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I am submitting herewith a dissertation written by Sangjun Chun entitled "Regulation of Virus induced Inflammatory Immune Responses with Cytokine DNA". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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REGULATION OF VIRUS INDUCED INFLAMMATORY IMMUNE RESPONSES WITH CYTOKINE DNA

A

Dissertation

presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Sangjun Chun

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DEDICATION

This dissertation is dedicated to my parents

Mr. Jongku Chun and Mrs. Jungsook Jang,

And to my wife

Sanghee Kim,

without whose help and understanding

I could never have reached for this moment.

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" In Everything Give Thanks", 1 Thessalonians 5 : 18. I shall always be thankful for His love of my family and His guidance to peace and honest.

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ABSTRACT

Viral infection frequently results in chronic inflammatory lesions the principal mediators of which are proinflammatory molecules from activated T cells. Controlling these events has been a focus of the clinical field. Although cytokine therapy using some regulatory cytokines such as IL-10 or TGFβ represents one of the most promising approaches, proteins have a short half-life and bioactivity in vivo, making the strategy inconvenient and costly. In this study, cytokines expressed by means of plasmid DNA were evaluated for their efficacy to modulate inflammatory responses and the mechanisms by which such modulation occurred were investigated. Both mucosal and systemic administration of plasmid DNA encoding IL-4 or IL-10 prior to HSV infection inhibited the development of cutaneous inflammatory responses. To achieve modulation of ocular inflammation administration close to the eye itself provided best results. In the case of cytokine DNA administration to already HSV-primed animals (therapeutic approach), only plasmid DNA encoding IL-10 showed a regulatory effect on the expression phase of the cutaneous DTH response. The mechanisms of immune modulation following prophylactic administration of plasmid DNA encoding IL-4 showed a shift the Th subset balance toward Th2 responses. In contrast, both prophylactic and therapeutic administration of plasmid DNA encoding IL-10 downregulated the activity of Th1 response. The delivery systems for the therapeutic modulation of cutaneous inflammatory response were also compared. Plasmid DNA encoding IL-10 had a delayed and milder, yet more persisting effect than that achieved by viral vectors expressing IL-10. Moreover, plasmid DNA was not affected by preexisting immunity as occurred following administration of viral vectors. Finally, the studies on the mechanism of modulation by plasmid DNA encoding IL-10 revealed that multiple mechanisms such as active suppression, induction of anergy or inhibition of APC appeared to be involved. Although wide dissemination, even to the inflammatory sites and transgene expression occur following in vivo inoculation of plasmid DNA, the central T cell regulation appeared to be the principal mechanism for immune modulation by IL-10 DNA.

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ABBREVIATION

IL- : Interleukin-

i.m (i/m). : intramuscular

i.n (i/n). : intranasal

i.oc (i/oc) : intraocular

SFC : spot forming cells

β-gal : β-galactosidase

GFP : green fluorescent protein

DTH : delayed type hypersensitivity

APC : antigen presenting cell

HSV : herpes simplex virus

VV : vaccinia virus

PART 1 : LITERATURE REVIEW

CHAPTER 1. DNA VACCINE

The finding that naked DNA injected into mice could be expressed was observed as long ago as 1962 (1). However, the observation by Wolff and colleagues in 1990 that prolonged protein expression following the intramuscular injection of naked plasmid DNA alerted the wise to the prospect of DNA immunization (2). Intramuscular injection of a DNA expression vector as a vaccine approach was described in a mouse influenza model (3,4). The reports demonstrated that the approach induced both viral-specific antibodies and cytotoxic T-lymphocytes following muscle injection of plasmid DNA encoding hemagglutinin antigen. Furthermore, DNA immunized mice have been protected against challenge with a high dose of heterologous influenza virus strain. Therefore, plasmid injