, several lines of evidence have supported an important role for circulating TGF-β1 in the mediation of its effects. In a model of SLE, the MRL/lpr autoimmunity-prone mouse, excess TGF-β1 in the circulation was found to dampen systemic immune responses (220). Interestingly, delivery of maternal TGF-β1 to homozygous TGF-β1 -/- mice resulted in a systemic supply of this cytokine capable of restoring growth and development (221). Exhaustion of this maternal TGF-β1 supply resulted in excessive inflammatory responses and subsequent premature death (221).

The biological functions of TGF-β1 depend highly on the contextual nature of its expression, secretion and activation. Wyss-Coray and co-workers (222) have recently demonstrated that mice overexpressing bioactive TGF-β1 within the CNS parenchyma, show an earlier onset of clinical symptoms, more severe disease, and an enhanced mononuclear infiltration in the CNS. This data is not consistent with the anti-inflammatory nature of TGF-β1 and many reasons may account for this discrepancy. Local overproduction of active TGF-β1 may cause activation of fibroblasts with a resulting excessive elaboration of extracellular matrix components (192,203). In contrast, circulating TGF-β1 (systemic) interacts preferentially with endothelial cells with a resulting reduction of adhesiveness for immune cells (192). This apparent difference between the preferred cellular targets for circulating versus local TGF-β1 emphasizes the contextual nature of TGF-β1 action and suggests that cellular responsiveness is defined not only by the presence of cell surface receptors, but may depend on whether TGF-β1 exposure is either local or systemic (192).

In summary, there is extensive literature suggesting that TGF-β1 plays a critical role in the mediation of a number of immune responses. Due to TGF-β1's many potential systemic effects, it is obvious that its synthesis, secretion, activation, and clearance must be tightly regulated, and that defects in this regulation may provoke the production of autoimmune pathological conditions.

D. Experimental models of autoimmune disease

Several clinical and experimental observations suggest that autoimmune diseases develop as a result of abnormalities in the immune response mediated by T cells and T cell-derived cytokines (119,201,223-225). Evidence is now accumulating in both experimental animal models and human pathological conditions to suggest that the relative contribution of either Th1- or Th2- dominated reactions can determine the development of a particular autoimmune response (125). In most cases, it appears that organ-specific autoimmune disease develops as a consequence of the development of self-reactive CD4+ Th1 cells (reviewed in 201).

There are several animal models of naturally occurring or autoantigen-induced organ-specific autoimmune diseases. Currently, those attracting the most attention include the non-obese diabetic mouse (NOD) and experimental allergic encephalomyelitis (EAE). In both of these experimental models, autoimmune disease manifestation is determined by the relative contribution of CD4+ T cell subsets in the induction and prevention of disease (119,125,201) .

D.1 Autoimmune diabetes and the NOD mouse

Type 1 insulin-dependent diabetes mellitus is an autoimmune disease, and both genetic and environmental factors strongly contribute to predisposition toward

this disease (226). The study of IDDM has been greatly facilitated through the use of the NOD mouse, which serves as an experimental animal model. IDDM in this model shares many characteristics with the human equivalent of the disease (227,228), including the polygenic influence as suggested by the inheritance of particular MHC class II alleles and multiple non-MHC loci as genetic risk factors; the transmission of disease by bone-marrow stem cells and splenocytes; the early appearance of an intrapancreatic inflammation (see below) and anti-islet cell antibodies; and the autoreactive T cell dependence of disease development and the capacity to intervene with disease activity by modulating T cell functions.

In 1980, the NOD model of spontaneous diabetes was originally developed by selective inbreeding from a subline of the ICR mouse strain (229). IDDM develops predominantly in female NOD mice and 80-90% of NOD mice become hyperglycemic by 30 weeks of age, depending on the mouse colony (228). This may due to local differences that have been attributed to viruses and diet. Indeed, infection with viruses such as the lymphocytic choriomeningitis virus has been shown to prevent diabetes in NOD mice (226). Diabetes also occurs in male mice but at a lower incidence (10-40%) (228). This pronounced, increased disease activity observed in female NOD mice is not seen in humans (228).

Compelling experimental evidence suggests that autoimmune diabetes in NOD mice is mediated by T cells (227,228). In particular, IDDM is prevented by neonatal thymectomy, by immunosuppressive agents that target T cells and by anti-

CD4 and anti-CD8 mAb treatments (199). In addition, autoimmune diabetes can be transferred to NOD-SCID or neonatal mice by CD4+ or CD8+ T cells isolated from spontaneously diabetic adult NOD mice (230). Cooperation between CD4+ and CD8+ T cells is required to initiate disease, and β islet cell destruction is CD4+ T cell dependent (231). The role of ICA in the development of IDDM in NOD mice is unclear. If autoantibody production is blocked in these mice, the incidence of IDDM remains unchanged. This indicates that T cells but not B cells play a critical role β-cell destruction (232). However, a recent study suggests that B cells may likely function as APCs (233).

The development of IDDM in the NOD mouse is preceded by an inflammatory lesion, termed insulitis (228). The cellularity of these infiltrates is characterized with the early arrival of dendritic cells and macrophages, and then by CD4+ and CD8+ T cells and B cells (226). This leukocytic infiltration occurs about the perivascular duct and peri-islet areas of the pancreatic β-islet cell of Langerhans beginning at 3-4 weeks of ages (228). This peri-insulitis is then followed by a progressive, slowly-evolving T-mediated destruction of the insulin-producing β islet cells by 20-30 weeks of age. These mice also have evidence of thyroiditis and sialadenitis, which may also be found with human IDDM (228).

The pathogenesis of IDDM in NOD mice involves complex interactions between several immune mechanisms in which β islet cells are destroyed by cellular and molecular effector mechanisms that are not completely understood. However, a

consensus view suggests that a dysregulation of the immune system is a critical factor. An important characteristic of the development of IDDM in NOD mice is that although a non-destructive insulitis (peri-insulitis) is seen in all NOD mice, only a fraction of these mice develop a destructive insulitis and IDDM (226,228). It appears that imbalance between pathogenic and immunoregulatory T cells determines the onset and progression of autoimmune disease in NOD mice (228).

NOD mice lack immunoregulatory T cells. Evidence supporting this notion came from studies showing that thymocytes from prediabetic NOD mice block disease transfer by T cells from diabetic mice, whereas thymocytes from diabetic NOD mice lack that suppressive function (231). TCR ligation of T cells or thymocytes from 3-5 week old mice induces a state of proliferative hyporesponsiveness which is mediated by a reduced IL-2 and IL-4 production (232,233). This hyporesponsiveness occurs in both CD4+ and CD8+ T cells from both male and female NOD mice (232). Anti-CD3 mAb or mitogen (PHA) stimulated T cells from new onset IDDM in humans also results in a decreased IL-4 production (234). Recent findings suggest the model that autoimmune diabetes in NOD mice results from an excessive Th1 response to β-islet cell antigens, perhaps as a direct consequence of defective regulatory Th2 cells (228,233). The balance of Th1 and Th2 CD4+ T cells seems to be decisive in determining whether autoimmune T cell reactivity will result in IDDM. In fact, recently it was shown that IL-4 expression at the onset of islet inflammation predicts non-destructive insulitis in

male NOD mice, whereas IFN-γ expression predicts destructive insulitis in NOD females (235). IL-4 restores TCR-dependent T cell proliferative responsiveness *in vitro* and prevents insulitis and IDDM when administered *in vivo* to prediabetic NOD mice (236). NOD mice transgenically expressing IL-4 in β islet cells also display a significantly reduced incidence in IDDM, paralleled with a restored T cell proliferative responsiveness (237). In addition, prevention from insulitis and diabetes is mediated by the modulation of homing of autoreactive T cells to the pancreas and the potentiation of regulatory Th2 cell function in the thymus, spleen and β islets (238). T cells from IL-4 treated NOD mice reduce and delay IDDM onset in NOD-SCID recipients (231). The proliferative hyporesponsiveness of regulatory Th2 cells in NOD mice may favor a Th1 environment in the pancreas and consequently lead to a loss of T cell tolerance to β islet autoantigens, including insulin, glutamic acid decarboxylase 65 (GAD65), carboxypeptidase and I-A2 tyrosine phosphatase (reviewed in 228).

A considerable amount of evidence supports the pathogenic role of Th1 cells and related cytokines in IDDM (239-243). In an islet antigen specific TCR trangenic model, Th1 cell transfer developed diabetes rapidly, whereas Th2 cells did not promote or induce diabetes in recipient mice (243). Furthermore, trangenic mice selectively expressing Th1 (IFN-γ) or Th2 (IL-4) cytokines in the β islets either promoted or prevented the onset of IDDM, respectively (reviewed in 244). While IL-12 administration provokes disease in NOD mice, diabetes is prevented with IL-12

antagonists (201,225,245). Interestingly, prophylactic treatment with an anti-IFN-γ mAb prevented spontaneous autoimmune disease, while IFN-γ gene deletion delayed disease in NOD mice (reviewed in 201).

D.2 Experimental allergic encephalomyelitis (EAE)

Multiple sclerosis (MS) is the most common autoimmune disease of the CNS (246). It is hall-marked by localized areas of demyelination and progresses in either a chronic/progressive or relapsing/remitting mode. In the United States, approximately 250,000 people are afflicted with this disease. MS preferentially affects females (2:1 over men) and usually begins between the ages of 20-40. The ethiology is not completely clear but both genetic and environmental factors appear to be involved (247).

EAE serves as a good experimental model for MS. EAE is an inflammatory, autoimmune disease of the central nervous system (CNS) sharing many clinical and pathological features with MS in humans (248). EAE is characterized by perivascular leukocytic infiltration (CD4+ T cells and macrophages) and demyelination of the CNS, with an acute or relapsing and remitting mode of progression (246). EAE can be induced in mice by immunization with either whole brain homogenate, purified myelin basic protein (MBP), or proteolipid protein (PLP) (248). The target of the autoimmune inflammatory response is the myelin sheath that envelops axons, as well as the

oligodendrocytes that produce the myelin (248). In mice and rats, paralysis of the hind legs begins 2 to 3 weeks after challenge. Within the brain, perivascular infiltration of inflammatory cells precedes the clinical signs (246,248,249).

EAE is a genetically controlled, T cell mediated autoimmune disease. Encephalitogenic T cells are I-A restricted (H-2^s in SJL/J mice) and CD4+, and they initiate the inflammatory lesions by recruiting both CD4+ and CD8+ effector cells to the brain (250). Astrocytes and oligodendrocytes express MBP on their surface and can express MHC class I and II Antigens by cytokines secreted by infiltrating macrophages and T cells (251). Endothelial cells of cerebral blood vessels also express MHC class II molecules (251). Treatment of mice with antibodies to MHC, CD3 or CD4 can prevent the induction of disease or even reverse established disease (reviewed in 252).

There is compelling evidence for a role of proinflammatory cytokines produced by Th1 cells and macrophages in the pathogenesis of EAE (252,253). MBP-specific T cell lines that adoptively transfer disease in mice produce the Th1 cytokines IFNγ, IL-2, TNFα and β, and these cytokines are also present in the CNS of animals with active disease (207,254). Conversely, spontaneous recovery of mice from EAE correlates with IL-4, IL-10 and TGF-β1 production, and with the development and expansion of CNS antigen-specific Th2 cells (125,253). Treatment of mice with IL-4, IL-10, IL-13 or TGF-β1 ameliorate inflammatory responses in EAE by either downregulating the encephalitogenic Th1 response, or inducing the development of protective Th2 or Th3

cells (204,248,255,256, and reviewed in 257). In accordance with these findings, mAbs directed against TGF-β1, IL-10, and IL-4 increase the encephalitogenicity of T cells and ultimately exacerbate EAE (202,224,258). Interestingly, orally administered autoantigens have been shown to tolerize mice to the induction of EAE (120,121,209). In this model, the mechanism of oral tolerance depends on the dose administered with lower doses favoring the induction of regulatory Th3 cells via the secretion of anti-inflammatory cytokines such as TGF-β1, IL-10 and IL-4 and mediate their effects by means of bystander suppression (120,121,209).

IL-12, a product of activated macrophages, has a determining role in the development of EAE effector T cells. The adoptive transfer of T cells cultured *in vitro* with MBP or PLP and IL-12 results in a more severe disease than that induced when T cells were cultured with antigen alone (259). In an adoptive transfer model of EAE, administration of IL-12 increased the severity and accelerated the progression of disease (225), while mAb against IL-12 completely prevented paralysis in mice (259). EAE cannot be induced in IL-12 deficient mice (260) and disease still occurs in IFN-γ deficient mice (261). Remarkably, a recent study demonstrated that IL-12 can unmask a latent (asymptomatic) form of autoimmunity in mice genetically resistant to EAE (262). These findings provide support for a critical role of IL-12 in the establishment of EAE T cell effector functions.

The pathogenic role of IFN-γ and/or TNF-α secreting T cells in the pathogenesis of EAE has been firmly established. As described above, IFN-γ and

TNF-α production is elevated at the height of active disease and either low or undetectable during periods of remission. Highly encephalitogenic T cells were generated when cultured in the presence of either TNF-α or IFN-γ and MBP (254). Transgenic mice expressing IFN-γ or TNF-α in the CNS have provided additional evidence for a direct and disease-promoting role of IFN-γ and TNF-α in EAE (263,264). Conversely, incubating T cells with either anti-IFN-γ or anti-TNF-α, in the presence of MBP, resulted in a drastically reduced encephalitogenic potential (254,265). Furthermore, attempts to interfere with the activity of TNF-α *in vivo* by using neutralizing antibodies, soluble TNF receptors or type 1 phosphodiesterase inhibitors lead to the prevention of EAE, while injection of TNF-α triggers relapses (254,265, and reviewed in 257). Despite this clear correlation between IFN-γ/TNF-α and disease progression, recent studies have suggested paradoxical roles for these cytokines in EAE. For example, antibodies against IFN-γ did not attenuate disease development (266), while disruption of the IFN-γ or TNF-α gene renders mice more susceptible to the induction of EAE (267). The reasons for these contradictory results are unclear, but IFN-γ and TNF-α may have different effects at afferent and efferent stages of the autoimmune process (266).

E. Hypothesis, rationale and objectives of research

Although central mechanisms are important for the establishment of T cell tolerance, autoreactive T cells can escape thymic selection, and persist in peripheral compartments (94). As described above, numerous clinical and experimental findings suggest that organ-specific autoimmune diseases develop as a result of an abnormal expansion of autoreactive/autoaggressive T cells with subsequent tissue destruction (201). Compelling evidence in both experimental animal models and human disease conditions suggests that the relative contribution of either CD4+ Th1 or Th2 cell subsets can determine either resistance or susceptibility to the onset of autoimmune disease (125). In most cases, organ-specific autoimmune disease results from the aberrant activation of autoreactive CD4+ Th1 cells and excessive elaboration of proinflammatory type 1 cytokines (125,201). Furthermore, the development and expansion of CD4+ Th2- or Th3-like responses can maintain tolerance to autoantigens (120-122). Therefore, suppressing the pathogenic activity of Th1 cells is likely to be therapeutically effective in these diseases.

Cytokine immunotherapy can affect autoimmune disease outcome by altering the balance between Th1 and Th2 cell activity or proinflammatory versus immunosuppressive cytokine profiles (218). In our studies, our experimental aim was to use somatic cytokine gene therapy, by means of direct i.m. injection of cytokine

plasmid vectors, as a novel method of cytokine delivery *in vivo*. We reasoned that somatic cytokine gene therapy could be an effective alternative to cytokine immunotherapy of autoimmune diseases.

In an attempt to test the applicability of i.m. cytokine gene therapy, we decided to study the immunoregulatory effects of TGF-β1 and IL-4 *in vivo* in two experimental models of Th1 cell-mediated autoimmune diseases: murine autoimmune diabetes (Chapter III) and EAE (Chapter IV). As described elsewhere, anti-inflammatory cytokines such as TGF-β1 and IL-4 can protect from autoimmune diseases. We hypothesize that i.m. injection of cytokine plasmid vectors would result in sufficient production of circulating cytokine to prevent autoimmune disease expression in these models by modulating T cell functions and endogenous cytokine production.

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Chapter II

Factors influencing foreign gene expression following injection of plasmid DNA into mouse skeletal muscle

by

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Abstract

Direct intramuscular (i.m.) injection of naked plasmid DNA is a novel and promising approach for the expression of a wide variety of proteins for the purpose of vaccination and therapeutic protein delivery. However, relatively little is known about the factors which limit the magnitude and duration of transgene expression in mouse skeletal muscle. In this study, we sought to elucidate the underlying factors which appear to influence reporter gene expression following i.m. injection of a luciferase-encoding plasmid DNA vector (pVR1255, VICAL Inc.). We demonstrate that i.m. injection of pVR1255 results in detectable luciferase expression by 24 hours post-injection, which rapidly rises to peak levels at 7 days, declines thereafter, and persists at a low level for at least 6 months. Similar experiments in severe-combined immunodeficient (SCID) mice result in peak day 7 levels and persistent high reporter gene expression for several months. Since immune responses to the transgene product appear to dramatically influence the stability of gene expression, we co-delivered the reporter gene with a plasmid expression vector encoding an immunosuppressive protein, TGF-β1. Co-injection of luciferase and TGF-β1 plasmid vectors greatly improves reporter gene expression levels. We also demonstrate that other factors such as age, gender, muscle type and muscle regeneration could influence peak level foreign gene expression.

In summary, this study demonstrates that the immune system is the major factor influencing prolonged foreign gene expression following plasmid DNA injection in

mouse skeletal muscle. These findings have important implications on the use of direct i.m. plasmid injection for the expression of foreign genes in the treatment of genetic and acquired diseases.

Introduction

Direct injection of naked plasmid DNA into skeletal muscle has proven to be an effective means of gene delivery *in vivo* (1,2). Previous studies by Wolff et al. (1) showed that plasmid DNA injected directly into skeletal muscles can be taken up by muscle cells adjacent to the site of injection and expressed for prolonged periods of time. This mode of gene transfer has encountered some success in the systemic delivery of therapeutic proteins (Chapter III, 3-5) and expression of foreign proteins for vaccination purposes (6).

As the potential use of naked plasmid DNA-mediated gene transfer becomes increasingly apparent, it is important to address some of the potential drawbacks of this gene delivery approach. Despite the apparent persistence in reporter gene expression in skeletal muscle (at least 19 months)(1), a substantial decrease in this expression is observed by 30 days post-injection. This decline in expression has been consistently observed with many heterologous reporter proteins, irrespective of the plasmid DNA construct used (Chapter III, 3,7-9). Many factors have been suggested to account for this lack of stability in foreign gene expression in skeletal muscle (8-10). In this study, we sought out to identify the factors which could influence this decline in foreign gene expression.

In mice, luciferase is a foreign antigenic protein to which host immune responses could be directed. This immune response may injure or kill transfected cells, prevent reporter gene expression and limit readministration of foreign protein-

encoding plasmid DNA vectors (10). Here, we demonstrate that i.m. injection of a luciferase DNA vector results in detectable luciferase expression by 24 hours post-injection, which rapidly declined by day 21 post-injection. Similar experiments in SCID mice resulted in day 7 peak levels that were sustained for the entire duration of the experiment. Since immune responses to the transgene product appeared to suppress luciferase levels, we then hypothesized that co-expression of luciferase with TGF-β1, an immunosuppressive cytokine, could alter the kinetics of reporter gene expression in skeletal muscle. Co-injection of pVR1255 and pVR-TGF-β1 greatly improved reporter gene expression levels. We also report that gender, age, muscle type and muscle state are important determinants limiting the magnitude of transgene expression.

Materials and Methods

Mice

BALB/c, CD-1 and BALB/c-SCID mice were obtained from Charles River (St-Constant, Quebec, Canada) and kept in a pathogen-free facility.

Plasmids

All firefly (*Photinus pyralis)* luciferase expression experiments were done using pVR1255 (VICAL Inc. San Diego, CA) (11). The Lux (Luc+) gene is under the transcriptional control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter, and downstream of the human CMV intron A. Transcription is terminated by the minimal rabbit β-globin terminator. Luc+ encodes a modified luciferase which preferentially localizes in the cytoplasm. pVR1255 has been shown to be far superior (over 150 times) to several other plasmid vectors for direct injection of naked plasmid DNA (11). A plasmid map of pVR1255 is shown in figure 1.

The mouse TGF-ß1 cDNA was previously isolated by RT-PCR (Chapter III, 3) and subcloned into compatible enzyme restriction sites of pVR1255, in place of the Luc+ gene, to generate pVR-TGF-ß1 which encodes the latent form of mTGF-β1. Expression was confirmed in supernatants collected from transiently transfected COS-7 cells. The latent TGF-β1 was activated by acidification, and detected by ELISA

(R&D, Minneapolis, MN), and its bioactivity was confirmed by the CCL64 mink cell line proliferation assay. Both the ELISA and bioassay detect only active TGF-β1.

Plasmid DNA Preparation

Large-scale plasmid DNA preparations were produced by the alkaline lysis method using a Qiagen giga kit (Qiagen Inc, Santa Clarita, Ca). All plasmid preparations for i.m. injections were resuspended in sterile 0.85% saline. Spectrophotometric analysis revealed 260/280 nm ratios to be 1.80 or higher. Plasmid DNA preparations were free of visible bacterial RNA or genomic DNA, as visualized on a 1% agarose gel. Gel electrophoresis was also used to confirm the percentage of supercoiled plasmid (greater than 95%). Plasmid identity was determined by restriction endonuclease analysis.

Intramuscular injection of plasmid DNA

Intramuscular injections of plasmid DNA were done as described (Chapter III, 3). Briefly, mice were anaesthetized by i.p. injection with xylazine (10mg/kg) and ketamine (200 mg/kg). The tibialis anterior (TA) muscles of each mouse were injected with a 0.5 cc sterile 29G1/2 insulin syringe, fitted with a plastic collar to limit needle penetration to 2 mm. Mice received 50 μg of pVR1255 in 50μl of sterile saline in each TA muscle, for a total of 100 μg of plasmid DNA per mouse.

Extraction of luciferase from skeletal muscles

Mice were killed at various time points post-injection in a CO_2 chamber. The entire tibialis anterior muscle was excised from each leg and immediately put in an ice-cooled 1.5 ml microcentrifuge tube. Tissue samples were then quickly stored at -80°C until needed. Frozen muscles were placed in frozen lysis reagent (Promega Cell Culture Lysis Reagent, Promega, Madison, WI), cut into small pieces with sterile scissors and completely homogenized with a hand-held tissue grinder. The entire homogenization process was conducted on ice. Sterile Milli-Q water was added to the mixture, vortexed briefly and centrifuged for 30 minutes at 4°C. The upper phase was transferred to a clean sterile 1.5 ml microcentrifuge tube and stored at -80°C until assayed.

Luciferase assays

Total luciferase activity was determined using a commercial kit by Promega. Briefly, one hundred microliters of luciferase substrate (Promega, Madison, WI) was added to fifty microliters of muscle extract, properly mixed and quickly placed in a luminometer. Light units were recorded many times within 10 seconds after addition of the substrate. The total luciferase content of the muscle sample was determined from relative light units using a standard curve of purified luciferase (Analytical Luminescence Labs, San Diego, CA) which was diluted in muscle extract from uninjected muscles.

Statistical analysis

All statistical analyses were done using the Student's t test.

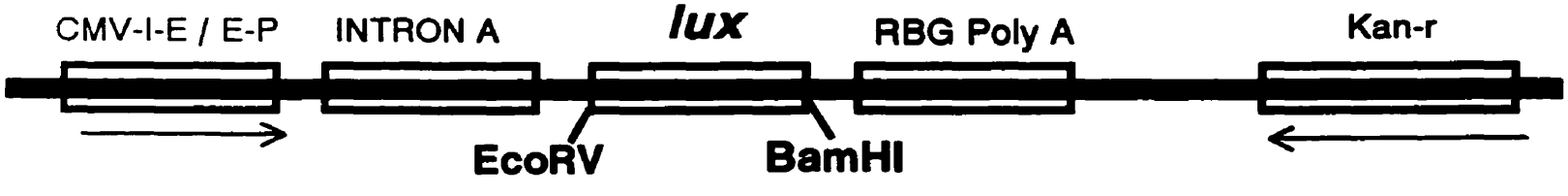
Results

DNA-dose dependent luciferase expression following i.m. injection

In order to determine the optimal dose of plasmid DNA to be given per injection site, increasing amounts of pVR1255 were administered into mouse TA muscles. Luciferase gene expression was measured 7 days following plasmid injection. Muscle luciferase expression increased with the amount of DNA injected, up to 100 μg, after which expression began to plateau and decline (Fig. 2). At 7 days post-injection, reporter gene expression ranged from 125 ng of luciferase/muscle when 10 μg of pVR1255 was given, to about 360 ng of luciferase/muscle when 50 μg of DNA was injected. Luciferase expression increased to about 420 ng of luciferase/muscle when 100 μg of pVR1255 was injected. Beyond 200 μg of plasmid DNA, luciferase expression declined (data not shown). Luciferase expression levels reported here are similar to those reported in previous studies (11).

Figure 1 Schematic diagram of the luciferase expression vector pVR1255.

pVR1255 6.4 kB



Effect of age and sex on reporter gene expression

We administered 50 μg of pVR1255 in each TA muscle of male and female CD-1 mice, at an age ranging from 3 weeks to 6 months. TA muscles were removed on day 7 post-injection and total luciferase activity was assayed. Three week old males and females, and 10 week old females displayed similar luciferase expression patterns (Fig.3). Interestingly, 10 week old males and 6 month old females expressed luciferase at higher levels compared to other age/sex groups ($p<0.005$). Mice of both sexes, at all ages, showed declines in reporter gene expression between days 14 and 21 post-injection (data not shown).

Effect of muscle group on reporter gene expression

TA and RF muscle groups are the most frequently targeted skeletal muscle tissues by naked plasmid DNA injection protocols. In order to determine whether these muscles provide similar plasmid uptake and expression potentials, TA and RF muscles were injected with 50 μg of pVR1255 and tissue extracts were assayed for luciferase enzyme activity 7 days post-injection. Remarkably, TA muscles consistently generated more luciferase than its RF counterpart (Fig.4). We find that TA muscles produce about 10-fold more enzyme than RF muscles.

Figure 2 Luciferase expression in skeletal muscle is a DNA dose-dependent process. Female BALB/c mice (4-6 weeks, n=8 per group) were injected i.m. with increasing amounts of pVR1255. Luciferase expression was measured 7 days following plasmid injection.

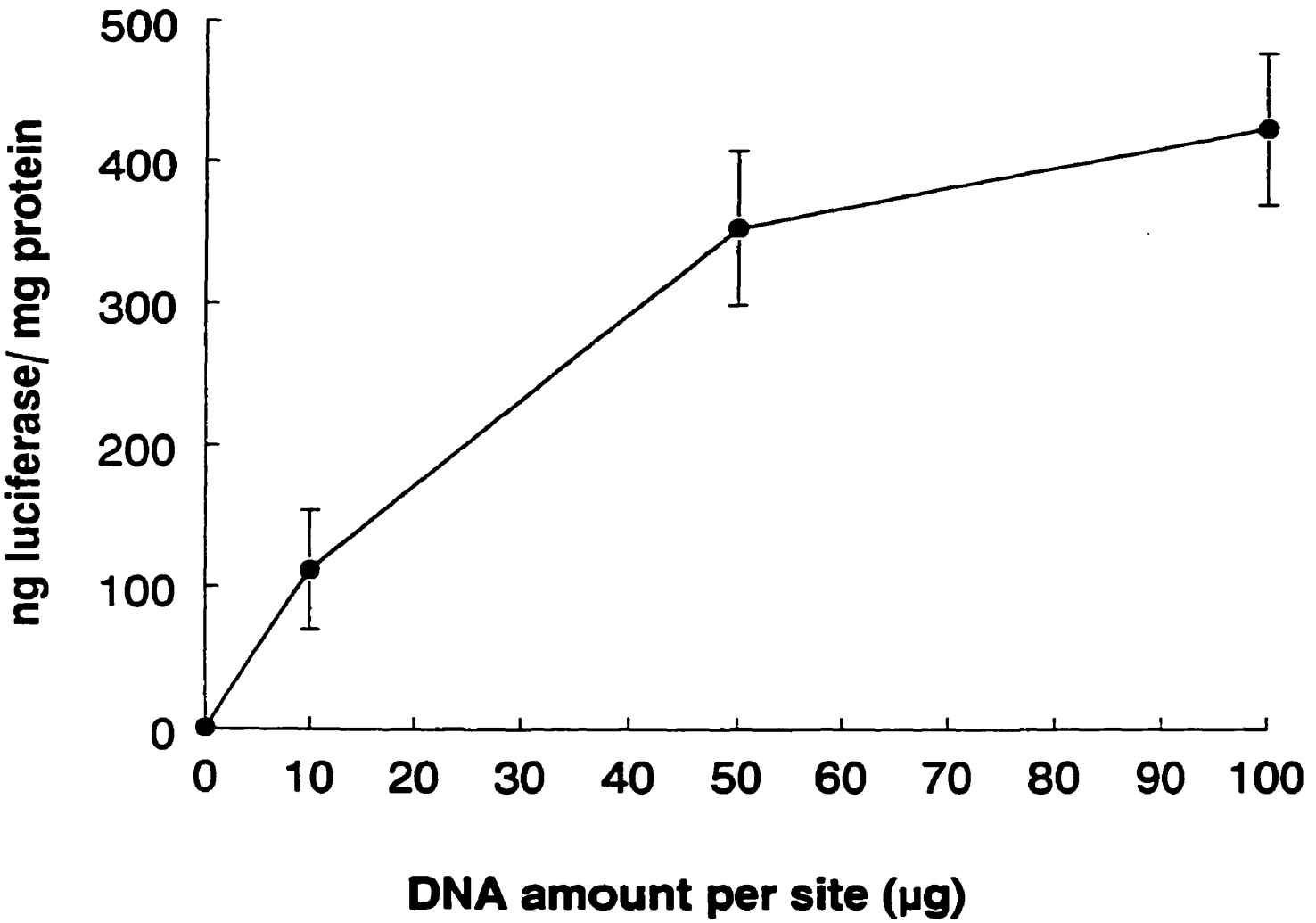

500
400
300
200
100
0
ng luciferase/ mg protein
0
10
20
30
40
50
60
70
80
90
100
DNA amount per site (µg)

Figure 3 Effect of age and sex on the magnitude of reporter gene expression. 50μg of pVR1255 in each TA muscle of male and female CD-1 mice, at an age ranging from 3 weeks to 6 months (n=10 per group). Tissue extracts were assayed for luciferase activity 7 days post-injection.

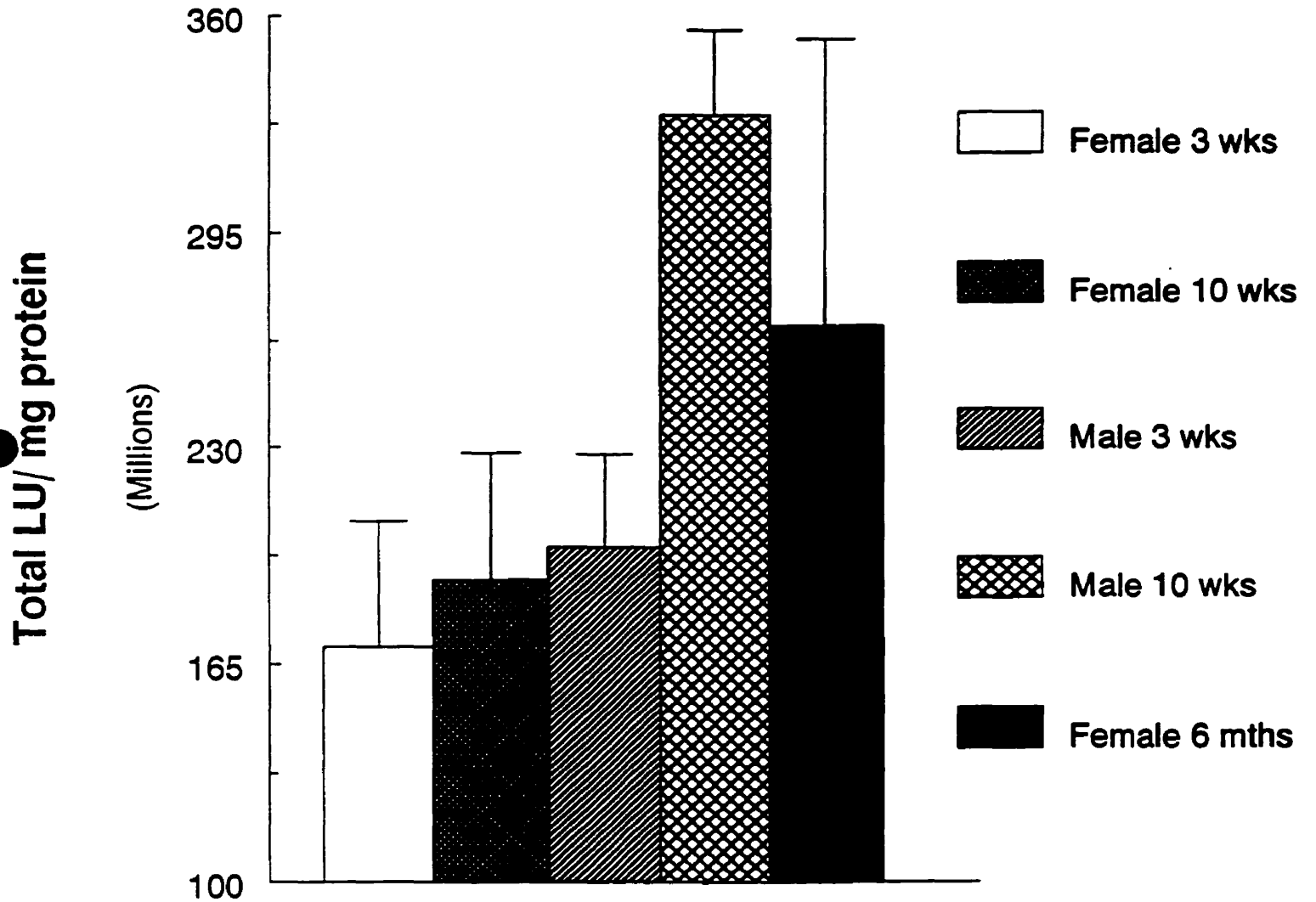
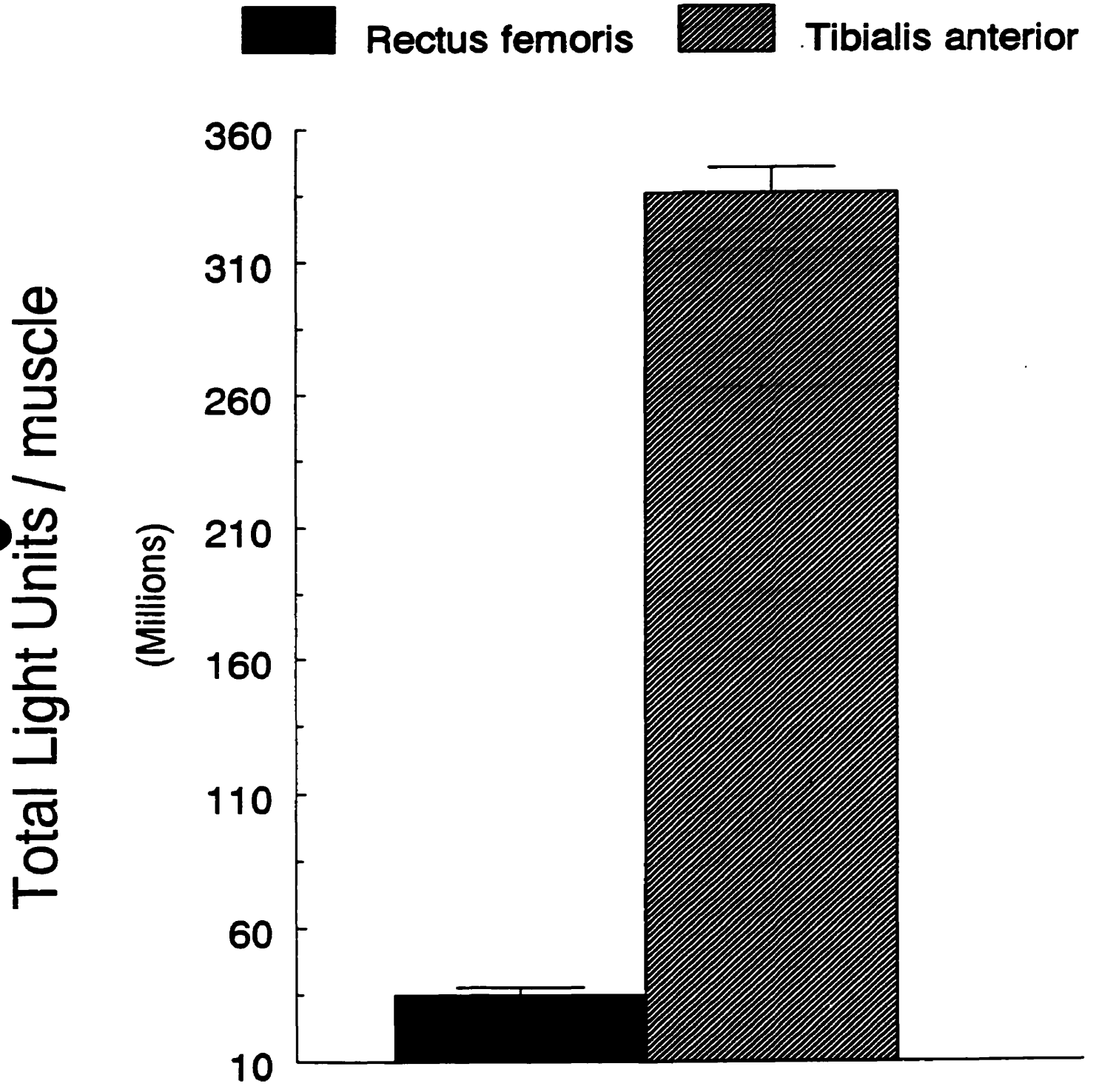

Total LU/ mg protein
(Millions)
360
295
230
165
100
Female 3 wks
Female 10 wks
Male 3 wks
Male 10 wks
Female 6 mths

Figure 4 Effect of muscle group on gene expression. TA and RF muscles from female BALB/c female mice (4-6 weeks, n=10 per group) were injected with 50μg of pVR1255 and tissue extracts were assayed for luciferase activity 7 days post-injection.

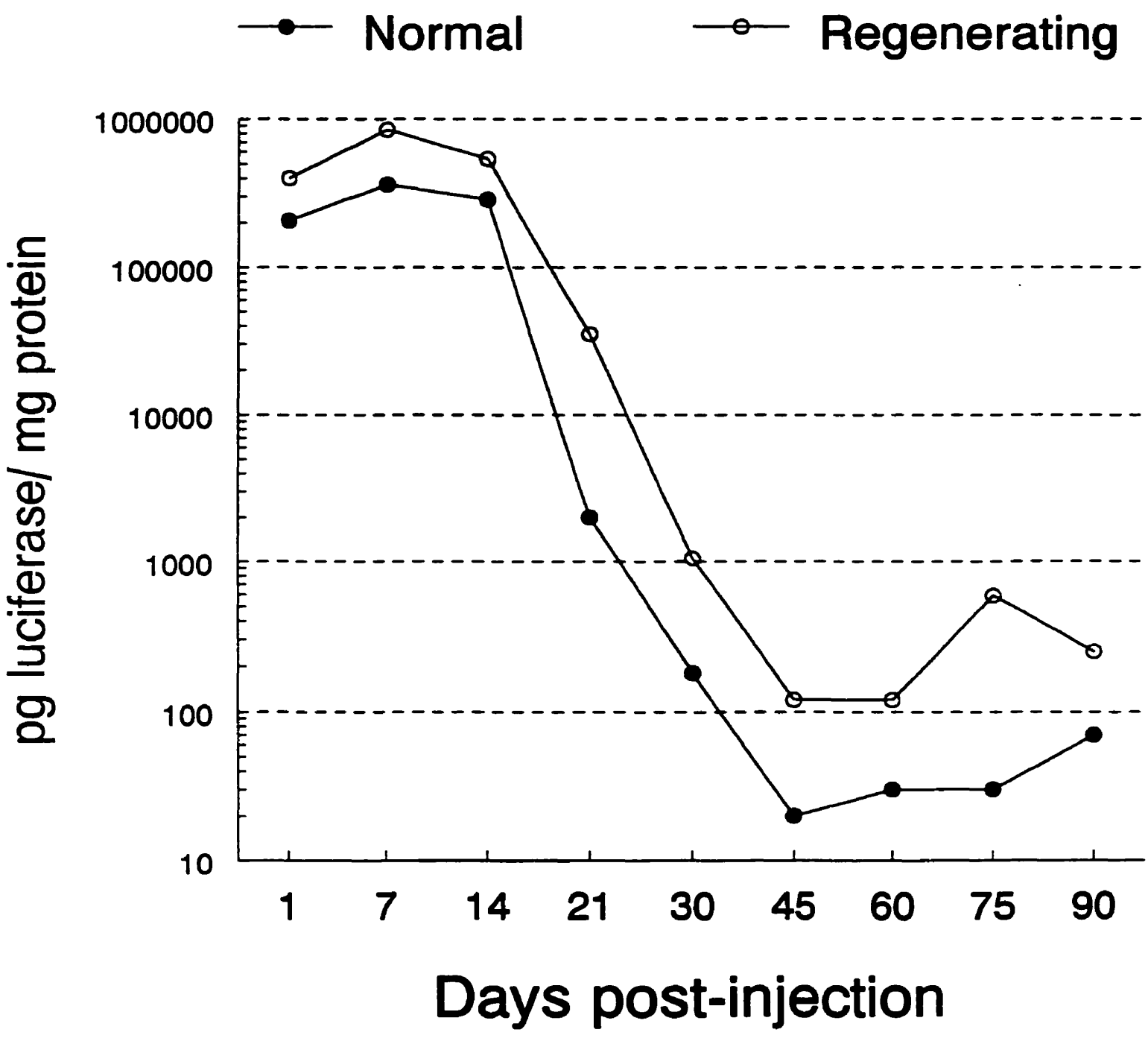

Rectus femoris
Tibialis anterior
Total Light Units / muscle
(Millions)
360
310
260
210
160
110
60
10

Time course of luciferase expression in normal and regenerating skeletal muscle

Muscle regeneration induced by some local anesthetic agents has been shown to greatly improve plasmid DNA uptake and expression in mouse skeletal muscles (12). We injected BALB/c TA muscles with 50 μg of pVR1255 7 days after a 0.4% bupivacaine muscle pre-treatment. As in previous studies (12), we determined that the time at which plasmid DNA was administered into muscle after bupivacaine treatment greatly influenced the magnitude of luciferase expression. The optimal time interval between bupivacaine and plasmid treatment was 7 days for BALB/c mice (data not shown). Treated muscles were then excised at various time points and total luciferase activity was assessed. Our results show that although bupivacaine pre-treatment markedly increased luciferase production at every time point, it did not prolong reporter gene expression significantly (Fig.5). In both normal and regenerating muscles, i.m. injection of pVR1255 resulted in detectable luciferase expression by 24 hours post-injection, which rapidly rose to peak levels at 7-14 days, declined thereafter, and persisted at a low level for up to 4 months. By day 7, there was a total of 3 times more luciferase produced in regenerating muscles, compared to normal muscles ($3x10^5$ pg/mg protein versus $9x10^5$ pg/mg protein, $p<0.005$). On day 21, reporter levels in normal muscle decreased to $2x10^3$ pg /mg protein of luciferase produced, compared to $3.5x10^4$ pg /mg protein in regenerating muscle. Although expression levels also declined in regenerating muscle by day 21 of the experiment,

this still represents a 17.5 fold increase in luciferase production compared to normal muscles ($p<0.001$). Between days 45 and 90, luciferase expression levels in normal muscles dropped below 100 pg/mg protein. Interestingly, during the same time period, bupivacaine-treated muscles continued to display a 2-30 fold increase in luciferase expression compared to normal muscles ($p<0.01$).

Figure 5 Time course of reporter gene expression in normal and regenerating muscle. Female BALB/c mice (4-6 weeks, n=6-8 per group) were injected with 50μg of pVR1255 7 days after a 0.4% bupivacaine muscle pre-treatment. Control mice did not receive a bupivacaine pre-treatment and were injected with equal amounts of pVR1255. Muscles were excised at various time points, tissue extracts were assayed for luciferase activity. Normal, untreated mice (closed circles); bupivacaine pre-treated mice (open circles). Data is presented as mean values ± SEM.


Normal
Regenerating
1000000
100000
10000
1000
100
10
pg luciferase/ mg protein
1
7
14
21
30
45
60
75
90
Days post-injection

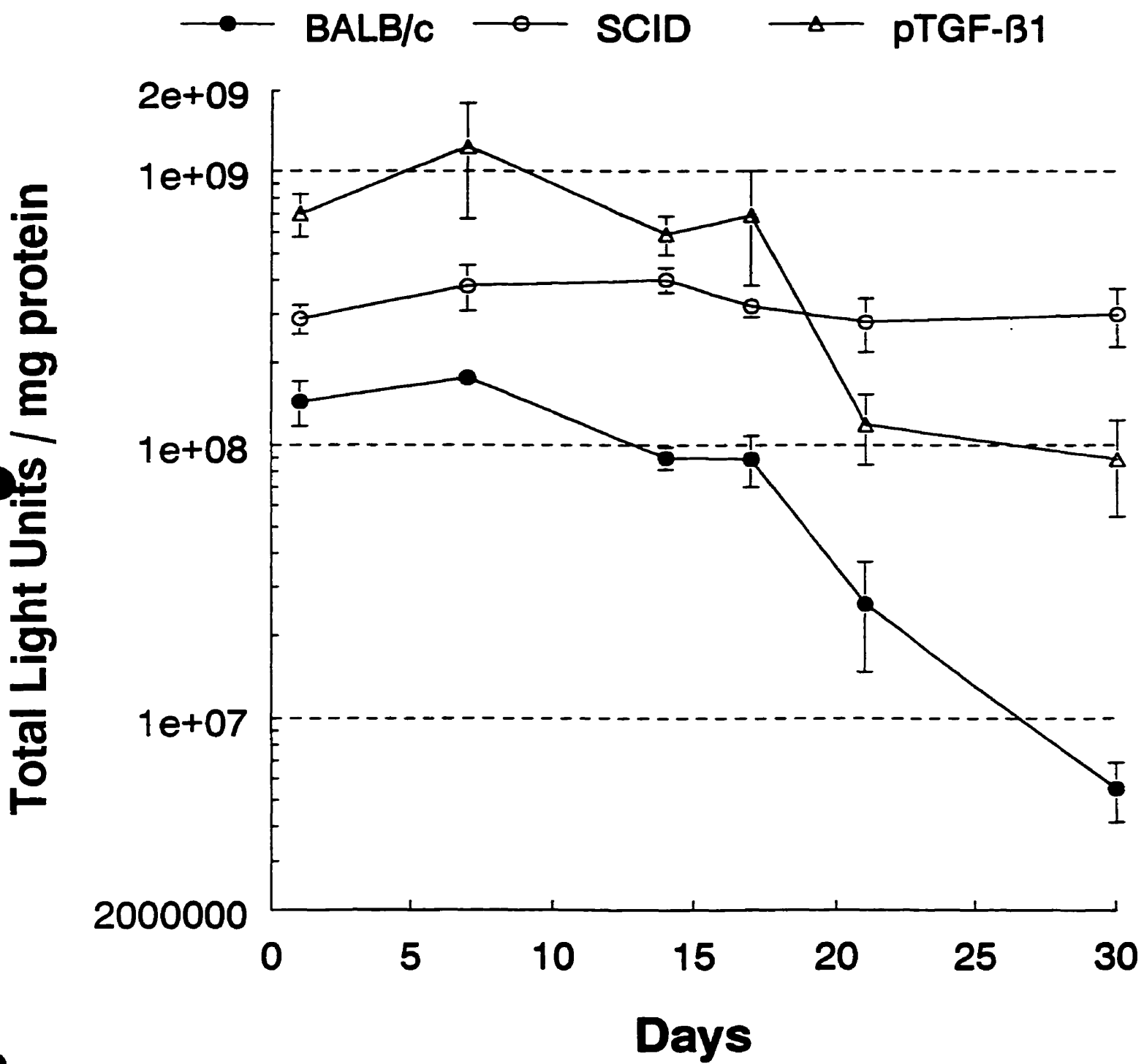
Involvement of immune system in limiting long-term luciferase expression

Since luciferase is a foreign antigenic protein (non-self), we determined if host immune responses were suppressing reporter gene expression in treated mice. Female BALB/c and SCID mice were injected with 50 μg of pVR1255 in TA muscles. Treated muscles were then removed at various time points and total luciferase activity was ascertained. In immunocompetent BALB/c mice, i.m. injection of pVR1255 resulted in a rapid rise in luciferase expression by 24 hours, which peaked by day 7 post-injection. Expression levels declined about 2-fold on day 14 and then dropped 31-fold on day 30 (Fig. 6). Remarkably, the temporal decline in luciferase expression was not observed in immunodeficient SCID mice, since peak day 7 reporter levels were sustained throughout the course of the experiment (Fig. 6). This represented about a 35-fold increase in luciferase levels in SCID mice on day 30 post-injection, compared to BALB/c mice ($p<0.0001$).

Previously, we demonstrated that i.m. injection of plasmid DNA encoding murine TGF-β1 resulted in increased plasma levels of TGF-β1 capable of immunosuppression (3, Chapter IV). We therefore postulated that TGF-β1/luciferase plasmids co-expression in skeletal muscle could dampen immune responses directed against luciferase and consequently prolong reporter expression. Interestingly, when immunocompetent BALB/c mice were co-injected with pVR1255 and pVR-TGF-β1, luciferase expression levels remained elevated on days 21 and 30 post-injection. On day 30, there was approximately an 18-fold increase in luciferase protein levels in

pVR1255/pVR-TGF-β1-treated mice compared to mice treated with pVR1255 alone ($p<0.005$) (Fig. 6).

Figure 6 **The immune system limits the persistence of luciferase gene expression.** Female BALB/c (group A) and SCID mice (group B) (4-6 weeks, n=6 per group) were injected with 50μg of pVR1255 in each TA muscle. Another group of female BALB/c mice (group C) (4-6 weeks, n=6) were co-injected with pVR1255 and pVR-TGF-β1 in each TA muscle. Treated muscle were then removed at various time points and total luciferase activity was ascertained.


BALB/c
SCID
pTGF-ß1
2e+09
1e+09
1e+08
1e+07
2000000
Total Light Units / mg protein
0
5
10
15
20
25
30
Days

Discussion

Intramuscular administration of naked plasmid DNA has been shown to be an effective route of gene transfer *in vivo* (1,2). The mechanism of plasmid DNA uptake and expression by muscle fibers is so far poorly understood. In this study, we investigated the factors which influenced the magnitude and persistence of foreign (non-self) gene expression following the injection of naked plasmid DNA in mouse skeletal muscle. Luciferase was chosen over other reporter genes because of assay sensitivity, and short T½. Thus, luciferase activity could accurately reflect subtle changes in gene transcription and protein production (13).

The uptake of plasmid DNA by skeletal muscle fibers appears to occur through a saturable process since luciferase expression levels increased to about 100μg of plasmid DNA, and then declined. Dowty and co-workers (14) have suggested that this saturation may be associated with a receptor-like uptake mechanism mediating the internalization of nucleic acids in muscle fibers.

Muscle regeneration induced by local anesthetic agents, such as bupivacaine (Marcaine), can greatly improve plasmid DNA uptake and expression in mouse skeletal muscles, presumably by increasing the proliferation of satellite cells in the muscle (12). This effect occurs primarily because bupivacaine selectively destroys myofibers while sparing extracellular matrix components and vascular endothelial cells. It appears that myotoxicity is due to increased intracellular calcium concentration (12,15). Following bupivacaine injection, muscle necrosis occurs within 48 hours, and

is followed by satellite cell proliferation and myotube formation (15). Although previous studies demonstrated significant effects of bupivacaine on i.m. plasmid DNA expression, little was known of its influence on the longevity of this expression compared to untreated muscles. We therefore postulated that induction of muscle regeneration could possibly increase the magnitude, stabilize and prolong foreign gene expression in skeletal muscle. Our results show that bupivacaine-treated muscles are superior to untreated muscles for foreign gene expression, since luciferase levels are significantly increased at every time point post-injection. This increase in expression may be due to a better spatial distribution of plasmid DNA in muscle tissue, increased DNA uptake or to an improved expression of the plasmid DNA (12,15). In support of this, a marked increase in lacZ-expressing muscle cells was observed in bupivacaine-treated muscles compared to normal untreated muscles (our unpublished data). However, this result also suggests that increased muscle cell uptake of plasmid DNA is not the major determinant for sustained foreign gene expression. Despite its obvious effect on peak luciferase levels compared to normal untreated muscle, bupivacaine-induced muscle regeneration did not significantly stabilize and prolong foreign gene expression (Fig.5). This may be attributed to the fact that plasmid transfection efficiency is increased, while other factors controlling expression are unaffected. This suggests that the positive effects of bupivacaine on plasmid DNA uptake and expression are not sufficient to sustain reporter gene levels in skeletal muscle.

As in previous studies (12), we determined that the time at which plasmid DNA was administered into muscle after bupivacaine treatment greatly influenced the magnitude of luciferase expression. The optimal time interval between bupivacaine and plasmid treatment was 7 days for BALB/c mice. This suggests that naked plasmid DNA is apparently unable to transfect mononuclear muscle progenitors in bupivacaine-treated muscles. It appears that transgene expression is feasible only when muscle fibers reach a certain stage of differentiation. Previous studies have suggested that this increase in expression correlates with the presence of T-tubules in bupivacaine-treated muscles (15). Incidentally, T-tubules are only found in skeletal and cardiac muscles and this may account for the preferential uptake of plasmid DNA in muscles compared to other tissues (16).

We show that i.m. injection of a luciferase plasmid DNA vector results in elevated luciferase expression within hours, which peaks at 7 days, and then declines to a low level, where it persists for many months. We hypothesized that this decline in expression was due to a host immune response directed at the transgene product, firefly luciferase. Similar time course experiments in immunodeficient SCID mice resulted in sustained levels for the entire duration of the experiment. Since SCID mice cannot generate immune responses to luciferase, we postulate that immunity to this protein accounts for the depressed expression in immunocompetent mice. Recent studies (10,11) have also correlated this observation with a mononuclear cell infiltration occurring 30 to 60 days post-injection and subsequent destruction of

reporter gene expressing myofibers. Muscle cells may be destroyed by a cytolytic T cell response against *lux*-postive myofibers expressing luciferase Antigens in the context of MHC class I molecules. Similarly, i.m. injection of plasmid DNA encoding autologous (self) proteins resulted in sustained transgene expression in immunocompetent mice (Chapter IV, 3,4,17,18).

TGF-β1 is a pleiotropic cytokine with multiple immunosuppressive effects on T cells and macrophages, including related cytokine production and adhesion molecule expression (reviewed in 19). We previously reported that i.m. injection of a plasmid vector encoding mouse TGF-β1 results in sustained muscle cell expression of this cytokine, with increased plasma levels of TGF-β1 capable of suppressing delayed-type hypersensitivity responses (Chapter III, 3). As well, mice were protected from autoimmune type 1 diabetes (Chapter III, 3) and encephalomyelitis (Chapter IV). In accordance with the above findings, we postulated that co-injecting pVR1255 with a TGF-β1 plasmid DNA vector (pVR-TGF-β1) would improve reporter gene expression levels in skeletal muscle, possibly by dampening immune responses directed against luciferase, and consequently prolong reporter expression. When immunocompetent mice were co-injected with pVR1255 and pVR-TGF-β1, luciferase expression levels remained elevated on days 21 and 30 post-injection. Although a moderate decline in luciferase expression was apparent by day 21, it was nevertheless significantly greater than the expression levels observed in mice treated with pVR1255 alone. Thus, it appears that host immune responses to the transgene product can be dampened by

gene-mediated immunosuppression. At the moment, it is unclear at which points TGF-β1 may be acting to mediate its effect. It is possible that TGF-β1 is blocking the access of infiltrating immune cells to transfected muscle fibers or suppressing the cytolytic effector phases. In fact, infiltrating immune cells are frequently seen in large numbers around reporter gene-expressing muscle fibers, and this parallels the temporal decline in luciferase expression. On the other hand, immune cells do not accumulate in muscles injected with a control plasmid. Interestingly, fewer mononuclear cells are observed in TGF-β1 plasmid-treated muscles (our unpublished results), supporting the notion that TGF-β1 protects muscle cells from cytolytic destruction, in part, by preventing immune cell accumulation at the site of foreign antigen expression. These observations are consistent with the inhibitory effects of TGF-β1 on adhesion molecule expression on a variety of cell types such as lymphocytes and vascular endothelial cells (20,21).

Although the immune response may represent an important factor regulating the stability of expression, down-regulation of the activity of the CMV-based luciferase plasmid in muscle cells can also account for the decrease in reporter expression. Manthorpe and co-workers have shown a marked temporal decline in luciferase expression 14 days after injection of a luciferase CMV promoter containing plasmid, whereas a luciferase plasmid containing the RSV promoter maintained almost constant, albeit lower levels of expression for at least 4 months (10). Furthermore, immune responses to foreign proteins following i.m. plasmid injection may result in

IFN-γ production which can down-regulate CMV promoter activity (23). Other factors which may contribute to this decline in reporter gene expression include the stability of the episomal plasmid in mammalian nuclei and toxicity resulting from i.m. transgene protein overload.

Numerous muscle-based DNA transfer systems have been developed to deliver genes *in vivo*, including adenoviruses and rAAV (24,25). Both adenoviral and rAAV vectors generate significant expression levels in skeletal muscle (15). Although these vectors have the capacity of infecting dividing and non-dividing cell types with high efficiency, these viral vehicles have been shown to be highly immunogenic, thus limiting sustained levels of expression and repeated administrations (26,27). The results presented here suggest that co-expressing immunosuppressive proteins with the transgene may offer a novel approach to attenuate host immune responses against transgenes and viral surface antigens in order to achieve long-term gene expression. A recent study has shown that, following adenoviral-mediated gene transfer, cellular and humoral immune responses to adenoviral proteins and dystrophin are contributing factors to the decline of dystrophin expression in dystrophic muscles (28). In these studies, transient immunosuppression prolonged transgene expression. Similarly, immunosuppression with cyclosporine allows for sustained transgene expression following readministration of rAAV vectors (27).

The mechanism of plasmid DNA uptake and expression by skeletal muscle fibers is a poorly understood process. Factors which may influence this process include

muscle type, age and gender. We report that the degree of uptake and expression is age- and sex-dependent. In our hands, old male mice (10 weeks old) repeatedly gave better results than young males and young/old females. This difference may be due to the type (slow versus fast) of muscle fiber transfected and to the overall muscle mass (9). Surprisingly, approximately 10-fold more luciferase activity was observed in T.A. muscles. Thus, different muscle groups express considerably different levels of reporter protein after injection of the same amount of plasmid DNA. The basis for this expression pattern may be related to the nature of the muscle fibers transfected, or to muscle physiology and architecture (11).

Direct DNA injection in skeletal muscle appears to be safe and is technically simple. Unlike most viral vectors, the plasmid vectors are maintained episomally, minimizing the risk of genomic insertional mutagenesis (1). Moreover, the use of plasmid vectors eliminates the possibility of immune responses to viral Antigens. The ease of preparation and relatively low production cost make plasmid an excellent therapeutic delivery system. A detailed understanding of the conditions which regulate the magnitude and persistence of foreign gene expression in skeletal muscle will establish the working basis for the application of i.m. plasmid DNA injections in the treatment of genetic and acquired diseases.

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Connecting text for Chapter III.

In the previous chapter, I characterized the i.m. somatic gene transfer approach and identified some of the factors which appear to influence long-term foreign gene expression in skeletal muscle. In Chapter III, I focused on TGF-ß1 for our *in vivo* cytokine gene transfer studies. TGF-ß1 is potent anti-inflammatory and immunosuppressive cytokine with numerous immunoregulatory functions in autoimmune disease. I reasoned that i.m. injection of a TGF-ß1-encoding plasmid DNA would result in sufficient production and secretion of TGF-ß1 by skeletal muscle fibers to influence autoimmune disease expression in an animal model of autoimmune diabetes.

CHAPTER III

Transforming growth factor β1 (TGF-β1) somatic gene therapy prevents autoimmune disease in NOD mice

by

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Abstract

NOD mice develop insulitis and diabetes through an autoimmune process. Since transforming growth factor beta 1 (TGF-β1) downregulates many immune responses, we hypothesized that TGF-β1 could prevent disease in NOD mice and that there would be several advantages to cytokine delivery by a somatic gene therapy approach. We opted for i.m. injection of a naked plasmid DNA expression vector encoding murine TGF-β1 (pCMV-TGF-β1). Treatment with pCMV-TGF-β1 resulted in the retention and expression of the vector in muscle cells, associated with a considerable elevation in the plasma levels of TGF-β1, that was not observed in control vector-treated mice. The levels of TGF-β1 produced were sufficient to exert immunosuppressive effects. DTH responses were suppressed and autoimmunity-prone NOD mice were protected from insulitis and diabetes in models of cyclophosphamide-accelerated and natural course disease. In pCMV-TGF-ß1 treated mice, pancreatic IL-12 and IFNγ mRNA expression was depressed, and the ratio of IFNγ to IL-4 mRNA was decreased, as determined by semi-quantitative RT-PCR. In contrast, NOD mice injected with a vector encoding the proinflammatory cytokine IFNγ developed diabetes earlier. Intramuscular administration of cytokine-encoding plasmid vectors proved to be an effective method of cytokine delivery in these mice, and altered autoimmune disease expression.

Introduction

The NOD mouse develops diabetes spontaneously, through an autoimmune process. This disease shares many features with human insulin-dependent diabetes mellitus (IDDM or type 1 diabetes) (1). There is a T-cell and macrophage-dependent progressive infiltration of islets of Langerhans (termed insulitis), with destruction of insulin-producing beta islet cells, occurring over a period of weeks or months. Strong experimental evidence suggests that proinflammatory cytokines produced by Th1 cells and macrophages play an important role in the development of these lesions (2,3). Therefore, suppressing the activity of these cells is likely to be therapeutically effective.

In this study, we focused on the potential immunosuppressive role of transforming growth factor beta (TGF-β1) in autoimmune murine diabetes. TGF-β1 is a pleiotropic cytokine with multiple anti-inflammatory effects. It suppresses the activity of T cells, macrophages, NK cells and B cells and inhibits the expression of many proinflammatory cytokines such as IFNγ, TNFα, IL-1 and IL-2 (4). We hypothesized that TGF-β1 could prevent IDDM in NOD mice, and that there would be several advantages to delivering this cytokine by a somatic gene therapy approach.

It has been shown that i.m. injection of naked plasmid expression vectors results in the cellular uptake of the plasmid DNA, which is maintained episomally for prolonged periods of time within skeletal muscle cells (5). Here, we demonstrate that

i.m. injection of a vector encoding mouse TGF-β1 cDNA, i.e., pCMV-TGF-β1, results in uptake, retention and expression of this vector. There is detectable vector-derived TGF-β1 mRNA in skeletal muscle cells, as well as increased levels of TGF-β1 in the plasma of treated mice. Administration of pCMV-TGF-β1 was effective at suppressing a DTH response, and at protecting NOD mice from insulitis and diabetes. There is a decreased expression of IL-12 and IFNγ mRNA in the pancreas of protected mice. In contrast, administration of an IFNγ encoding vector accelerated disease.

Materials and Methods

Animals

Female NOD mice (8-10 weeks) were purchased from Taconic Farms (Germantown, NY) and female BALB/c mice (substrain AnNCrlBR, 4-6 weeks) were purchased from Charles River Canada (St-Constant, Quebec, Canada). Mice were kept in a pathogen-free facility. These NOD mice exhibit mild insulitis as early as 4 weeks of age and become diabetic starting at about 12 weeks of age.

Expression Vector Construction and TGF-β1 assays

The mouse TGF-β1 (mTGF-ß1) cDNA was produced by RT-PCR from ConA stimulated BALB/c splenocytes and cloned into compatible enzyme restriction sites of pCI-neo (Promega, Madison, WI) to generate pCMV-TGF-β1. The mTGFβ1 cDNA is under the transcriptional control of a cytomegalovius (CMV) immediate-early enhancer/promoter, and downstream of a chimeric intron. pCMV-TGF-β1 encodes the latent form of mTGFβ1, and expression was confirmed using a TGF-β1 ELISA (R&D, Minneapolis, MN) in supernatants collected from transiently transfected COS-7 cells. The TGF-β1 ELISA only detects active TGF-β1. The latent TGF-β1 was activated to its biologically active form by acidification for 10 minutes and its bioactivity was confirmed by the CCL64 mink cell line proliferation assay. Mouse IFNγ cDNA was subcloned into pCI-neo, as described above, to generate pCMV-

IFNγ. pCI-neo, henceforth referred to as pCMV-null, was used as control vector in all the experiments.

Plasmid DNA Preparation

Large-scale plasmid DNA preparations were produced by the alkaline lysis method using a Qiagen giga kit (Qiagen Inc., Santa Clarita, CA). All plasmid preparations for i.m. injections were resuspended in sterile 0.85% saline. Spectrophotometric analysis revealed 260/280 nm ratios ≥ 1.80. Purity of DNA preparations and conformations were confirmed on a 1% agarose gel.

Determination of plasma TGF-β1 levels

In order to minimize the activation of platelets and subsequent release of endogenous TGF-β1, platelet-poor plasma was obtained as follows: whole blood was mixed with a 1.5% EDTA solution, mixed thoroughly and put on ice immediately after blood collection. The blood/EDTA mixture was then layered gently on 20% sucrose and centrifuged for 30 minutes at 12000g. The upper two thirds of the upper phase (platelet-poor plasma fraction) was collected without disturbing the interface. Once separated, the platelet-poor plasma was frozen at -80°C until assayed for TGF-β1 by ELISA. Statistical analysis was performed by Student's t test.

Induction and measurement of delayed-type hypersensitivity reaction in mice

On day 1, BALB/c mice were immunized with 200 μg of OVA in CFA, or with CFA alone. On day 6, mice were injected with OVA in PBS in the right footpad. DTH responses were measured, with calipers, as the increase in footpad thickness 24, 36, and 48h after OVA antigen recall immunization. Statistical analysis was performed with the Student's t test.

Intramuscular injection of plasmid DNA

Intramuscular injections of plasmid DNA were done as described (6). Briefly, mice were anaesthetized by i.p. injection with xylazine (10mg/kg) and ketamine (200 mg/kg). The rectus femoris (RF) and tibialis anterior (TA) muscles of each mouse were injected with a 0.5 cc sterile 29G1/2 insulin syringe, fitted with a plastic collar to limit needle penetration to 2 mm. Mice received 100 μg of pCMV-TGF-β1 in 50μl of sterile saline in each RF or TA muscle, for a total of 400 μg of plasmid DNA in each treatment session, unless stated otherwise. Control mice received equivalent amounts of pCMV-null control vector in each muscle group. A vector encoding mouse IFNγ, pCMV-IFNγ, was administered in a similar fashion.

Induction and diagnosis of diabetes

Cyclophosphamide (CYP, Sigma Chemical Co., St-Louis) which accelerates the onset of diabetes in NOD mice (7), was administered i.p. twice, 14 days apart, at a

dose of 200 mg/kg in PBS to 8-10 weeks old female NOD mice. Diabetes was diagnosed by regular urinary glucose analysis and confirmed by blood glucose determination. Mice were considered diabetic when sequential blood glucose measurements were shown to be equal to, or above 16.7 mmol/L (300 mg/dl) as determined by the Accu-ChekIII glucometer (Boehringer Mannheim).

Statistical analysis was done using the SAS software (version 6.12) for Windows 95. The incidence of diabetes was plotted using the Kaplan-Meier method (nonparametric cumulated survival plot). The statistical comparison between the curves obtained was performed using the Wilcoxon log rank test.

Histological analysis

The pancreas was excised immediately after CO_2 asphyxiation, fixed in 10% buffered formalin, and hematoxylin and eosin stained histological slides were prepared. Insulitis was graded as follows: grade 0, normal islet totally free of any peri-islet mononuclear cells; grade 1, focal peri-islet lymphocytic infiltration <25% of islet circumference; grade 2, peri-islet lymphocytic infiltration >25% of islet circumference; grade 3, mild insulitis, intra-islet infiltration with good retention of islet cell morphology, grade 4, severe insulitis with significant destruction of β-islet cells. Three randomly obtained levels of pancreas were analyzed in double-blind fashion by two observers. Statistical analysis was performed with the Chi-squared test.

PCR and RT-PCR analysis

Mice were killed and their TA muscles were excised, immediately frozen in liquid nitrogen and stored at -80°C. Total genomic DNA was isolated from thawed muscle specimens as described (8). The PCR reactions were performed in a 50 µl reaction volume containing 2.5 µl of genomic DNA, 10mM Tris-HCl pH 8, 50mM KCl, 2 mM dNTP, 5 mM $MgCl_2$ 2µM of each primer and 1.5 units of Taq DNA polymerase. The primer sequences amplifying vector-encoded TGF-β1 were 5'-AGAGAAGAACTGCTGTGTGCGGCAG-3' (sense) and 5'-CGCTTCCCTTTAGTGAGGGTTAATG-3' (antisense). The TGF-β1 primer set amplified a TGF-β1 product from pCMV-TGF-β1 DNA, or cDNA derived from that vector, but not from genomic DNA or endogenous TGF-β1 cDNA. PCR cycling conditions were as follows: one cycle at 94°C; 40 cycles at 94°C for 1 minute, at 55°C for 2 minutes and 72°C for 2 minutes; and one final extension cycle at 72°C for 10 minutes. The PCR amplimers were analyzed on a 1.5% agarose gel containing 0.5µg/ml ethidium bromide. The TGF-β1 mRNA in treated muscle was detected by reverse transcription PCR (RT-PCR). Total RNA was extracted from entire TA muscles as described (8) and reverse transcribed with the Superscript preamplification system (Gibco BRL). 2µls of the reverse transcription reaction were used for PCR amplification using the above-mentioned vector-specific primers for pCMV-TGF-β1, or G3PDH primers (sense 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' and antisense 5'-CATGTAGGCCATGAGGTCCACCAC-3'). Optimal amplification

conditions were carried out for 40 cycles and amplifiers were labeled with [α-32P]dCTP (5μCi) (ICN, Mississauga, ON). The PCR products were analyzed on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide, transferred onto Hybond-N+ nylon membrane (Amersham Canada, Oakville, ON) and either exposed to autoradiographic film or subjected to PhosphorImager analysis.

For intrapancreatic cytokine mRNA analysis, total RNA was isolated from snap-frozen pancreas and quantification of specific cytokine gene expression was performed by RT-PCR, as described above. For this purpose, specific primers were used in PCR for IL-12 p40 (9), IL-4 (10), IFNγ (10) and ß-actin (11). Analysis of cytokine PCR products was performed as previously described (11). Briefly, PCR reactions were terminated in the linear portion of the amplification reaction (which extended up to 35 cycles). The ^{32}P-labeled PCR products were analyzed on a 2% agarose gel, transferred onto a nylon membrane and subjected to PhosphorImager analysis. Semi-quantitative mRNA analysis was performed by calculating relative quantities of RT-PCR signals for each cytokine, normalized to the ß-actin signal of each sample. The ratio of IFNγ to IL-4 mRNA (for a mouse) was derived after normalization of these cytokines with their ß-actin signal, as described above :

IFNγ / IL-4 ratio = (IFNγ / ß-actin ratio) ÷ (IL-4 / ß-actin ratio).

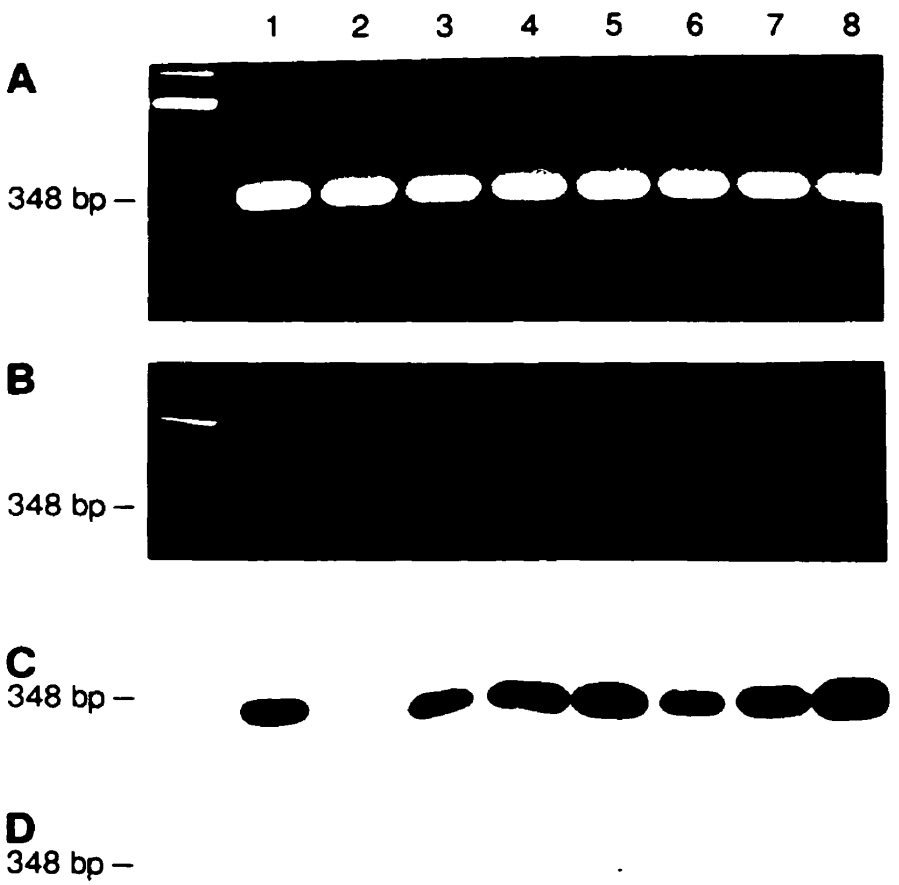
In all these experiments, specificity of PCR products was confirmed by restriction enzyme analysis. Statistical analysis was performed with Student's t-test.

Results

Detection of pCMV-TGF-β1 plasmid DNA and TGF-β1 gene expression in skeletal muscle

To determine if plasmid DNA is successfully retained and expressed by treated muscle tissue, each TA muscle was injected with 100μg of pCMV-TGF-β1, followed by total DNA or RNA extraction, and PCR or RT-PCR analysis, respectively. We found that 14 days following i.m. injection of pCMV-TGF-β1, a 348 bp amplimer, specific for this vector, could readily be detected by PCR analysis from all treated muscle samples (Fig. 1A), and was undetectable in null-vector treated mice (Fig. 1B). Furthermore, RT-PCR analysis of RNA samples extracted from these muscles revealed the presence of vector-derived TGF-β1 transcripts (Fig. 1C), which were absent in pCMV-null treated mice (Fig. 1D). No product was identified from RNA preparations when reverse transcription was omitted, and the PCR primers could not amplify endogenous TGF-β1 from either genomic DNA or cDNA (not shown).

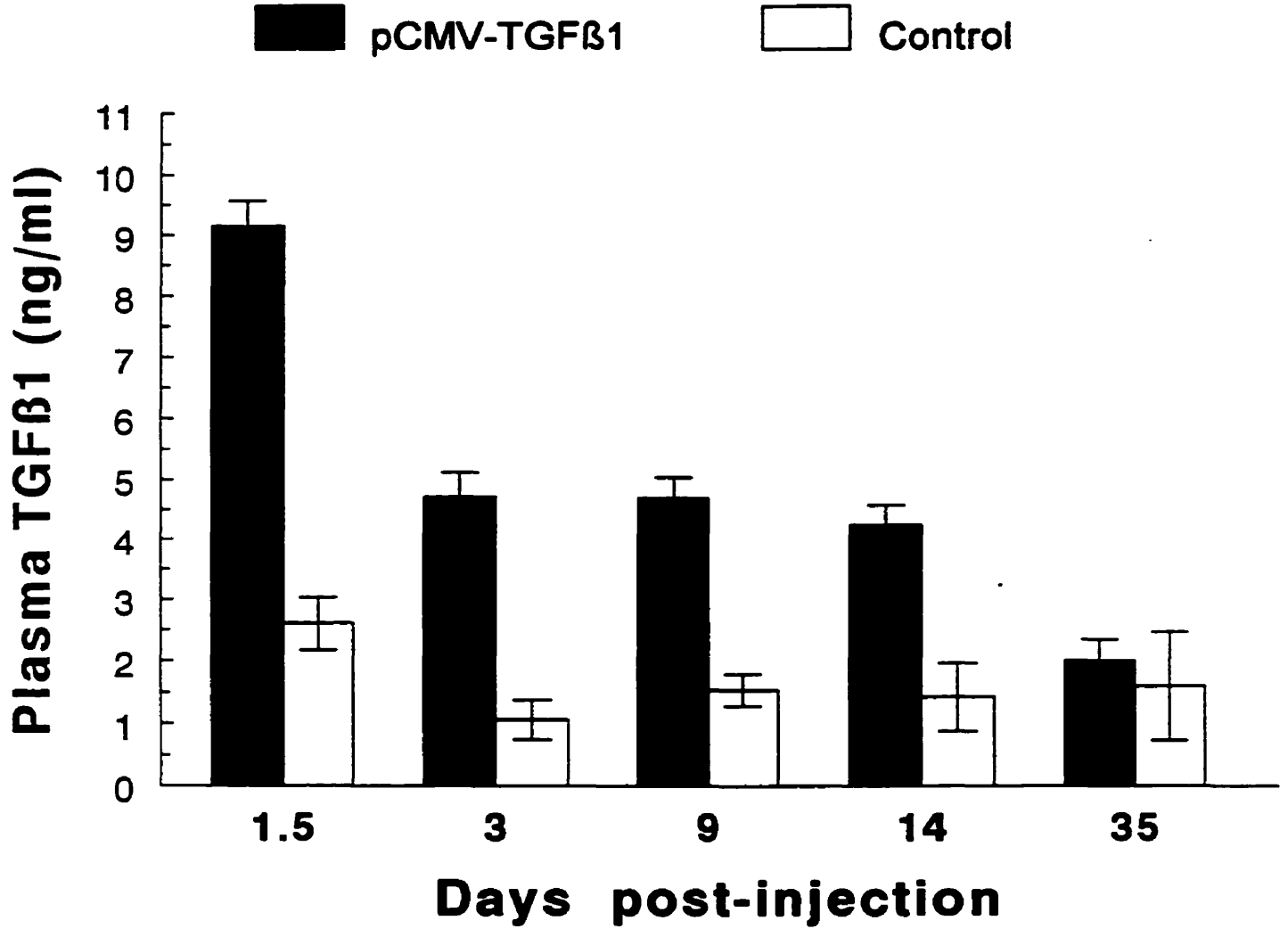
Figure 1 PCR and RT-PCR analysis following i.m. injection of plasmid DNA. A. Detection of a TGF-β1 PCR product following amplification of DNA from muscles of mice injected with pCMV-TGF-β1. B. No TGF-β1 PCR product is detected following amplification of DNA from muscles of mice injected with pCMV-null. C. Detection of vector-derived TGF-β1 transcripts by RT-PCR in mice injected with pCMV-TGF-β1. D. Absence of TGF-β1 transcripts by RT-PCR in mice injected with pCMV-null vector. Each TA muscle was injected with 100μg of pCMV-TGF-β1 or pCMV-null and total DNA or RNA was extracted from muscles 14 days later. In panels A and B, PCR products were stained with ethidium bromide. In panels C and D, RT-PCR products were labeled with [α32P] dCTP in the PCR reaction mixture, electrophoresed, and exposed to autoradiographic film. Each lane is from a separate mouse. In all cases, a unique primer set which amplified a 348 bp amplifier from pCMV-TGF-β1 was used.


1 2 3 4 5 6 7 8
A
348 bp –
B
348 bp –
C
348 bp –
D
348 bp –

Increased TGF-β1 plasma levels

To establish the optimal dose of plasmid DNA, increasing amounts of pCMV-TGF-β1 were injected at different muscle sites and TGF-β1 levels in platelet-poor plasma were measured by TGF-β1 ELISA, 75 hours post-injection. We found that vector expression, as determined by plasma TGF-β1 levels, increases with the number of injection sites and amount of DNA injected per site, up to 100μg of DNA per injection site (data not shown). To determine if the elevation of TGF-β1 levels was sustained, female NOD mice were injected with a total of 200 μg of plasmid DNA (100μg in each TA muscle). TGF-β1 levels of 9.2 ± 0.4 ng/ml were observed at 36h post-injection, compared to 2.6 ± 0.4 ng/ml in pCMV-null-treated mice (Fig. 2). These levels decreased to a mean plasma level of 4.3 ± 0.3 ng/ml on day 14 ($p < 0.05$ at 36h and 14 days versus null-vector treated mice). At day 35, TGF-β1 was still slightly higher in the pCMV-TGF-β1 treated mice, but this was no longer statistically significant. TGF-β1 plasma levels in age-matched, untreated NOD mice were 2.3 ± 0.5 ng/ml, a value statistically different from the pCMV-TGF-β1 treated mice 36h and day 14 ($p < 0.05$), but not statistically different from pCMV-null treated mice at any time point. In all cases, active TGF-β1 was either absent or present at undetectable levels in the plasma of treated mice (not shown).

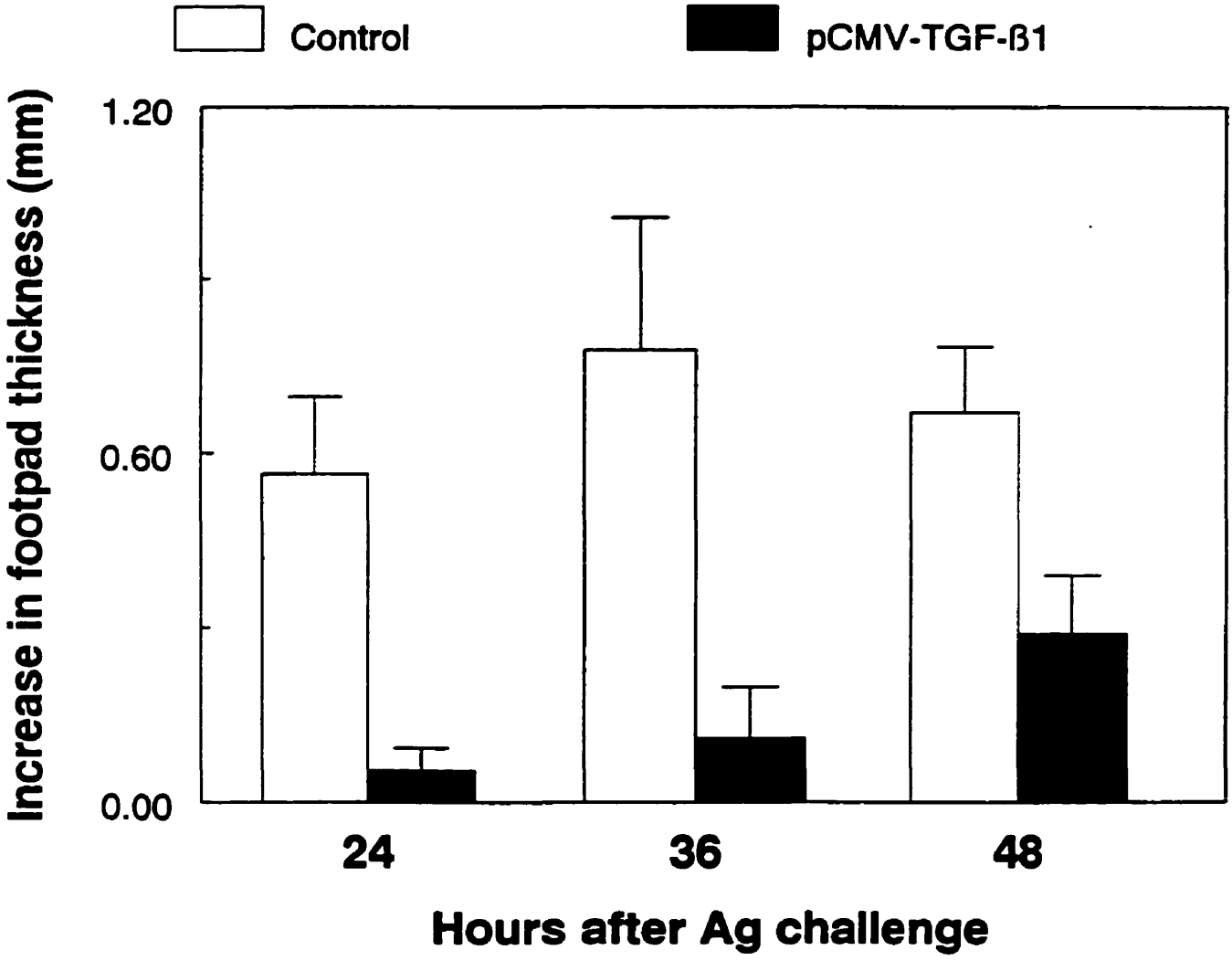
Figure 2 Time course of TGF-β1 protein expression in plasma. Each mouse received a total of 200μg of pCMV-TGF-β1 or pCMV-null injected into TA muscles, and plasma samples were assayed for TGF-β1 at the indicated times post-injection. Each group represents the mean of 3-5 mice ±SEM. TGF-β1 plasma levels in untreated mice were 2.3 ± 0.5 ng/ml (not shown). Closed bar, pCMV-TGF-β1 treated mice; open bar, pCMV-null treated mice.


pCMV-TGFß1
Control
Plasma TGFß1 (ng/ml)
11
10
9
8
7
6
5
4
3
2
1
0
1.5
3
9
14
35
Days post-injection

Suppression of DTH responses

To examine whether i.m. injections of plasmid DNA result in sufficient production of TGF-β1 to exert biological effects, we used a DTH model. DTH responses were assessed by footpad swelling 24-48h following recall immunization with OVA in female BALB/c mice. We found that pCMV-TGF-β1 treated mice had significantly suppressed DTH responses (Fig. 3). These mice had a 7-fold reduction in DTH responses observed at 36h, versus pCMV-null treated mice, consistent with the *in vivo* production and activation of TGF-β1.

Figure 3 Suppression of DTH responses by i.m. injection pCMV-TGF-β1 plasmid DNA. On day 0, mice were injected s.c. at 2 different sites with a total of 200μg of OVA in CFA, on day 5, injected i.m. with 200μg of pCMV-TGF-β1 and on day 6, were injected with OVA in PBS in the right footpad. DTH responses were detected by measurement of footpad swelling. Control mice received equivalent amounts of pCMV-null plasmid DNA at each time point. The results represent the mean increase in footpad thickness ±SEM (n=5 per group). Closed bar, pCMV-TGF-β1 treated mice; open bar, pCMV-null control mice.


Control
pCMV-TGF-ß1
Increase in footpad thickness (mm)
1.20
0.60
0.00
24
36
48
Hours after Ag challenge

Prevention of CYP-induced diabetes by pCMV-TGF-β1 administration

To determine if administration of pCMV-TGF-β1 could prevent autoimmune disease expression, we administered the TGF-β1 plasmid expression vector to adult NOD female mice. The autoimmune basis of this disease is well established (12). To accelerate disease expression, female NOD mice were injected with cyclophosphamide (CYP), as described (13). CYP has been reported to accelerate disease in NOD mice by enhancing intra-islet production of IFNγ and other inflammatory mediators (14). Briefly, CYP was administered i.p., on days 3 and 16, at a dose of 200 mg/kg. Animals received a total of 100 μg of pCMV-TGF-β1 in sterile saline in each RF and TA muscle for a total of 400 μg of plasmid DNA, 48h before each CYP injection. Control animals received equivalent amounts of pCMV-null control vector in each muscle. The incidence of IDDM was evaluated by sequential measurements of blood glucose levels.

Administration of pCMV-TGF-β1 significantly reduced the incidence of diabetes in NOD female mice (Fig. 4A). The first case of diabetes occurred 14 days later in the pCMV-TGF-β1 group, compared to control group. By day 32 of the experiment, the incidence of diabetes was four times higher in pCMV-null treated mice compared to mice receiving pCMV-TGF-β1 ($p < 0.001$). In diabetic mice, the hyperglycemia ($\geq$ 300 mg/dl) was maintained two or more weeks following onset of disease (data not shown).

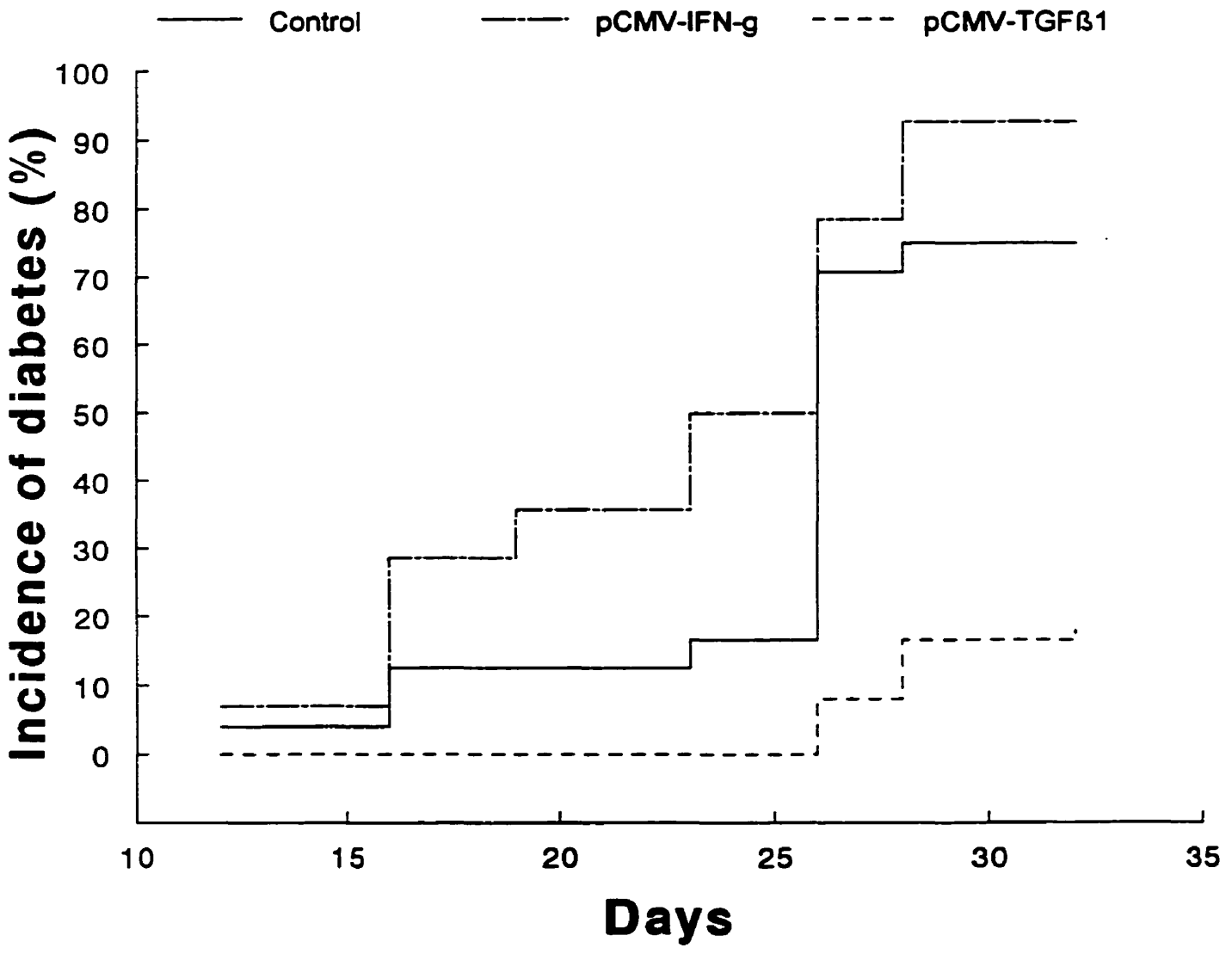
Acceleration of CYP-induced diabetes by pCMV-IFNγ administration

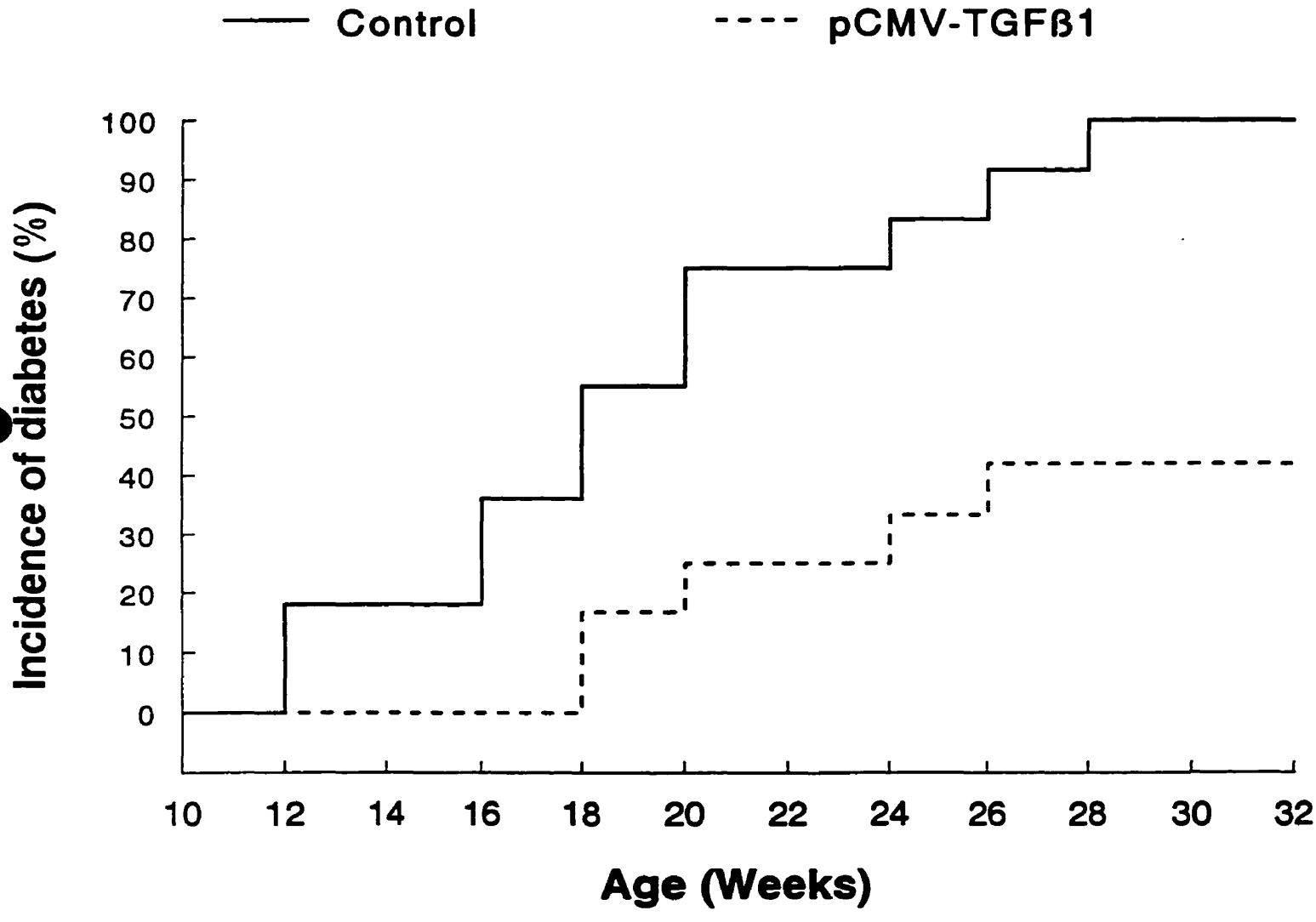
Diabetes occurred earlier in pCMV-IFNγ treated mice compared to control mice (Fig. 4A, $p = 0.05$, versus null-vector treated mice). The pCMV-mIFNγ treated mice had increased serum levels of IFNγ (up to 200 pg/ml, as determined by ELISA), while this cytokine was undetectable in the serum of control mice (data not shown).

Protective effect of pCMV-TGF-β1 treatment on the natural course of diabetes

To determine an effect on the natural course of disease, we injected female NOD mice (9-11 weeks) with pCMV-TGF-β1 in the absence of CYP (Fig. 4B). Under these conditions, the incidence of diabetes was again significantly reduced, to approximately 50% of control values over the course of several weeks ($p < 0.002$). Thus, TGF-β1-mediated protection is not a feature unique to the CYP-accelerated diabetes model.

Figure 4 Administration of pCMV-TGF-β1 reduces the incidence of diabetes. A. Diabetes was induced in 8-10 week old female NOD mice by administration CYP (200mg/kg) on days 2 and 16. Intramuscular administration of 400μg of pCMV-TGF-β1 (n=27 per group) on days 0 and 14, significantly reduced the incidence of diabetes compared to pCMV-null treated mice (n=27 per group, $p<0.001$). NOD mice injected with pCMV-IFNγ (n=14 per group) became diabetic earlier ($p=0.05$, compared to pCMV-null treated mice). B. To demonstrate an effect on the natural course of disease, NOD female mice (9-11 weeks, n=12) were injected with 200μg of pCMV-TGF-β1 every 2 weeks until the age of 32 weeks. Under these conditions, the incidence of diabetes was again markedly reduced ($p<0.002$). Panels A and B, pCMV-TGF-β1 treated mice (dotted line); pCMV-null treated mice (solid line); Panel A, pCMV-IFNγ treated mice (intermittently dotted line).

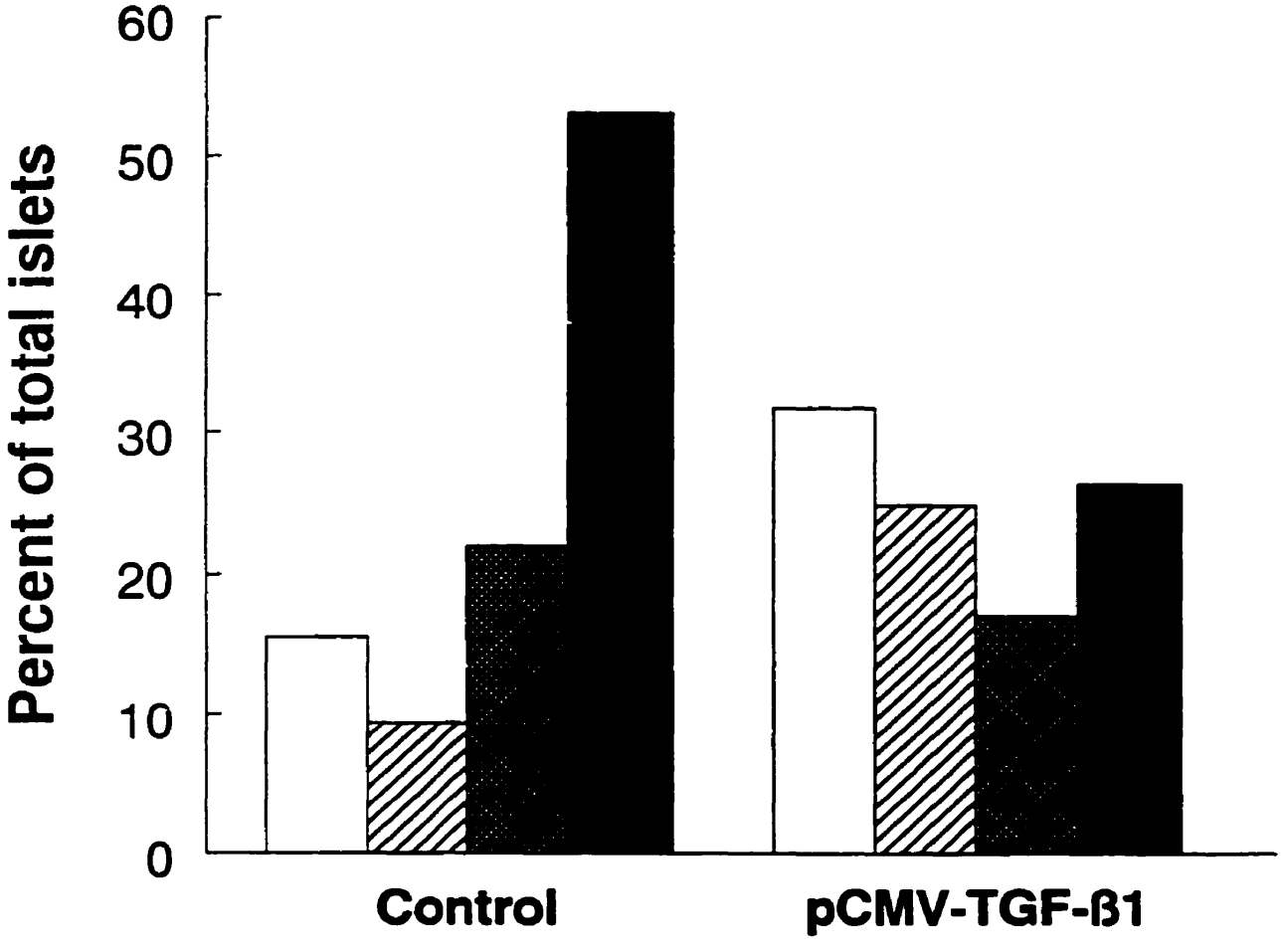

Control
pCMV-IFN-g
pCMV-TGFß1
Incidence of diabetes (%)
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
35
Days

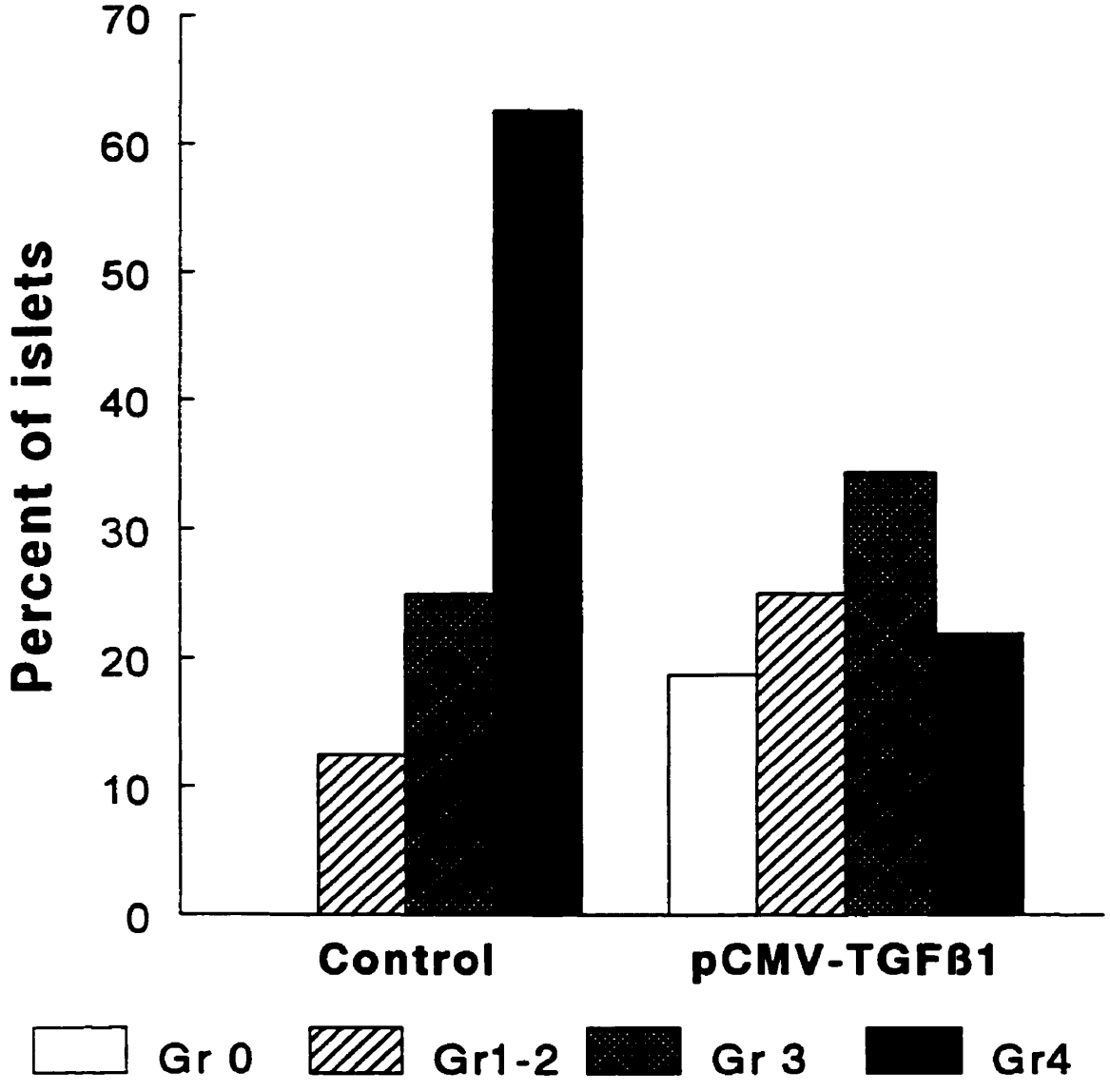

Control
pCMV-TGFß1
Incidence of diabetes (%)
100
90
80
70
60
50
40
30
20
10
0
10
12
14
16
18
20
22
24
26
28
30
32
Age (Weeks)

Protection from insulitis in pCMV-TGF-β1 treated NOD mice

Insulitis was graded histologically based on mononuclear cell infiltration of pancreatic islets. In Fig.5, we compare non-diabetic mice necropsied prior to the development of overt diabetes. In CYP-accelerated disease (Fig.5A), administration of pCMV-TGF-β1 reduced the mean insulitis score from 2.91 in control mice to 1.92 in treated mice ($p<0.001$). Protection was also observed in natural course disease (Fig.5B), where the mean insulitis score was 3.44 in control mice and 2.25 in treated mice ($p<0.01$). Thus, pCMV-TGF-β1 treatment induced a shift to lower grade lesions, including increased number of normal islets (Fig. 5A, B). This shift was more marked in CYP-induced disease, than in natural course disease, but was highly significant in both cases.

Figure 5 Insulitis scores in treated NOD mice. A. NOD mice were injected with CYP to induce diabetes and were treated with either pCMV-TGF-β1 or pCMV-null, as described in the legend to figure 4A. Mice were killed prior to the onset of diabetes, and insulitis was graded as described in Materials and Methods. The mean grade of insulitis was 1.9 in pCMV-TGF-β1 treated mice and 2.9 in null-vector treated mice ($p<0.001$). In each group, 15 mice were examined and 12-15 islets per pancreas were scored. B. Female NOD mice were treated with pCMV-TGF-β1 or pCMV-null, in the absence of CYP, as described in the legend to figure 4B. Non-diabetic mice ($n = 4$-6 per group) were killed at week 22 and insulitis was graded as described above. The mean grade of insulitis was 2.2 in pCMV-TGF-β1 treated mice and 3.4 in null-vector treated mice ($p<0.01$).

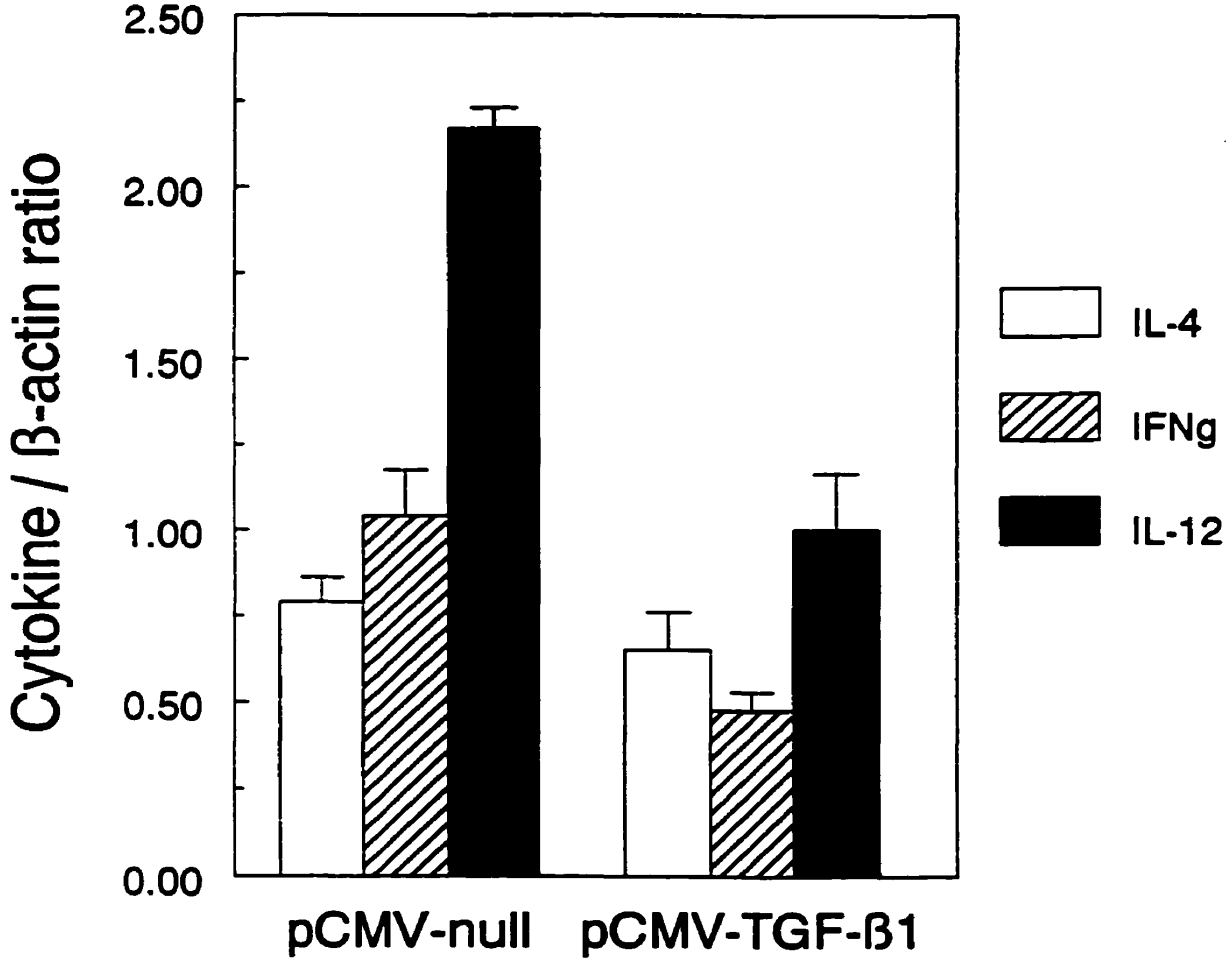

Percent of total islets
60
50
40
30
20
10
0
Control
pCMV-TGF-ß1

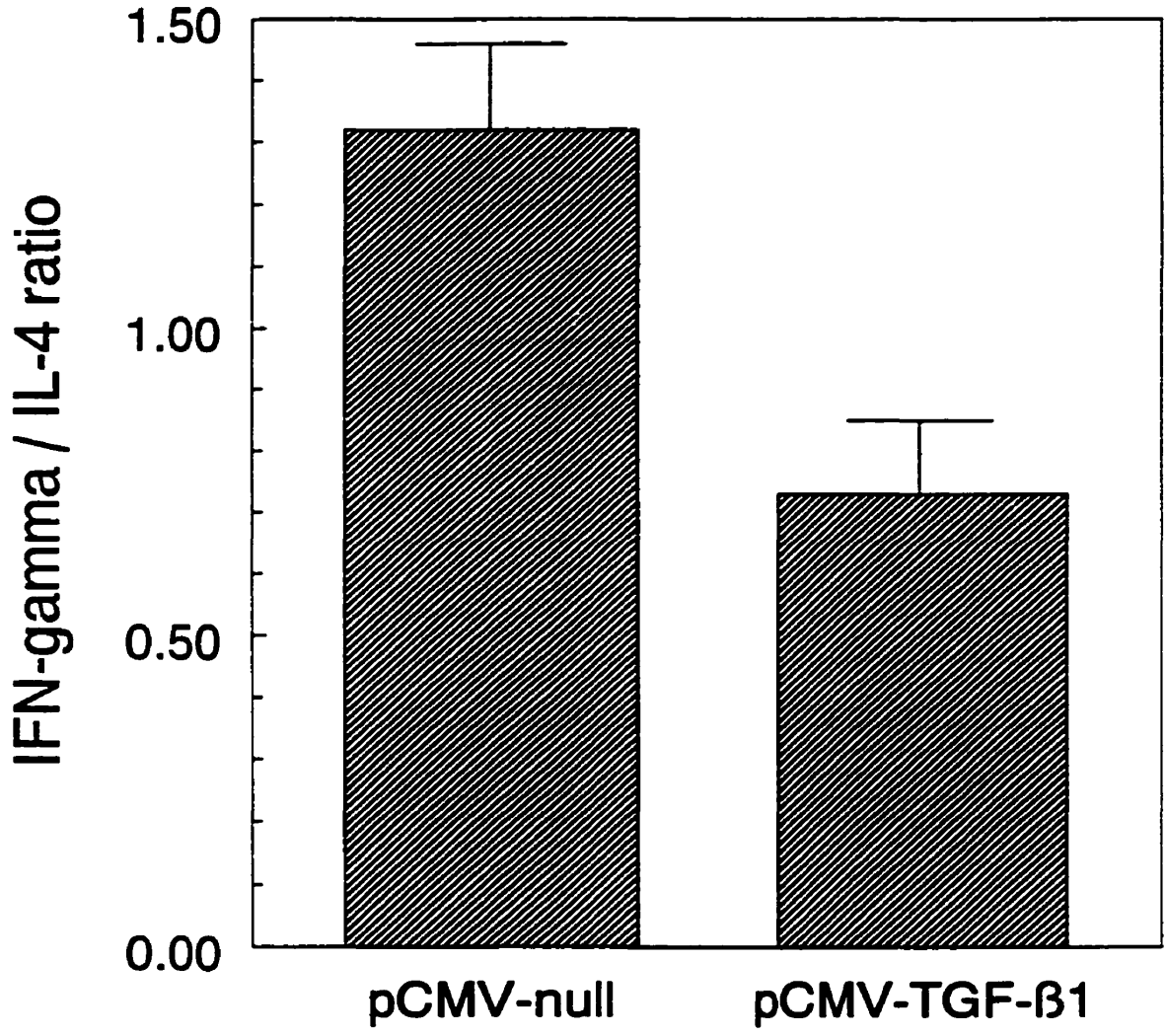

70
60
50
40
30
20
10
0
Percent of islets
Control
pCMV-TGFß1
Gr 0
Gr1-2
Gr 3
Gr4

Reduced pancreatic IL-12 and IFNγ mRNA expression in pCMV-TGF-β1 treated NOD mice

Analysis of intrapancreatic cytokine expression patterns in CYP-treated mice showed marked differences between the prediabetic pCMV-TGF-ß1-treated and control mice. Compared to pCMV-Null control mice, pCMV-TGF-ß1-treated mice had lower levels of both IFNγ ($p = 0.002$) and IL-12 ($p < 0.001$) mRNA (Fig.6A). Mean IL-4 mRNA levels were not significantly altered by pCMV-TGF-ß1 administration (a slight decrease versus control mice was not statistically significant) (Fig.6A). Consequently, there was a considerable decline of the ratio of IFNγ to IL-4 mRNA levels ($p < 0.05$) (Fig.6B).

Figure 6 Decreased pancreatic IL-12 and IFNγ mRNA expression in pCMV-TGF-β1 treated mice. Female NOD mice (70-80 days) were injected with a single dose of CYP (250 mg/kg, i.p.) to induce diabetes, and were treated with either pCMV-TGF-β1 (n=12) or pCMV-null (n=7) 48 hours prior to CYP administration. Mice were killed 10 days after CYP injection, and prior to the onset of diabetes. Reverse transcription was performed on pancreas total RNA. ^{32}P radiolabelled PCR products were electrophoresed and transferred to a nylon membrane. A. Relative quantities of RT-PCR signals for IL-12 p40 (dark bar), IFNγ (hatched bar) and IL-4 (open bar) were calculated by PhosphorImager and normalized to the ß-Actin PCR product. B. Ratio of IFNγ / IL-4 mRNA, calculated as described in Materials and Methods. In panels A and B, mean values were calculated and are presented with their SEM.


Cytokine / β-actin ratio
2.50
2.00
1.50
1.00
0.50
0.00
pCMV-null
pCMV-TGF-β1
IL-4
IFNg
IL-12


1.50
1.00
0.50
0.00
IFN-gamma / IL-4 ratio
pCMV-null
pCMV-TGF-ß1

Discussion

Cytokine therapy can influence the outcome of autoimmune diseases, by altering either Th1/Th2 balance, macrophage activity, or inflammatory versus suppressive cytokine production (15,16). In this study, we focused on the potential immunosuppressive role of TGF-β1 in autoimmune murine diabetes. TGF-β1 is a multifunctional cytokine with numerous anti-inflammatory effects (reviewed in ref. 4, 17). Therefore, we hypothesized that TGF-β1 could prevent IDDM in NOD mice, and we opted for delivery of this cytokine by a somatic gene therapy approach, consisting of i.m. injection of TGF-β1-encoding plasmid expression vector. Direct i.m. administration of naked plasmid DNA has been shown to be an effective route of gene delivery *in vivo* (5, 6, 18-20), and our objective was to use skeletal muscle as a source of TGF-β1.

Here, we demonstrate that i.m. injection of plasmid DNA encoding latent TGF-β1 (pCMV-TGF-β1), results in uptake, retention and expression of this vector by muscle cells. As demonstrated by RT-PCR, there is detectable vector-derived TGF-β1 mRNA in skeletal muscle cells, which is not seen in null-vector treated muscles. Moreover, plasma samples collected from pCMV-TGF-β1 treated mice show significantly elevated levels of TGF-β1 for well over two weeks. Over that time period, the TGF-β1 levels were 2-4 fold higher than those of control mice.

We found that DTH responses to OVA are markedly suppressed in pCMV-TGF-β1 treated mice. Thus, it is apparent that sufficient quantities of TGF-β1 are produced to exert as immunosuppressive effect. Moreover, the production of TGF-β1 protects NOD mice from an autoimmune disease.

NOD mice spontaneously develop an autoimmune form of insulitis, with destruction of beta-islet cells (reviewed in Ref. 12). Insulitis is the precursor lesion, leading to diabetes when it is sufficiently severe. This disease is clearly T-cell and macrophage dependent, although the mechanism of islet cell destruction are not fully elucidated. Islet cells may be killed by infiltrating CTLs, NK cells and macrophages (12). Proinflammatory cytokines (IL-1, IFNγ, TNFα), as well as nitric oxide (NO), are toxic to islet cells (14, 21). Th1-dependent immunity appears to be a key pathogenic factor, since, for example, administration of IL-12 enhances Th1 reactivity to islet-cell antigens and rapidly induces diabetes (22). IL-12 and IFNγ are produced locally in inflamed islets, and insulitis can be induced by adoptive transfer of islet-reactive Th1 clones (23). The administration of CYP to NOD mice stimulates intra-islet production of IFNγ and other inflammatory mediators, and results in rapid onset of diabetes (14).

We found that administration of pCMV-TGF-β1 considerably reduced the incidence of diabetes in NOD mice. In CYP-induced diabetes, there was a four-fold reduction in incidence (d 32 of the experiment). In non-CYP-treated mice (natural course), treatment delayed the appearance of the first case of diabetes, and subsequently reduced the incidence of disease by approximately 50 % over the course

of several weeks. In the latter mice, treatment was begun at 9-11 weeks, i.e., at a time when insulitis is already apparent, suggesting that an ongoing autoimmune response was suppressed.

pCMV-TGF-β1-treated mice had low insulitis scores, with markedly increased numbers of normal, or islets with mononuclear cell infiltrates limited to the peri-islet was (peri-insulitis). In TGF-ß1-treated mice following CYP administration, analysis of pancreatic cytokine mRNA expression by semi-quantitative RT-PCR revealed depressed IL-12 and IFNγ levels. IL-4 levels were not significantly altered. Consequently, the ratio of IFNγ to IL-4 mRNA was reduced by TGF-ß1 plasmid DNA therapy. Protection from insulitis was somewhat greater in CYP-accelerated than in natural course disease, but was statistically significant in both cases. Taken together, our results reveal that TGF-β1 plasmid therapy had an anti-inflammatory or immunosuppressive effect. As expected, mice that were protected from insulitis were also protected from diabetes.

The immunoregulatory influence of TGF-β1 has been studied in many *in vivo* models including experimental allergic encephalomyelitis (EAE) (24), collagen-induced arthritis (CIA) (25) and allograft rejections (26). In all of these instances, microgram amounts of TGF-β1 were administered, over a period of a few days, in order to achieve a significant suppression of autoimmune responses. Since we could suppress DTH and prevent autoimmune disease expression with i.m. injections of

pCMV-β1, it appears that we are achieving results equivalent to administration of microgram quantities of TGF-β1 protein.

TGF-β1 may act at multiple levels to block inflammatory reactions and/or prevent autoimmune disease. The potent immunosuppressive effects of TGF-β1 were clearly demonstrated in the TGF-β1 knock-out mice which died at an early age with a multi-organ inflammatory syndrome (27). The absence of TGF-β1 was associated with increased production of inflammatory cytokines such as IFNγ and TNFα, and an increased number of activated immune cells in peripheral lymphoid organs.

Two recent studies (9,28) demonstrate that TGF-ß (ß1 or ß2) can modulate macrophage activity in a way that favors Th2 over Th1 differentiation. Our results are consistent with these observations, since with TGF-ß1 therapy we see suppression of the type 1 proinflammatory cytokines IL-12 and IFNγ, with minimal alteration of IL-4 gene expression. Based on published data, the regulatory activity of TGF-ß1 may result from a direct action on macrophages or may be a consequence of decreased Th1 reactivity. In fact, this cytokine could be blocking several steps of an immune reaction, including antigen processing/presentation by APCs (29), activation/differentiation of Th1 cells (30), production of inflammatory cytokines and NO (30,31), and activation of effector cells (CTLs, macrophages, NK cells) (32). TGF-β1 blocks Jak-STAT signaling in T cells by preventing tyrosine phosphorylation and activation of Jak-1 and Stat-5 (33). It inhibits IL-2R expression, and can also induce apoptosis in T cells (33). These mechanisms are not mutually exclusive, and it would be difficult to ascertain

their relative importance *in vivo* in NOD mice. Interestingly, the increased number of normal islets in TGF-β1-treated mice suggests that either fewer islet-reactive T cells are activated or that their migration to islets of Langerhans is impeded. The site of activation of latent TGF-β1 is unclear, as discussed below.

Previously, others found that i.m. injection of TGF-β1 expression vectors was therapeutically effective in models of inflammatory bowel disease (34) and SLE (35). These investigators administered plasmid vectors with a Rous sarcoma virus enhancer/promoter (compared to a CMV enhancer/promoter in our study), and either did not report an increase in plasma TGF-β1 (34), or observed a smaller increase than in our experiments (35). It appears that we achieved a higher level of TGF-β1 production, possibly because the CMV enhancer/promoter of our vector generates a higher transcriptional activity (5, 8).

Adoptive transfer of TGF-β1 producing islet-reactive CD4+ T cells prevents diabetes in NOD mice (36). However, transgenic NOD mice producing active TGF-β1 in their islets (rat insulin promoter) developed insulitis and marked fibrosis in the pancreas (37). Immunoprotection may depend on the TGF-β1 levels achieved locally or systemically, and/or the state of TGF-β1 activation.

The ability of TGF-β1 to induce fibrosis and extracellular matrix formation (38) is a potential concern. In mice treated with pCMV-TGF-β1, we did not observe fibrosis or inflammation in muscles, kidneys, lungs, liver, heart or pancreas. We speculate that the latent TGF-β1 is not activated at the site of plasmid administration,

since active TGF-β1 was not detectable in plasma. Others have shown (26) that administration of latent TGF-β1 is probably an advantageous feature, since it may become biologically active only at distant inflammatory sites, through the action of macrophages, low pH or other factors (26).

As mentioned above, IFNγ may contribute to islet cell injury, since it has direct toxic effects on islet cells, and may also act by activating macrophages and stimulating NO production. In accord with this view, disease is delayed in IFNγ gene knockout NOD mice (39), and prevented by anti-IFNγ mAb treatment (40). To confirm the detrimental effects of IFNγ, as well as the efficacy of our gene delivery method, we administered an IFNγ expressing vector (pCMV-IFNγ) to CYP-treated NOD mice. The serum levels of IFNγ were increased in pCMV-IFNγ treated mice (up to 200 pg/ml), and this was sufficient to induce an earlier onset of diabetes. Acceleration of this disease by administration of IFNγ had not been reported before.

To our knowledge, we are the first to demonstrate that IDDM can be prevented by cytokine i.m. somatic gene therapy, and to demonstrate that TGF-β1 is therapeutically effective in this disease. Clinically, the use of cytokines has been limited by their short half-life and the necessity to administer relatively large quantities (often in boluses) of recombinant proteins, with considerable associated toxicity (41). Somatic cytokine gene therapy has the potential of circumventing these problems by minimizing the need for frequent protein injections, producing more constant blood levels, reducing side effects, and increasing therapeutic efficacy. Furthermore, direct

DNA injection in skeletal muscle appears to be safe and is technically simple. Unlike some viral vectors, the plasmid vectors are maintained episomally (5), minimizing the risk of genomic insertional mutagenesis. Moreover, the use of plasmid vectors eliminates the possibility of immune responses to viral antigens.

Various non-viral means of introducing DNA into cells have been developed. Although cationic liposomes usually enhance DNA uptake by cells, i.v. injection of DNA/liposome complexes has met with limited success. In that case, the cells of several organs are transfected (particularly endothelial cells) (42, 43), but the persistence of gene expression is usually much less than after i.m. delivery (43; and our unpublished observations). The potential toxicity of liposomes must also be considered (43). Thus, the muscle cell is an excellent target for gene therapy, and in these cells injection of naked plasmid DNA has proven to be an effective method.

Acknowledgments

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Connecting text for Chapter IV.

In the previous chapter, I demonstrated that i.m. administration of a TGF-ß1 plasmid expression vector resulted in significant elevations of latent TGF-ß1 in plasma capable of suppressing a DTH response, and at protecting NOD mice from autoimmune insulitis and diabetes. In Chapter IV, I then decided to demonstrate applicability of our cytokine gene therapy approach to other diseases by administering plasmid expression vectors encoding either IL-4 or TGF-ß1 in a model of CD4+ Th1 cell mediated disease : EAE. In this chapter, I hypothesized that TGF-ß1 or IL-4 plasmid therapy could suppress autoantigen-induced autoimmune responses.

EAE is an inflammatory autoimmune disease of the CNS sharing many clinical features to multiple sclerosis in humans. EAE can be experimentally induced in mice by immunizing with MBP in CFA. In addition to *in vivo* findings, this provides us with the possibility of examining specific immunological parameters such as autoantigen-induced T cell proliferation and cytokine expression *in vitro*. These studies would have been difficult to perform in the NOD mouse model, where autoantigens are poorly defined and/or not easily purified. Thus, EAE serves as an ideal experimental model of autoimmunity for our cytokine plasmid therapy.

CHAPTER IV

Prevention of experimental allergic encephalomyelitis by intramuscular gene therapy with cytokine-encoding plasmid vectors

by

Ciriaco A. Piccirillo and Gérald J. Prud'homme

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Abstract

Anti-inflammatory cytokines such as transforming growth factor beta 1 (TGF-β1) and IL-4 can protect from autoimmune diseases. To study the immunoregulatory effects of these cytokines *in vivo*, we used a method of gene therapy which permits continuous cytokine delivery over a period of weeks. We injected naked plasmid DNA expression vectors encoding either TGF-β1 (pVR-TGF-β1) or an IL-4-IgG1 chimeric protein (pVR-IL-4-IgG1) intramuscularly (i.m.). Treatment with pVR-TGF-β1 and pVR-IL-4-IgG1 resulted in elevated circulating levels of TGF-β1 or IL-4-IgG1, respectively. These levels were sufficient to exert anti-encephalitogenic effects in mice with myelin basic protein (MBP)-induced experimental allergic encephalomyelitis (EAE). TGF-β1 gene delivery had pronounced downregulatory effects on T cell proliferation and production of IFN-γ and TNF-α, upon *in vitro* restimulation with MBP. IL-4-IgG1 also suppressed these responses, though much less than TGF-β1, and enhanced secretion of IL-4. Therapy resulted in a significant decrease in the severity of histopathologic inflammatory lesions. With either vector, CNS IL-12 and IFNγ mRNA expression was significantly diminished, while IL-4 and TGF-β1 mRNA levels were increased compared to control mice. Thus, cytokine plasmid treatment appeared to suppress MBP-specific pathogenic Th1 responses, while enhancing endogenous secretion of protective cytokines. We demonstrate that gene therapy with these vectors is an effective therapeutic strategy for EAE.

Introduction

EAE is an inflammatory, autoimmune disease of the central nervous system (CNS) sharing many clinical and pathological features with multiple sclerosis in humans (1). EAE is characterized by perivascular leukocytic infiltration (CD4+ T cells and macrophages) and demyelination of the CNS, with a relapsing and remitting mode of progression (2). EAE can be induced in mice by immunization with either whole brain homogenate, purified myelin basic protein (MBP), or proteolipid protein (PLP) (1). The target of the autoimmune inflammatory response is the myelin sheath that envelops axons, as well as the oligodendrocytes that produce the myelin (2).

There is compelling evidence for a role of proinflammatory cytokines produced by Th1 cells and macrophages in the pathogenesis of EAE (3). MBP-specific T cell lines that adoptively transfer disease in mice produce the Th1 cytokines IFNγ, IL-2, TNFα and β, and these cytokines are also present in the CNS of animals with active disease (4,5). Conversely, spontaneous recovery of mice from EAE correlates with IL-4 and TGF-β1 production, and with the development and expansion of CNS antigen-specific Th2 cells (6,7). As well, a class of protective T cells secreting TGF-β1 (Th3 cells) have been described (8,9). Treatment of mice with IL-4, TGF-β1 or mAbs against IL-12 ameliorate inflammatory responses in EAE by either downregulating the

pathological Th1 cell-mediated response, or inducing the development of protective Th2 or Th3 cells (10-12). In accordance, mAbs directed against TGF-β1 and IL-4 exacerbate EAE (13,14).

In this study, we examined and compared the immunoregulatory effects of TGF-β1 and IL-4 in murine EAE. Cytokines were delivered an by intramuscular (i.m.) gene therapy approach. Gene therapy obviates the need for frequent and costly protein administration, and provides relatively constant cytokine delivery over a period of weeks (15,16,17). We constructed vectors that encode latent TGF-β1 or an IL-4-IgG1 (heavy chain) fusion protein.

We found that administration of plasmid DNA encoding either TGF-β1 or IL-4-IgG1 delayed the onset and considerably reduced the severity of EAE. Gene transfer of either cytokine had suppressive effects on MBP-stimulated T cell proliferation and cytokine production. Protection from EAE correlated with a significant decrease in CNS histopathology and inflammatory cytokine production (IL-12 and IFN-γ), with upregulation of endogenous TGF-β1 and IL-4. Though both cytokines were protective, TGF-β1 had much greater suppressive effects on several parameters. Intramuscular injection of cytokine-encoding plasmid DNA vectors represents a novel therapeutic strategy for the treatment of EAE and other autoimmune diseases.

Materials and Methods

Mice

Female SJL/J mice (6-8 weeks) were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were kept in a pathogen-free facility.

Plasmid DNA Vector Construction

We subcloned the full-length mouse TGF-β1 cDNA was into pVR1255 (VICAL, San Diego), whose characteristics have been previously described (18). The resulting plasmid expression vector, pVR-TGF-β1, is under the transcriptional control of a cytomegalovius (CMV) immediate-early enhancer-promoter. pVR-TGF-β1 encodes the latent form of mTGF-β1. Expression was confirmed in supernatants collected from transiently transfected COS-7 cells. The latent TGF-β1 was activated by acidification, and detected by ELISA (R&D, Minneapolis, MN), and its bioactivity was confirmed by the CCL64 mink cell line proliferation assay. Both the ELISA and bioassay detect only active TGF-β1.

The mIL-4 cDNA was isolated by RT-PCR from an IL-4-producing cell line, and fused to an mIgG1 heavy chain segment (part of CH1, hinge, CH2 and CH3) by

overlapping PCR gene assembly. The resulting mIL-4/IgG1 fusion product was then subcloned into the EcoRV/BamH1 sites of pVR1255, to produce pVR-IL-4-IgG1. COS-7 cells transfected with this plasmid secrete a disulfide-linked homodimer of 100-105 kD, as depicted in Fig.1 (G.Prud'homme and Y.Chang, manuscript in preparation). IL-4 activity was confirmed in the CT.4S IL-4-dependent cell line proliferation assay. pVR1012 (VICAL, San Diego, CA), which does not encode any gene product in mammalian cells, was used as a control vector in all the experiments. pVR1012 is hereafter referred to as pVR-null.

Plasmid DNA Preparation

Large-scale plasmid DNA preparations were produced by the alkaline lysis method using a Qiagen giga kit (Qiagen Inc., Santa Clarita, CA). All plasmid preparations for i.m. injections were resuspended in sterile 0.85% saline. Spectrophotometric analysis revealed 260/280 nm ratios to be 1.80 or higher. Plasmid DNA preparations were free of bacterial RNA or genomic DNA, as visualized on a 1% agarose gel. Gel electrophoresis was also used to confirm the percentage of supercoiled plasmid (greater than 95%).

Intramuscular injection of plasmid DNA

Intramuscular injections of plasmid DNA were done as described previously (15). Briefly, mice were anaesthetized by i.p. injection with xylazine (10mg/kg) and

ketamine (200 mg/kg). The tibialis anterior (TA) muscles of each mouse were injected with a 0.5 cc sterile 29G1/2 insulin syringe, fitted with a plastic collar to limit needle penetration to 2 mm. Mice received 50 μg of pVR-TGF-β1 or pVR-IL4-IgG1 in 50μl of sterile saline in each TA muscle, for a total of 100 μg of plasmid DNA, 48 hours before each MBP immunization session. Control mice received equivalent amounts of pVR-null control vector in each muscle group.

Induction of EAE

EAE was induced as previously described (5). Briefly, 6-8 week old SJL/J mice were injected with two s.c. injections 7 days apart, in the base of the tail and flank, respectively, of 400 μg purified myelin basic protein (MBP, Sigma Chemicals Co., St. Louis, MO) in CFA (Difco, Detroit, MI), containing 50 μg H37RA *Mycobacterium tuberculosis* (Difco). Symptoms were first observed 12 days after the initial injection.

Clinical evaluation of EAE

Mice were monitored up to day 21 post-immunization with MBP and clinical signs of disease were assessed daily, as described (5) : 0 = normal, no sign of disease; 1 = flaccid tail; 2 = hind limb weakness; 3 = Moderate hind limb paralysis with mild forelimb weakness; 4 = Total paralysis of hind limbs with moderate forelimb weakness; 5 = death. A clinical index was established by determining the mean (± SEM) of the clinical scores of all the mice in each experimental group. Clinical

evaluation of disease was done in double-blind fashion by two observers. Statistical analysis was performed with the Student's t test.

Lymphocyte proliferation

Lymph node cells (LNC) were prepared from draining lymph nodes of treated mice immunized with MBP/CFA. Proliferative responses were assessed by incubating LNC (8×10^5 cells/well) with MBP (50 μg/ml), Concanavalin A (ConA)(5 μg/ml), or no antigen. The cultures were maintained in 96-well, flat-bottom microtiter plates for 96 hours at 37° C in humidified 5% CO_2 air. The wells were pulsed with 1μCi/well of [^{3}H]thymidine (NEN) for the final 16 hours of culture. Results are reported as the mean cpm of triplicate cultures ± SEM.

Cytokine ELISA

Commercial ELISAs (R&D Systems, Minneapolis) were used to measure IFNγ, IL4, TNFα and TGF-β1 in culture supernatants. The ELISA detection limits were < 2 pg/ml for IFNγ, <2 pg/ml for IL4, <5 pg/ml for TNFα and <5pg/ml for TGF-β1.

Histological analysis

On day 21, the spinal cord was excised immediately after CO_2 asphyxiation, and fixed in 10% buffered formalin. Histological slides were prepared and stained

with hematoxylin and eosin (H/E), and luxol fast blue (LFB). Sections from three randomly obtained levels of spinal cord were examined by light microscopy and scored for leukocytic inflammation, as previously described (19). Briefly, 0, no inflammatory cells; 1, mild inflammation; 2, moderate inflammation; 3, extensive inflammation into the parenchyma. Statistical analysis was performed with the Chi-squared test.

PCR and RT-PCR analysis

Total RNA was isolated from snap-frozen CNS tissue, and was reverse transcribed with the Superscript preamplification system (Gibco BRL). 2μls of the reverse transcription reaction were used for PCR amplification using cytokine-specific primers, namely IL-12 p40 (20), IL-4 (21), IFNγ (21), TGF-β1 (21) and 22ß-actin (5). PCR cycling conditions were as follows: one cycle at 94°C; 28-30 cycles at 94°C for 1 minute, at 60°C for 2 minutes and 72°C for 2 minutes; and one final extension cycle at 72°C for 10 minutes. Analysis of cytokine PCR products was performed as previously described (5). Briefly, PCR reactions were terminated in the linear portion of the amplification reaction. The PCR products were analyzed on 2% agarose gel containing 0.5 μg/ml ethidium bromide, transferred onto Hybond-N+ nylon membrane (Amersham Canada, Oakville, ON) by vacuum blotting (Pharmacia, Baie d'Urfe), and hybridized by incubation with ^{32}P-labeled cDNA probes. Cytokine probes consisted of 25 nucleotide oligonucleotides which bound to internal portions of PCR amplimers. Probes were prepared by labeling 50 ng DNA with [α-^{32}P]dCTP using a T4

polynucleotide kinase kit (Pharmacia, Baie d'Urfe). Blots were either exposed to autoradiographic film or subjected to PhosphorImager analysis.

Semi-quantitative analysis of mRNA levels was performed by calculating relative quantities of RT-PCR signals for each cytokine, normalized to the ß-actin signal of each sample. The ratio of IFNγ to IL-4 mRNA (for each individual mouse) was derived after normalization of these cytokines with their ß-actin signal, as described above : IFNγ / IL-4 ratio = (IFNγ / ß-actin ratio) ÷ (IL-4 / ß-actin ratio). Statistical analysis was performed with Student's t-test.

Results

Circulating cytokine levels following i.m. gene transfer

We previously established the optimal dose of plasmid DNA for our experiments by injecting increasing amounts of TGF-β1 plasmid DNA at different muscle sites and measuring TGF-β1 levels in platelet-poor plasma by ELISA. We found that plasma TGF-β1 levels increase with the number of injection sites and amount of DNA injected per site, up to 100μg of DNA per injection site (15). We further determined that latent TGF-β1 plasma levels were increased by at least 2-4 fold for well over two weeks following a single injection of plasmid DNA (15). In accord with this, by day 21 of the current study, TGF-β1 plasma levels were 7.1 ± 0.3 ng/ml, compared to 3.1 ± 0.5 ng/ml in control mice (n= 3 per group) ($p<0.05$).

Similarly, following i.m. injection of pVR-IL-4-IgG1, the IL-4-IgG1 fusion protein was detectable in serum, with a specific ELISA assay. From day 7 to 21 of the experiment, mean serum IL-4-IgG1 levels ranged between 0.4-2 ng/ml, and as expected the molecule was undetectable in control mice (n= 3 per group).

Prevention of EAE by pVR-TGF-β1 and pVR-IL-4-IgG1 administration

To determine if pVR-TGF-β1 and pVR-IL-4-IgG1 could prevent the induction of EAE, we administered these expression vectors to adult SJL/J female mice. Mice received a total of 50 μg of either pVR-TGF-β1 or pVR-IL-4-IgG1 in sterile saline in

each TA muscle for a total of 100 μg of plasmid DNA, 48h before each MBP immunization. Control mice received equivalent amounts of pVR-null control vector. Mice were graded daily for clinical signs of EAE.

By day 21 of the experiment, the incidence of EAE was lower by approximately 70% in both pVR-TGF-β1 and pVR-IL-4-IgG1 groups, compared to mice receiving pVR-null ($p<0.001$) (Fig.2). All mice in the control group manifested signs of disease throughout the course of the experiment. Cytokine vector-treated mice that developed EAE only displayed a mild form of the disease, with the exception of one mouse in each group. Compared to pVR-null control mice, the first case of EAE occurred 6 days later in the pVR-TGF-β1 group and 4 days later in pVR-IL-4-IgG1 treated mice. The mean clinical score at day 21 post-immunization was 0.3 in pVR-TGF-β1 mice, 0.5 in pVR-IL-4-IgG1 mice and 2.6 in pVR-null control mice ($p<0.001$ versus null vector control mice in both cases). Thus, the disease severity of pVR-IL-4-IgG1 and pVR-TGF-β1 treated mice was significantly lower than control mice. There were no deaths in any group.

Figure 1 Schematic diagram of the IL-4-IgG1 fusion protein. See Materials and Methods for vector construction and properties of the protein.

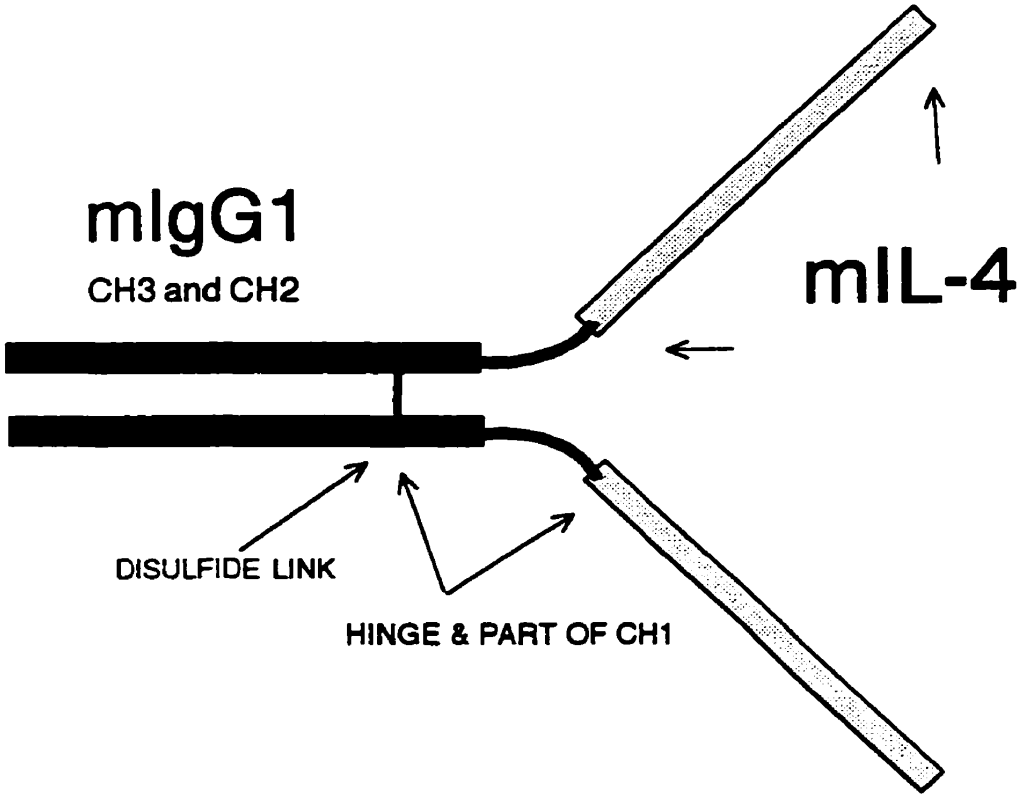
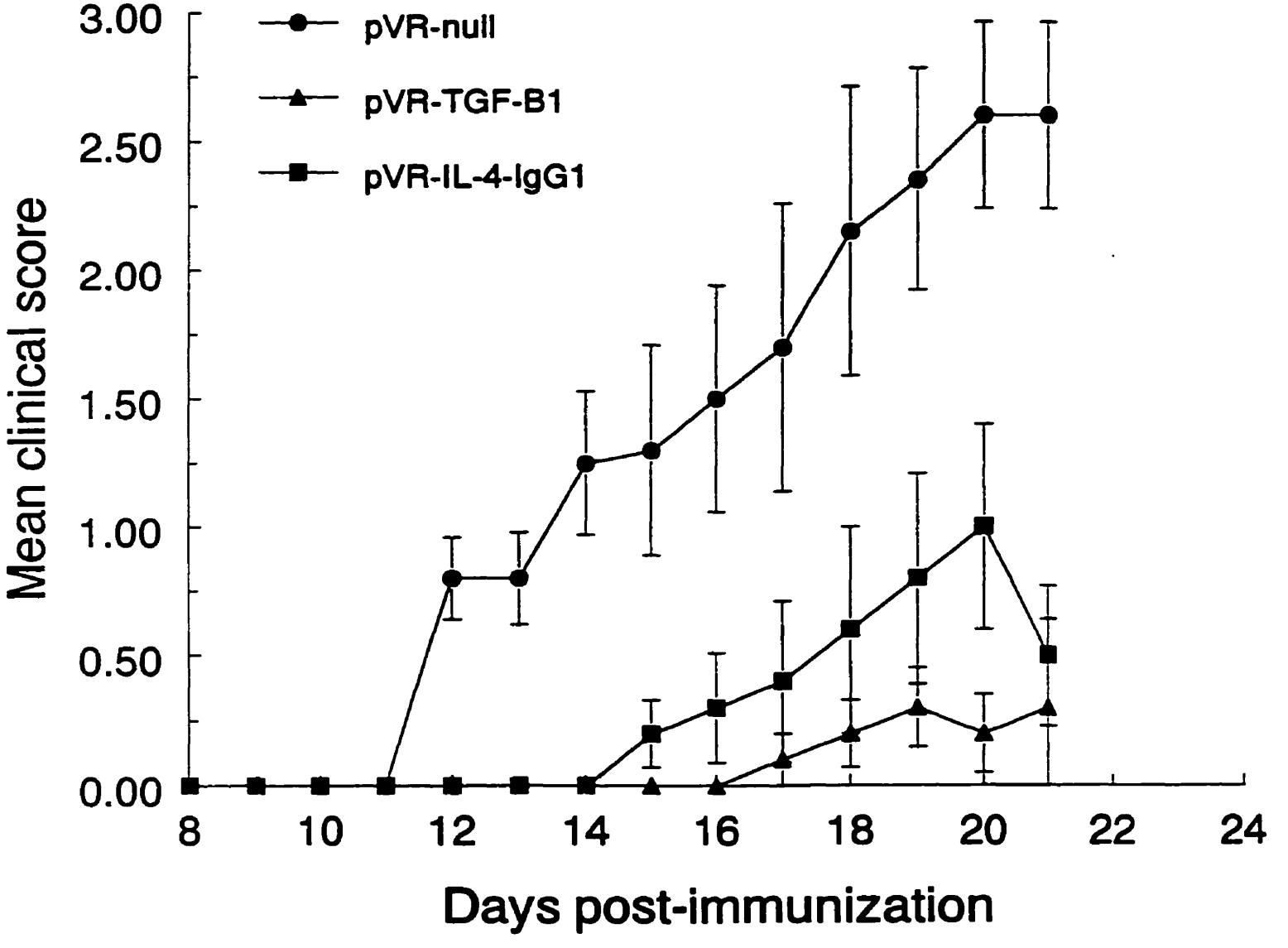

mIgG1
CH3 and CH2
mIL-4
DISULFIDE LINK
HINGE & PART OF CH1

Figure 2 Administration of pVR-TGF-β1 and pVR-IL-4-IgG1 reduces the incidence of autoimmune encephalomyelitis. EAE was induced in 6-8 week old female SJL/J mice by MBP immunization on days 0 and 7. Intramuscular administration of 100μg of pVR-TGF-β1 or pVR-IL-4-IgG1 (n=10 per group) on days -2 and 5, significantly reduced the severity of EAE compared to pVR-null treated mice (n=10). pVR-TGF-β1 (triangles); pVR-IL-4-IgG1 (squares); pVR-null treated mice (circles).


pVR-null
pVR-TGF-B1
pVR-IL-4-IgG1
Mean clinical score
3.00
2.50
2.00
1.50
1.00
0.50
0.00
8
10
12
14
16
18
20
22
24
Days post-immunization

CNS leukocytic infiltration is reduced in pVR-TGF-β1 treated mice

CNS inflammation was graded histologically. Administration of pVR-TGF-β1 resulted in a reduction of the mean histological score from 1.77 in control mice to 0.55 in TGF-β1-treated mice ($p<0.001$) (Fig.3). Similar treatment with pVR-IL-4-IgG1 reduced the clinical score to 0.88 ($p<0.001$ versus null-treated mice). Thus, TGF-β1 and IL-4-IgG1 plasmid gene therapy induced a shift to lower grade lesions.

Reduced MBP-stimulated T cell proliferative responses in pVR-TGF-β1 and pVR-IL-4-IgG1 treated mice

We stimulated freshly isolated LNC from MBP-immunized mice with MBP *in vitro*. While control T cells (pVR-null-treated mice) from draining lymph nodes proliferated as expected in response to MBP (mean SI = 10), LNC from TGF-β1 and IL-4-IgG1 plasmid treated mice had responses that were 94% and 36% lower, respectively ($p<0.01$, versus control mice in both cases) (Fig.4). There was no significant difference in the proliferative response to antigen in mice treated with pVR-null versus those receiving only sterile saline (data not shown).

Interestingly, responses to ConA stimulation were not statistically different between control and cytokine plasmid treated mice (Fig.4), suggesting that inhibition was restricted to antigen-induced responses.

Figure 3 Reduction of EAE histopathological scores in cytokine-vector treated mice. Female SJL/J mice were treated on day -2 with either pVR-TGF-β1, pVR-IL-4-IgG1, or pVR-null, and 48 hours later were immunized with MBP to induce EAE (day 0). The entire process was repeated one week later (day 5 and day 7, respectively). Mice were killed during active EAE, on day 21 of the experiment, and CNS inflammatory infiltrates were graded as described in Materials and Methods.

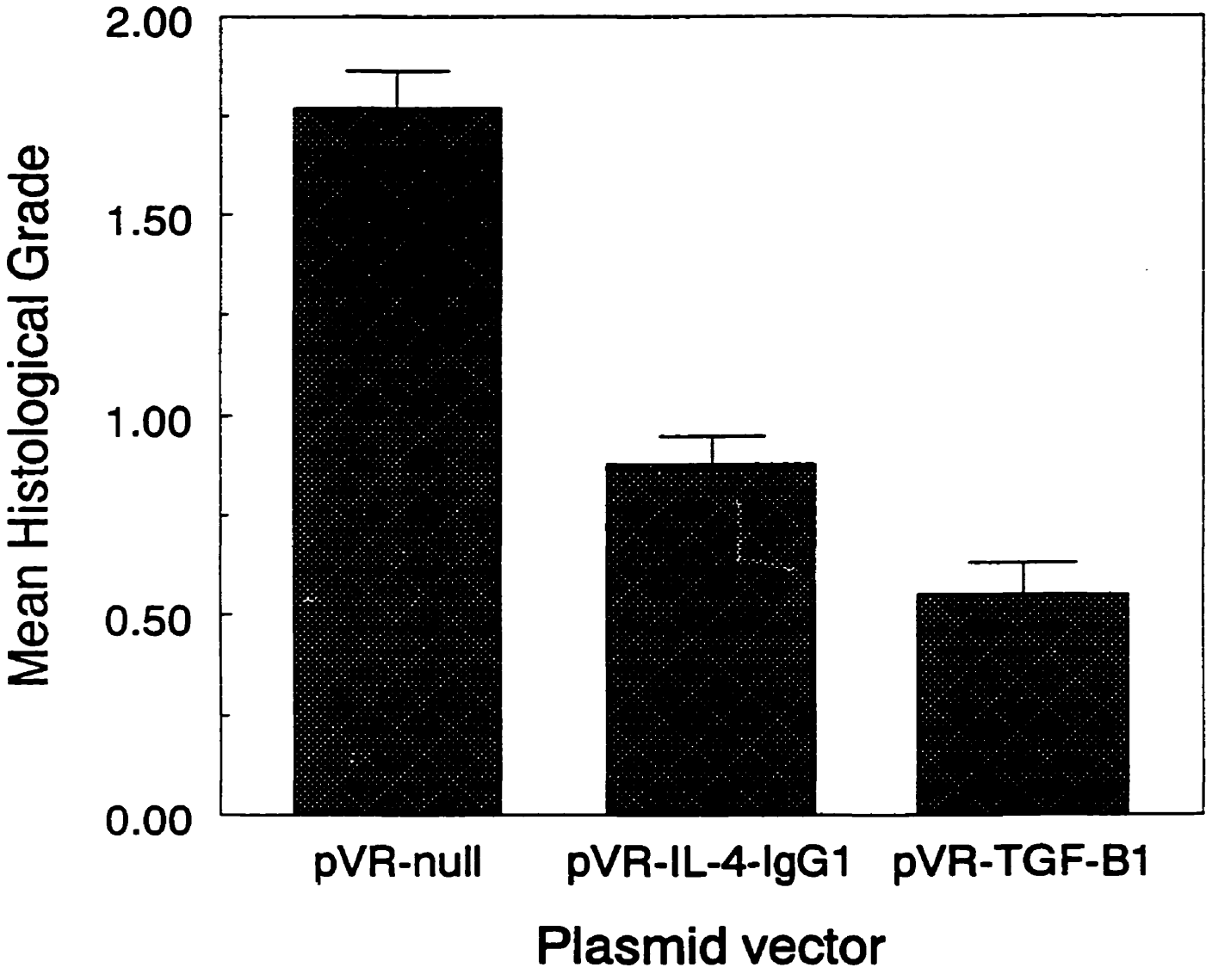
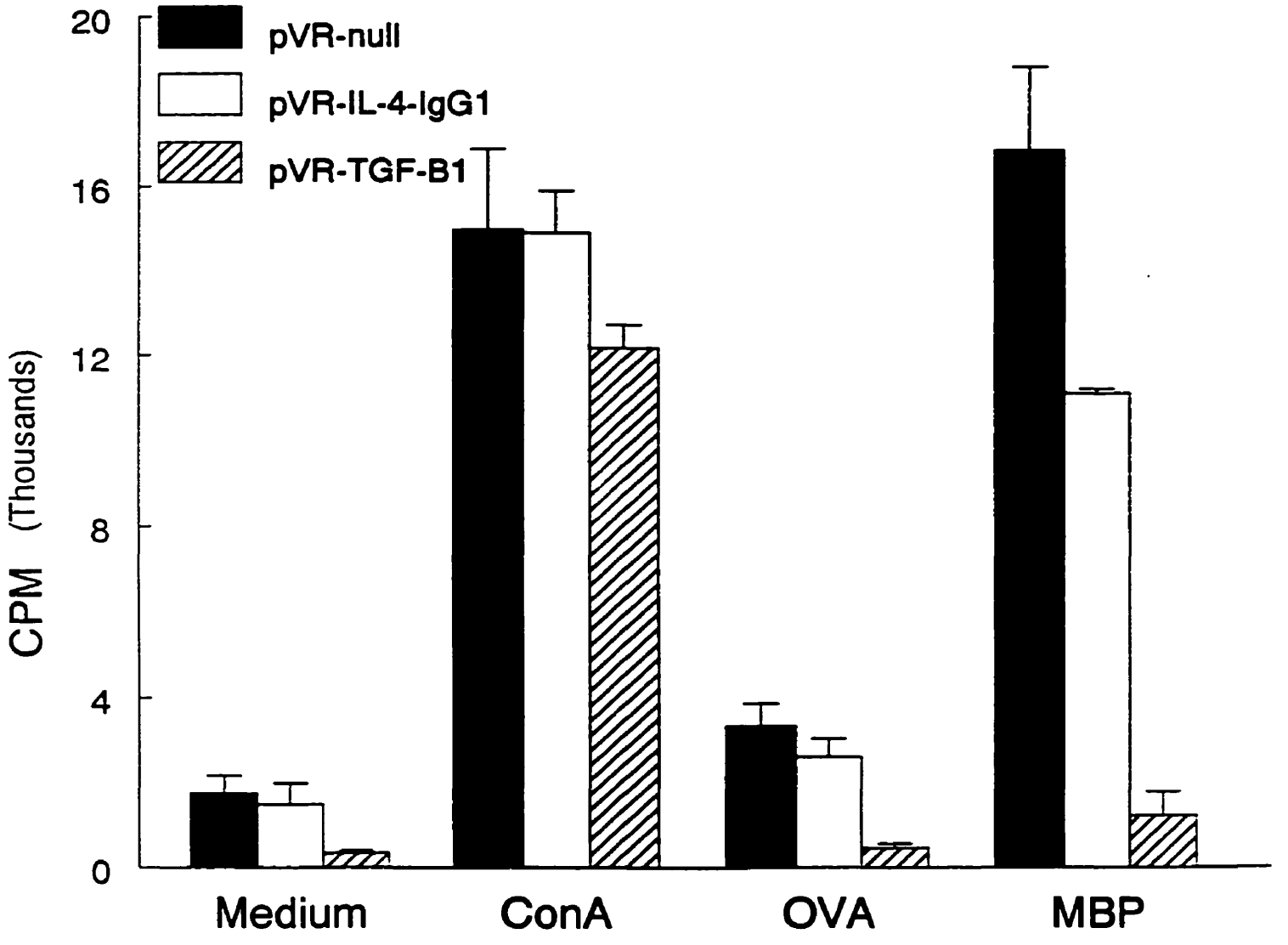

2.00
1.50
1.00
0.50
0.00
Mean Histological Grade
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1
Plasmid vector

Figure 4 Suppressed *in vitro* LNC proliferation to MBP after TGF-β1 and IL-4-IgG1 plasmid therapy. LNC were prepared from draining lymph nodes of plasmid treated mice that had been immunized with MBP/CFA. Immunization (day 0) followed injection with either pVR-TGF-β1, pVR-IL-4-IgG1, or pVR-null by 48 h. The cells were recovered on day 21 and were stimulated with MBP, OVA, or ConA *in vitro* (see text). The results represent the mean cpm ± SEM of triplicate wells (n=10 per group), in a 4-day culture.

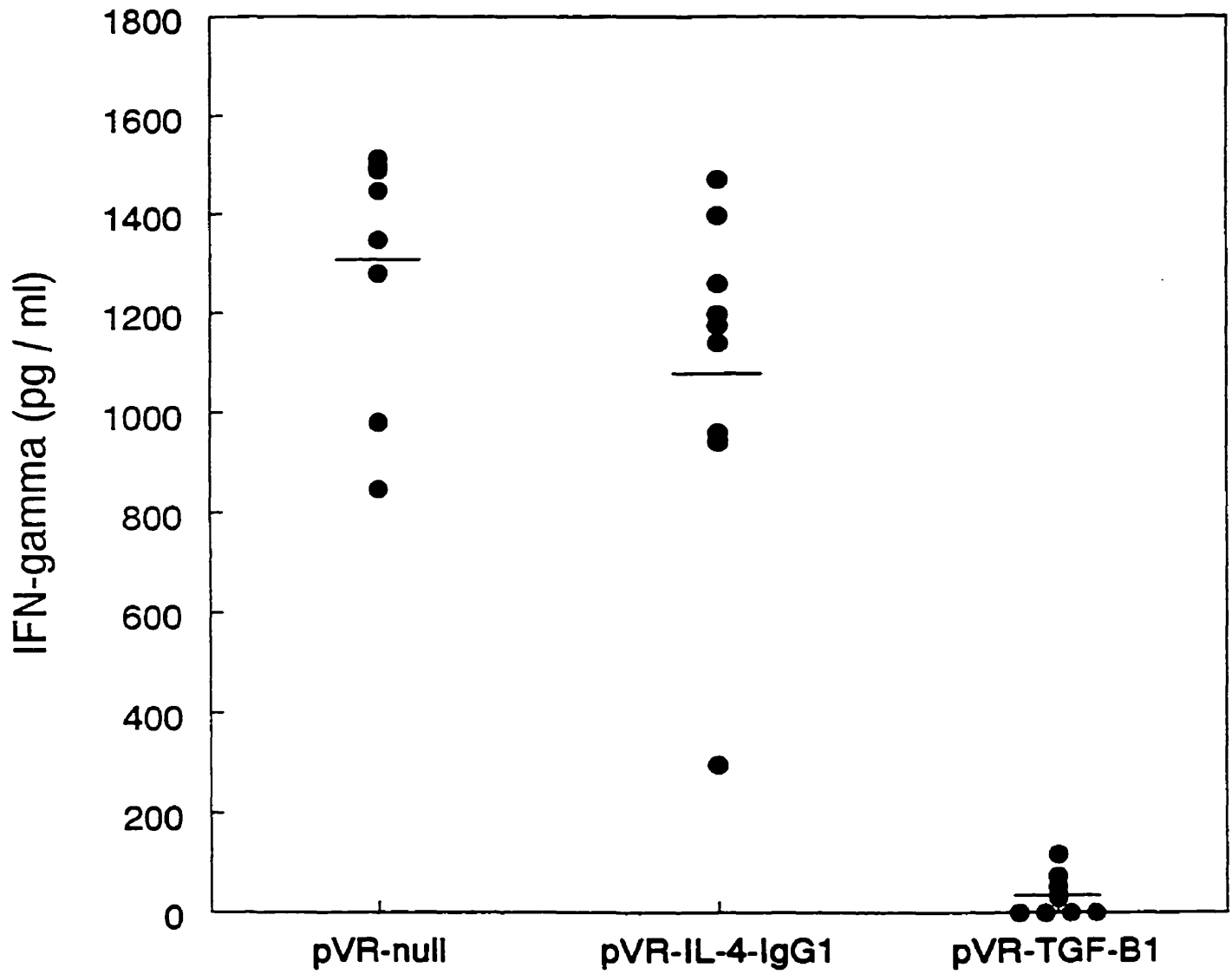

20
16
12
8
4
0
CPM (Thousands)
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1
Medium
ConA
OVA
MBP

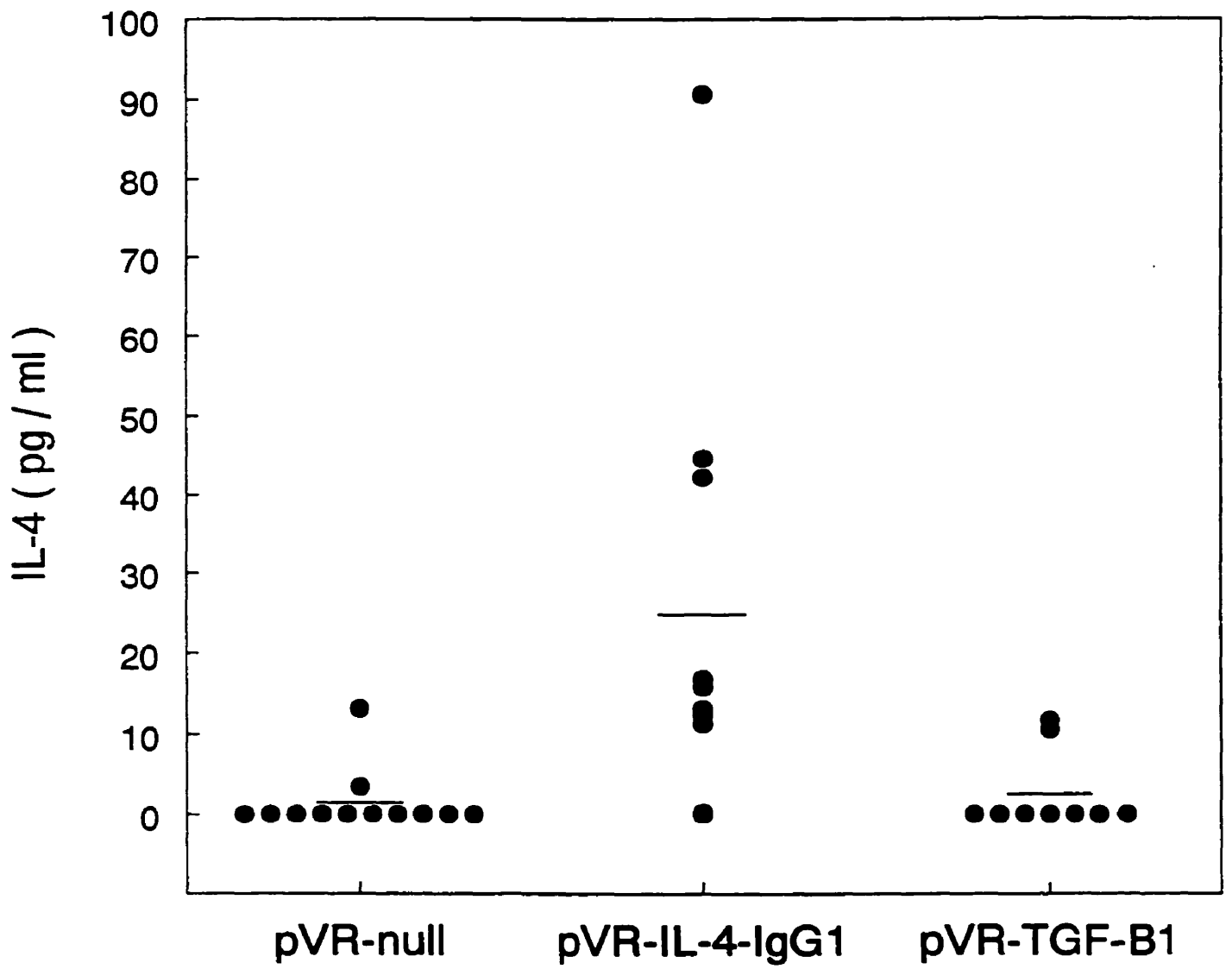
MBP-reactive LNC from pVR-TGF-β1- and pVR-IL-4-IgG1-treated mice display suppressed type 1 cytokine production

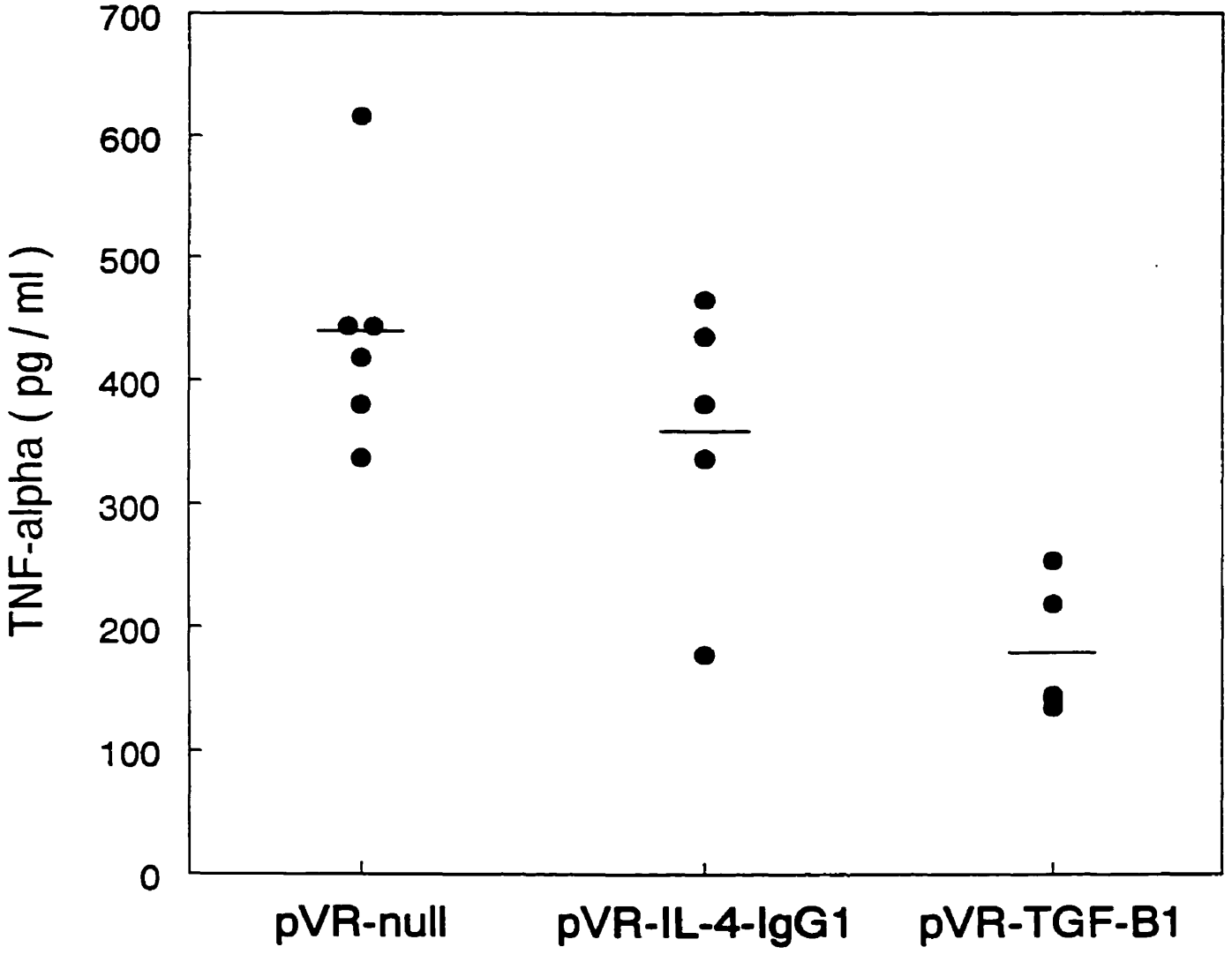
LNC from draining lymph nodes of MBP immunized mice were assayed for the amount of IFNγ, TNFα, IL-4 and TGF-β1 secreted in response to MBP stimulation *in vitro*. IFNγ production was drastically reduced in pVR-TGF-β1 treated mice (35 pg/ml versus 1,260 pg/ml in pVR-null vector controls, $p<0.001$) and only slightly lower than controls in the pVR-IL-4-IgG1 treated mice (1,080 pg/ml, $p<0.05$) (Fig.5A). Similarly, TNFα production was suppressed by pVR-TGF-β1 treatment ($p<0.01$ versus null vector), but much less by pVR-IL-4-IgG1 treatment, and this was not statistically significant (Fig.5B).

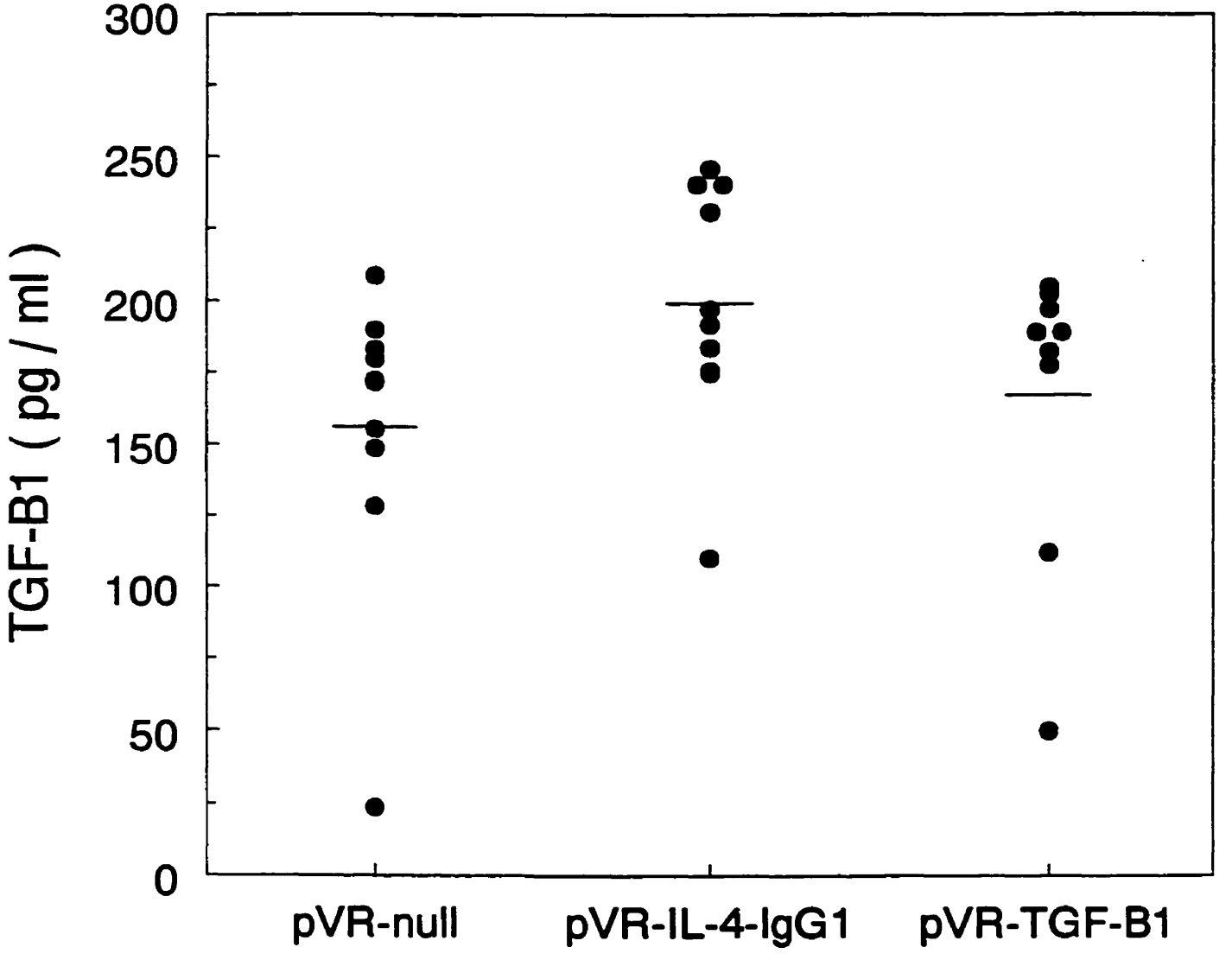
Interestingly, there was no statistically significant difference in the amount of IL-4 produced by LNCs of pVR-TGF-β1-treated mice compared to control LNC (Fig.5C). However, IL-4 production was upregulated significantly in the IL-4-IgG1-treated mice ($p<0.01$ versus null vector) (Fig.5C). In addition, the amount of TGF-β1 produced by LNCs was indistinguishable in all experimental groups (Fig.5D). There was no difference in cytokine production levels in response to MBP in LNC isolated from mice treated with pVR-null or saline (data not shown).

Figure 5 Cytokine-vector treatment alters MBP-stimulated cytokine secretion. We isolated LNC from draining lymph nodes of plasmid-vector treated and MBP-immunized mice, as described in the legend to Fig.4. The results represent the levels of IFNγ, TNFα, IL-4 and TGF-β1 secreted in response to MBP stimulation *in vitro* (day 4 of culture), determined by ELISA. Horizontal bars represent the mean value of each group. A. IFN-γ; B. IL-4; C. TNF-α; D. TGF-β1.


1800
1600
1400
1200
1000
800
600
400
200
0
IFN-gamma (pg / ml)
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1


100
90
80
70
60
50
40
30
20
10
0
IL-4 (pg / ml)
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1


700
600
500
400
300
200
100
0
TNF-alpha (pg / ml)
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1


300
250
200
150
100
50
0
TGF-B1 (pg / ml)
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1

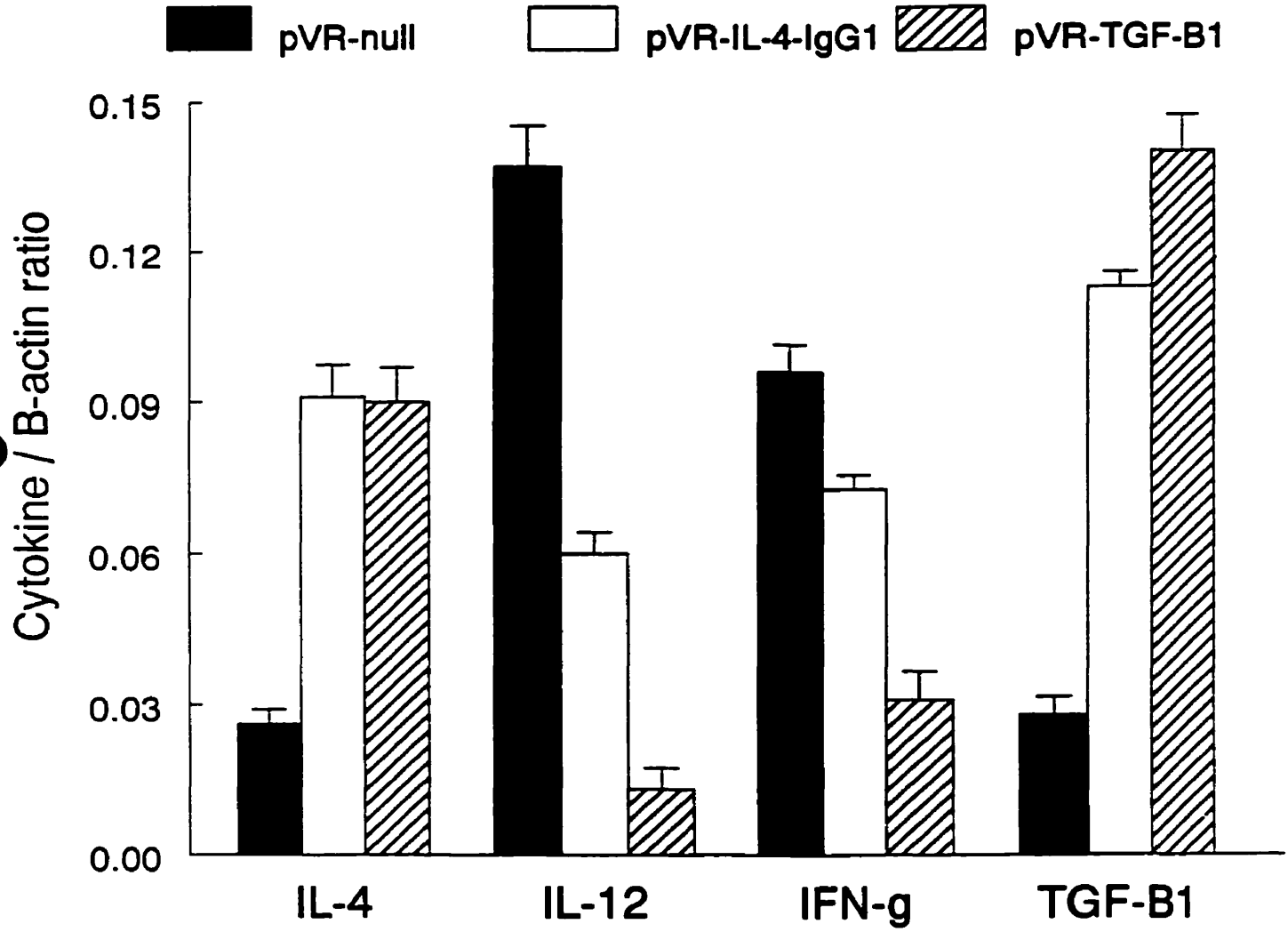
IL-12 and IFNγ transcripts are reduced while IL-4 and TGF-β1 transcripts are increased in the CNS of treated mice

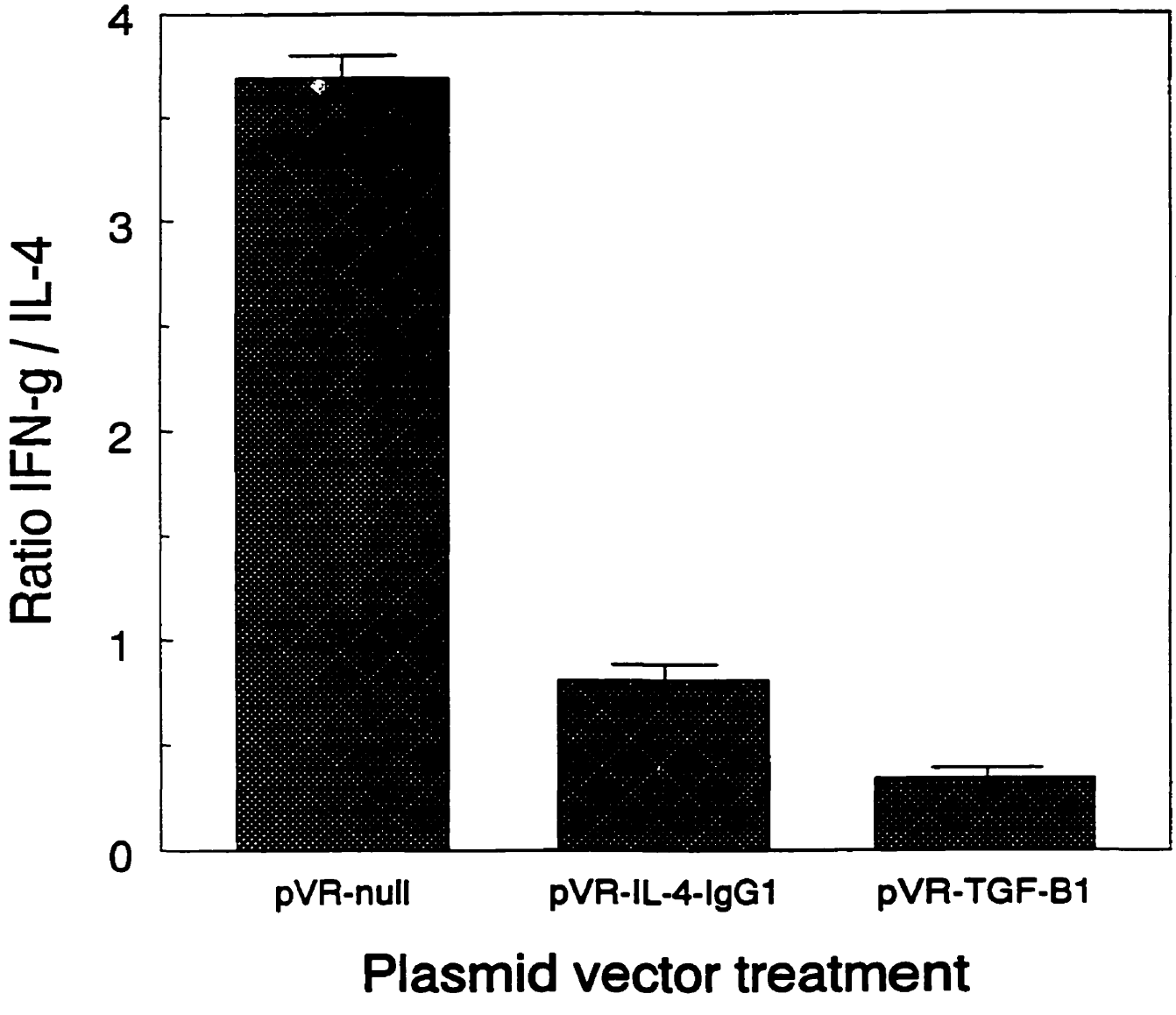
To examine the expression of cytokine mRNA in relation to disease, perfused brains were obtained on day 21 post-immunization with MBP and CFA. Total RNA was isolated and analyzed for cytokine transcripts by semi-quantitative RT-PCR coupled with Southern blotting. In TGF-ß1-treated mice, there were suppressed IL-12p40 and IFNγ mRNA levels, but increased TGF-β1 and IL-4 mRNA levels ($p<0.01$ for each cytokine versus null vector-treated mice) (Fig.6A).

IL-4-IgG1 vector therapy also depressed IL-12p40 and IFNγ mRNA levels ($p< 0.01$ in both cases versus null vector-treated mice), but the suppressive effect was much less than with TGF-β1 (Fig.6A).

Increased CNS IL-4 and TGF-β1 mRNA levels were observed in pVR-IL-4-IgG1 treated mice ($p<0.01$). In fact, TGF-ß1 or IL-4-IgG1 vector treatment upregulated production of endogenous TGF-ß1 and IL-4 with equal efficiency. Reflecting the results noted above, the ratio of IFNγ to IL-4 mRNA was reduced by either TGF-ß1 or IL-4-IgG1 plasmid DNA therapy ($p<0.01$, Fig.6B).

Figure 6 Decreased CNS IL-12 and IFNγ and upregulated IL-4 and TGF-β1 mRNA expression in cytokine-vector treated mice. Female SJL/J mice (6-8 weeks) were treated with either pVR-TGF-β1 (n=10), pVR-IL-4-IgG1 (n=10) or pVR-null (n=10) on day -2 and were immunized with an emulsion of MBP/CFA to induce EAE, 48 hours later (day 0). Reverse transcription was performed on CNS total RNA, recovered on day 21, and mRNA levels were analyzed by semi-quantitative RT-PCR. A. Relative quantities of RT-PCR signals for IL-12 p40, IFNγ, IL-4 and TGF-β1 were calculated by PhosphorImager and normalized to the ß-Actin PCR product. Dark bars = pVR-null; open bars = pVR-IL-4-IgG1; hatched bars = pVR-TGF-β1. B. Ratio of IFNγ / IL-4 mRNA, calculated as described in Materials and Methods. In panels A and B, mean values are presented with their SEM.


pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1
Cytokine / B-actin ratio
0.15
0.12
0.09
0.06
0.03
0.00
IL-4
IL-12
IFN-g
TGF-B1


4
3
2
1
0
Ratio IFN-g / IL-4
pVR-null
pVR-IL-4-IgG1
pVR-TGF-B1
Plasmid vector treatment

Discussion

Cytokine therapy can, in part, protect from autoimmune responses by shifting the balance between inflammatory and regulatory cytokines (6,10). In this study, we focused on the anti-inflammatory role of TGF-β1 and IL-4 in autoimmune encephalomyelitis. TGF-β1 is a pleiotropic cytokine with multiple immunosuppressive effects on T cells, macrophages and related proinflammatory cytokine production (22,23). IL-4 promotes Th2 cell differentiation and, like TGF-β1, suppresses Th1 cytokine production and inhibits macrophage activity (10,24,25).

Here, we analyzed cytokine effects *in vivo* by cytokine gene transfer into skeletal muscle. It is well established that i.m. administration of naked plasmid DNA is an effective route of gene delivery (26,27,28), and our objective was to use skeletal muscle as a source of systemically active TGF-β1 and IL-4. We injected vector encoding TGF-β1 (pVR-TGF-β1) or an IL-4-IgG1 chimeric protein (pVR-IL-4-IgG1) into EAE-susceptible SJL/J mice.

Previously, we demonstrated that i.m. injection of plasmid DNA encoding murine TGF-β1 resulted in secretion of this cytokine by muscle cells, with increased plasma levels of TGF-β1, and protection of NOD mice from autoimmune diabetes (15). Here we report that i.m. administration of either TGF-β1 or IL-4-IgG1 plasmid expression vectors protects mice from EAE, and downregulates inflammatory cytokine production.

To assess the effects of cytokine treatment on T-cell responses to MBP, we recovered the LNC of immunized mice and restimulated them with MBP *in vitro*. LNCs of pVR-TGF-β1-treated mice had dramatically reduced proliferative responses to this antigen, and secreted much lower levels of IFN-γ and TNF-α, compared to null vector-treated mice.

On the other hand, treatment with IL-4-IgG1, though also suppressive in these assays (proliferation, IFN-γ and TNF-α secretion), had a much less marked effect. However, a notable result of IL-4-IgG1 treatment was an upregulation of IL-4 secretion in response to MBP stimulation.

Therapy with either vector considerably suppressed histopathologic inflammatory CNS lesions. Moreover, analysis of CNS cytokine mRNA expression by semi-quantitative RT-PCR revealed that pVR-TGF-β1 treated mice had depressed IL-12 and IFNγ mRNA levels, and marked upregulation of IL-4 and TGF-β1 transcripts. A similar pattern was observed in pVR-IL-4-IgG1 treated mice. It is apparent, however, that while IL-4-IgG1 treatment suppressed IL-12 and IFNγ, this effect was much less important than with TGF-β1 therapy. On the other hand, in the CNS, the upregulatory effects of the two therapeutic cytokines on IL-4 and TGF-β1 mRNA expression were similar.

Taken together, our results demonstrate that TGF-β1 and IL-4 plasmid therapy exerts an anti-inflammatory effect on the encephalitogenic process by suppressing

pathogenic Th1 responses. This protective process may be amplified by the stimulation of endogenous anti-inflammatory cytokine production.

The immunoregulatory properties of TGF-β1 and IL-4 have been demonstrated in several models of autoimmunity, including EAE (10,11,29) and type 1 diabetes (30,31). In these studies, microgram doses of purified TGF-β1 or IL-4 were administered over several days, in order to achieve significant suppression of autoimmune responses. We are achieving similar results with i.m. injections of pVR-TGF-β1 or pVR-IL-4-IgG1.

TGF-β1 may act at multiple levels to prevent autoimmune encephalomyelitis. TGF-β1-null mice succumb to a multi-organ inflammatory syndrome, associated with production of inflammatory cytokines (such as IFNγ and TNFα), and increased numbers of activated immune cells in peripheral lymphoid organs (32). Since TGF-ß1 can impede the adhesion of lymphoid cells to blood vessel endothelia, it has been suggested that TGF-ß1 may be preventing EAE by inhibiting the entry of immune effector cells into CNS (11,29). TGF-ß1 can also inhibit the activation of Th1 cells and the production of Th1 pro-inflammatory cytokines (22,23). The suppressed *in vitro* production of IFN-γ and TNF-α by TGF-β1-treated LNC suggests that the encephalitogenic potential of these cells is diminished. Since IFN-γ and TNF-α induce apoptosis in oligodendrocytes (33,34), one can speculate that TGF-β1 therapy reduces the susceptibility of oligodendrocytes to autoimmune destruction. Furthermore, TGF-ß can influence T cell and APC functions in a way that favors Th2 over Th1

differentiation (20,35). Our results are consistent with these observations, since TGF-ß1 therapy suppressed IL-12 and IFNγ, with upregulation of IL-4 gene expression. Thus, the regulatory activity of TGF-ß1 could result from either a direct action on macrophages (36,37), decreased Th1 cell reactivity, or increased Th2 activity. In fact, these possibilities are not mutually exclusive.

Recently, it has been proposed that there is another subset of regulatory T cells referred to as Th3 (38). These cells may produce varying amounts of IL-4 and IL-10, but they differ from classical Th2 cells in their ability to produce large amounts of TGF-β1. Cells with Th3 features have been described in EAE, NOD mice, and other models of autoimmunity (38). Th3 differentiation appears to be promoted by TGF-β1, IL-4 and IL-10 (38,39). Generally, these cells are protective in autoimmune diseases. Though we have not addressed this question, it is tempting to speculate that systemic TGF-β1 and IL-4 therapy promotes differentiation of Th3 cells in the CNS or lymphoid tissues. In any case, the upregulation of TGF-β1 mRNA expression that we observe is compatible with this hypothesis. Furthermore, it is well established that TGF-β1 has the ability to induce its own expression through a process of autoinduction (22). In accordance with this observation, the increase in CNS TGF-β1 gene expression may also be due to this self-induction process, in either lymphoid or non-lymphoid cells.

As shown by our study, and other reports (10,40), IL-4 administration protects mice from EAE, and this could result from a variety of mechanisms. It has been

demonstrated that IL-4 is a critical factor for the generation of Th2-like cells. T cells activated with MBP in the presence of IL-4 had markedly reduced encephalitogenicity, while T cells cultured with MBP and anti-IL-4 were strongly encephalitogenic (10). Furthermore, it was reported that IL-4 administration induced the development of MBP-specific Th2 cells, but without a downregulation of Th1 cytokine production (10,40). These findings are in agreement with our observations, since pVR-IL-4-IgG1 therapy promoted IL-4 production upon antigen stimulation *in vitro*, while only mildly suppressing IFN-γ secretion.

It is recognized that IL-4 antagonizes IFN-γ-mediated stimulation of macrophages (24), suppresses nitric oxide (NO) production by these cells, and inhibits production of TNF-α, IL-12 and other monokines (10,24,36). In the CNS, IL-4 may influence the capacity of CNS macrophages to produce proinflammatory mediators such as TNF-α (10). Thus, as in the case of TGF-β1 treatment, the protective effects of IL-4 may relate to upregulation of Th2 activity and/or suppression of macrophage activity. However, it would appear from our results that the downregulatory effects on macrophage activity are less than with TGF-β1 treatment.

The pathogenic role of IFN-γ-secreting T cells in the pathogenesis of EAE has been firmly established. As described above, IFN-γ production is elevated at the height of active disease and either low or undetectable during periods of remission (6). Highly encephalitogenic T cells were generated when cultured in the presence of IFN-γ and MBP (10). Transgenic mice expressing IFN-γ in the CNS have provided

additional evidence for a direct and disease-promoting role of IFN-γ in EAE (41). Conversely, incubating T cells with either anti-IFN-γ or anti-IL-12, in the presence of MBP, resulted in a drastically reduced encephalitogenic potential (10). However, despite this clear correlation between IFN-γ and disease progression, recent studies have suggested paradoxical roles for IFN-γ in EAE. For example, antibodies against IFN-γ did not attenuate disease development (42). Furthermore, disruption of the IFN-γ gene render mice more susceptible to the induction of EAE (43). The reasons for these contradictory results are unclear, but IFN-γ may have different effects at afferent and efferent stages of the autoimmune process (43).

Our results are in agreement with recent reports of prevention of autoimmune disease by somatic cytokine gene therapy, particularly in models of insulin-dependent diabetes (15), inflammatory bowel disease (44) and SLE (16). Ludovic-Croxford and co-workers (45) recently reported that direct i.m. injection of plasmids encoding TGF-β1 and IL-4 failed to influence the clinical course of EAE. This discrepancy may be due to the fact that no detectable plasmid-derived cytokine production was observed in their experiments. Also, they administered plasmid DNA as a single dose in TA muscles, concurrently with MBP immunization. In our study, plasmid DNA was administered 48 hours before both the initial MBP priming and recall immunizations. Finally, we used a plasmid vector, pVR1255, selected for high expression in skeletal muscle, and probably superior for this purpose to other vectors in the literature (18).

The fibrogenic nature ability of TGF-β1 (46) is a potential drawback. In pVR-TGF-β1 treated mice, we did not observe either fibrosis or inflammation in brain, muscles, kidneys, lungs, liver, heart or pancreas. We speculate that the latent TGF-β1 encoded by our vector is not activated at the site of plasmid administration. Consistent with this, no active TGF-β1 was detectable in plasma (data not shown). It has also been shown that systemic administration of latent TGF-β1 is potentially an advantageous feature, since it may become biologically active only at distant inflammatory sites, through the action of macrophages, low pH or other factors (48). Whether latent TGF-β1 is activated in peripheral lymphoid or CNS tissues is not clear.

To our knowledge, this is the first study to demonstrate that EAE can be prevented by i.m. cytokine plasmid vector therapy. Conventional cytokine therapy has been limited by the need to administer large doses of cytokine protein with temporarily high systemic levels and ensuing toxicity. Most cytokines have a short $T_{1/2}$, resulting in poor therapeutic efficiency. Somatic cytokine gene therapy has the potential of overcoming these drawbacks, by allowing long-term constant delivery of cytokines at therapeutic levels. As well, cytokine-immunoglobulin fusion proteins, such as IL-4-IgG1, have a much longer T½ than native cytokines. Direct DNA injection in skeletal muscle appears to be safe and is technically simple. Contrary to some viral vectors, plasmid DNA vectors are maintained episomally (28,48), minimizing the risk of genomic insertional mutagenesis. Moreover, the use of plasmid vectors eliminates the possibility of immune responses to viral antigens. The ease of preparation and

relatively low production cost make plasmid DNA an excellent drug delivery system. Skeletal muscle represents an attractive target for somatic gene therapy, due to the large muscle mass, easy access, and non-dividing nature of the cells. The latter feature may account, at least in part, for the long-term persistence of vector expression reported in these cells (26-28,48).

Acknowledgments

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CHAPTER V

General Discussion and Conclusions

There is strong experimental evidence that many organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus and multiple sclerosis, are associated with immune dysregulation and cytokine imbalances (1). Cytokine therapy can protect from autoimmune responses by shifting the balance between inflammatory and regulatory cytokines (2,3). Alternatively, cytokines can modulate autoimmune responses to self antigens by altering the balance between pathogenic (Th1) and regulatory (Th2 or Th3) T cell populations (4), APC functions (5,6), or co-stimulatory activity (7).

Clinically, cytokine therapy is applied in a variety of neoplastic, inflammatory and autoimmune disorders (8). This form of therapy has been limited by the need to administer large doses (often in boluses) of purified cytokine protein because of their short half-lives in serum. Such administration of recombinant cytokines results in transient high systemic cytokine levels and is often associated with untoward toxicity, including fever, cachexia, leukocytosis, pulmonary hemorrhaging, splenomegaly, and hepatic dysfunction (9,10). In addition, production of purified cytokine protein is a laborious and expensive process. Somatic cytokine gene therapy could circumvent the above-mentioned drawbacks, by eliminating the need for frequent protein injections, and by generating more constant cytokine levels (11). This may reduce toxicity and increase therapeutic efficacy. With this objective in mind, we investigated the

applicability of somatic cytokine gene therapy for the treatment of 2 models of organ-specific autoimmune disease: IDDM in NOD mice and EAE.

Several methods of introducing genes into mammalian cells *in vivo* have been developed in recent years (12,13). Viral vectors have dominated gene therapy protocols in disease models and in human clinical trials (14). However, viral vectors all induce an immunological response to some degree and may have safety risks such as insertional mutagenesis and viral reactivation (15). Although less widespread compared to its viral counterpart, non-viral methods of DNA transfer offer advantageous features which circumvent many drawbacks of viral vectors. Non-viral vectors, such as plasmid DNA, are essentially non-immunogenic, can accommodate any DNA fragment size, have no infectious or mutagenic capability (16). Plasmid DNA vectors are relatively simple to construct and large scale production is feasible and inexpensive (17).

In recent years, numerous non-viral strategies of *in vivo* gene transfer have been proposed, including particle bombardment (gene gun), cationic liposomes, receptor-mediated gene transfer (molecular conjugates), jet injection and direct injection (16). Of these, direct i.m. injection of naked plasmid DNA represents a novel and promising approach to cytokine gene immunotherapy. Compared to other tissues, skeletal muscle represents an attractive site for *in vivo* gene transfer for several reasons (18,19) :

1) Injection of plasmid DNA expression vectors into skeletal muscle has previously been shown to result in the uptake of the plasmids, which are

maintained episomally for prolonged periods of time within skeletal muscle cells with expression of encoded genes. Similar results could not be obtained in other tissues. This may reflect a unique property of skeletal muscle and may be due to muscle physiology and tissue architecture.

2) Skeletal muscles consist of non-dividing, multi-nucleated cells which are highly vascularized. Muscle cells possess an increased capacity for protein synthesis compared to several cell types and many proteins synthesized in muscle are capable of entering the systemic circulation.

3) Direct DNA injection in skeletal muscle appears to be safe and is technically simple.

4) Skeletal muscles are easily accessible tissues thus direct DNA injection could readily be used to treat large numbers of patients without resorting to invasive interventions.

In our preliminary experiments, we established that plasmid DNA expression was a DNA dose-dependent process (Chapter II). Our studies suggest that reporter gene expression is dependent on the amount of DNA injected in muscle, up to 100μg per injection site. The uptake of plasmid DNA by skeletal muscle fibers appears to occur through a saturable process since reporter expression levels increased to about 100μg of plasmid DNA, and then declined. The stabilization in expression at superior DNA doses suggested that factors regulating the uptake and expression of plasmid DNA in skeletal muscle were saturated at these doses. Previous studies have reported

that this saturation may be associated with a receptor-like uptake mechanism mediating the internalization of nucleic acids in muscle fibers (20).

Despite numerous advantages, the use of naked plasmid DNA expression vectors to treat serum protein deficiencies and inflammatory conditions has the limitation that plasmid DNA is inefficiently taken up by muscle cells (3-5% of muscle cells)(our unpublished observations; 21). However, with proteins that exert their effects at relatively low concentrations, such as cytokines, this does not appear to be a problem. Thus, although direct i.m. injection of naked plasmid DNA transfects fewer muscle cells compared to common viral vectors, it nevertheless results in a more prolonged level of expression which could mediate long-term biological effects (22-24). It has recently been demonstrated that naked plasmid DNA expression vectors are superior to adenoviral and retroviral viral vectors for direct i.m. injection (21).

Our results (Chapters II and IV; 24), and those of others (11,22,23), show that direct i.m. injection of naked plasmid DNA is an effective approach for the *in vivo* expression of a wide variety of proteins. However, little is known about the factors which determine the magnitude and duration of foreign (non-self) transgene expression in skeletal muscle. In our studies (Chapter II), we sought to elucidate the underlying factors which appear to influence reporter gene expression following i.m. injection of a luciferase plasmid DNA expression vector, pVR1255. We demonstrate that i.m. injection of pVR1255 results in detectable luciferase expression by 24 hours post-injection, which rapidly rose to peak levels at 7 days, declined thereafter, and

persisted at a low level for several months. This observation corroborates the findings of others (17) who have demonstrated that reporter gene expression is sustained for many months following a single injection of plasmid DNA. Interestingly, luciferase expression was easily detectable at 30 minutes after DNA injection suggesting that muscle cell uptake and expression of plasmid DNA molecules is a rapid process. Similar experiments in immunodeficient SCID mice resulted in peak day 7 levels for several months. In addition, when pVR1255 was co-delivered with pVR-TGF-β1, we observed greatly improved reporter gene expression levels compared with mice treated with pVR1255 alone. These studies provide us with strong evidence that immune responses to the transgene product dramatically reduced the persistence of gene expression.

The mechanism of plasmid DNA uptake and expression by skeletal muscle fibers is poorly understood and several factors have been postulated to influence this process. We have shown that factors such as age, sex, muscle type and muscle regeneration could influence peak level foreign gene expression in mouse skeletal muscle. We report that the degree of plasmid uptake and expression is age- and sex-dependent. In our hands, old male mice (10 weeks old) repeatedly gave better results than young males or young and old females. This difference may be due to the type (slow versus fast) of muscle fiber transfected and to the overall muscle mass. Surprisingly, approximately 10-fold more luciferase activity was observed in TA muscles compared to RF muscles. Thus, different muscle groups express considerably

different levels of reporter protein after injection of the same amount of plasmid DNA (17, 25). The basis for this expression pattern may be related to the nature of the muscle fibers transfected, or to muscle physiology and architecture (26).

For our *in vivo* cytokine gene transfer studies (Chapter III), we focused on TGF-β1. TGF-β1 is a pleiotropic cytokine with multiple anti-inflammatory and immunosuppressive effects on T cells, macrophages, NK cells and B cells (reviewed in 27). TGF-β1 also inhibits the expression of many proinflammatory cytokines such as IL-1, IL-2, IL-12, IFNγ, and TNFα (28). TGF-β1 was our candidate cytokine for 2 main reasons:

1. TGF-β1 has critical and fundamental roles in the induction/maintenance of immunological tolerance. In addition, the immunoregulatory properties of TGF-β1 have been demonstrated in several models of autoimmunity (3,29-33).
2. In order to achieve significant suppression of autoimmune responses, microgram doses of purified recombinant TGF-β1 are required (31,33). Since TGF-β1 isolation and purification are particularly difficult and expensive processes, TGF-β1 gene therapy could alleviate these burdens by providing relatively constant TGF-β1 delivery *in vivo*.

We determined that i.m. injection of a plasmid DNA vector encoding latent mouse TGF-β1 (pCMV-TGF-β1), resulted in uptake, retention and expression of this vector by skeletal muscle cells (24, Chapter III). Plasmid DNA persisted in skeletal

muscle for at least 2 weeks post-injection and there was detectable vector-derived TGF-β1 mRNA in skeletal muscle cells. We then determined the optimal dose of plasmid DNA by injecting increasing amounts of TGF-β1 plasmid DNA at different muscle sites and measuring TGF-β1 levels in platelet-poor plasma by ELISA. Latent TGF-β1 plasma levels increase with the number of injection sites and amount of DNA injected per site, up to 100μg of DNA per injection site. We also found that once we reached DNA amounts of 100μg per muscle, any further increase of DNA dose had either no additional effect, or in some cases, expression declined drastically. TGF-β1 levels of 9.2 ± 0.4 ng/ml were observed at 36h post-injection, compared to 2.6 ± 0.4 ng/ml in pCMV-null-treated mice. These levels decreased to a mean plasma level of 4.3 ± 0.3 ng/ml on day 14. At day 35, TGF-β1 was still slightly higher in the pCMV-TGF-β1 treated mice, but this was no longer statistically significant. Collectively, our results demonstrate that latent TGF-β1 plasma levels were increased by at least 2-4 fold for well over two weeks following a single injection of plasmid DNA. These studies confirmed the usefulness of skeletal muscles as a target for our cytokine gene therapy approach.

To examine whether i.m. injections of plasmid DNA result in sufficient production of TGF-β1 to exert biological effects, we used a DTH model of inflammation (Chapter III). pCMV-TGF-β1-treated mice had significantly suppressed DTH responses to foreign antigen. Thus, it was apparent that a few i.m. injections of as little as 50μg of pCMV-TGF-β1 in mice produced physiologically significant

elevations in latent TGF-β1 plasma levels, and that this cytokine was subsequently activated *in vivo* to exert immunosuppressive effects.

In an attempt to demonstrate that i.m. gene transfer could influence the expression of autoimmune disease, we tested this approach in a model of experimental autoimmune diabetes, the NOD mouse (Chapter III). NOD mice develop diabetes spontaneously, through an autoimmune process (34,35). This disease shares many features with human IDDM and is characterized by a T-cell and macrophage-dependent progressive infiltration of islets of Langerhans (insulitis), with destruction of insulin-producing beta islet cells, occurring over a period of weeks or months. Strong experimental evidence suggests that proinflammatory cytokines produced by Th1 cells (IFNγ, TNFα or ß) and macrophages (IL-12, TNFα and IL-1) play an important role in the development of these lesions (34,35).

Treatment with pCMV-TGF-β1 was effective at protecting NOD mice from autoimmune insulitis and diabetes (Chapter III). In CYP-accelerated disease, there was a decreased expression of IL-12 and IFNγ mRNA and IL-4 mRNA was slightly upregulated in the pancreas of protected mice. In contrast, we found that administration of an IFN-γ encoding vector to CYP-treated NOD mice accelerated disease. TGF-β1 plasmid treatment also protected NOD mice from spontaneous insulitis and diabetes in natural course disease (without CYP treatment). In the CYP-accelerated model of disease, TGF-β1 plasmid expression vector treatment appeared to divert the cytokine environment in the target tissue, namely from a

pathogenic/destructive Th1 cytokine expression profile to a more protective/nondestructive Th2 cytokine profile. In accordance with our findings, King and co-workers (36) have recently shown that TGF-β1 expressed in the pancreatic islets protected NOD mice from IDDM by altering APC function and consequently polarizing islet-antigen responses toward a Th2 phenotype. In another recent study, autoimmune diabetes was abrogated in NOD mice overexpressing TGF-β1 in pancreatic islets (37).

We then analyzed if cytokine plasmid therapy could also prevent the expression of EAE, a model of autoantigen-induced autoimmunity in mice (Chapter IV). EAE is an inflammatory autoimmune disease of the CNS mediated by Th1 cells and characterized by perivascular leukocytic infiltration and demyelination (reviewed in 38). There is compelling evidence for a disease-promoting role of Th1 cytokines while Th2 cytokines are associated with disease recovery and protection (38). Specifically, IL-4 and TGF-β1 correlate with disease remission and the development of CNS antigen-specific Th2 cells (1,3,4). We therefore hypothesized that i.m. injection of naked plasmid DNA expression vectors encoding either TGF-β1 or an IL-4-IgG1 chimeric protein would protect mice from EAE.

In an attempt to increase the expression of our cytokines *in vivo*, we subcloned the TGF-β1 and IL-4-IgG1 cDNAs into pVR1255 (pVR-TGF-β1 and pVR-IL-4-IgG1), a plasmid DNA expression vector optimized for high-level expression in skeletal muscle, and shown to be far superior (over 150 times) to commercially

available plasmid vectors intended for direct injection of naked plasmid DNA (17). Our cytokine genes are under the transcriptional control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter, and downstream of the human CMV intron A. Transcription is terminated by the minimal rabbit β-globin terminator. Furthermore, pVR1255 does not encode any foreign protein (such as neomycin phosphotransferase, Neo ^{R}) to which host immune responses may be directed and ultimately affect the magnitude or persistence of cytokine expression.

Treatment with pVR-TGF-β1 and pVR-IL-4-IgG1 resulted in elevated circulating levels of TGF-β1 or IL-4-IgG1 (by day 21 post-immunization, 7.1 ± 0.3 ng/ml and 2.0 ± 0.4 ng/ml, respectively) which were sufficient to exert anti-encephalitogenic effects in mice with MBP-induced EAE (Chapter IV). The onset of disease was delayed and the severity of the clinical manifestations was reduced. TGF-β1 gene delivery had pronounced downregulatory effects on T cell proliferation and production of IFN-γ and TNF-α, upon *in vitro* restimulation with MBP. IL-4-IgG1 also suppressed these responses, but much less than TGF-β1. In addition, IL-4-IgG1-treatment enhanced secretion of IL-4. The clinical improvement brought by therapy was accompanied by a significant decrease in the severity of histopathologic inflammatory lesions. With either vector, CNS IL-12 and IFNγ mRNA expression was significantly diminished, while IL-4 and TGF-β1 mRNA levels were increased compared to control mice. Thus, TGF-β1 or IL-4 cytokine plasmid treatment appeared to suppress MBP-specific pathogenic Th1 responses, while enhancing endogenous

secretion of protective cytokines. Collectively, these results support the notion that cytokine gene therapy, with the intent of inducing immune deviation or immunosuppression, may be most effective in preventing destructive inflammatory responses when initiated prior to the onset of such responses (Chapter IV, 35).

In summary, we have successfully shown that i.m. administration of cytokine-encoding plasmid vectors is a novel approach to cytokine immunotherapy of autoimmune diseases. Cytokine gene therapy generates a microenvironment that promotes immune tolerance and suppresses pathogenic autoimmune T cell responses. These findings provide the basis for the application of i.m. somatic gene therapy for the modulation and suppression of cellular immunity in many immunological diseases. This approach may also find applicability for the delivery of many other protein drugs. However, certain problems must be overcome in applying the direct i.m. DNA injection method. One important problem is that cytokine expression levels vary significantly among experimental mice. Improvements in plasmid DNA injection and pretreatment techniques are anticipated to increase muscle cell uptake and minimize expression variability (22). In addition, we need to develop plasmid expression vectors which can function in a regulatable or muscle-specific manner such that high-level transgene expression can be achieved for long-term therapeutic benefits.

The ease of vector construction and the feasibility of inexpensive large-scale production make this technology attractive for immunotherapy. With further refinements of the technique, and adaptation to larger mammals and humans, it is

likely that this technology will find widespread application in biomedical research and eventually in the clinic.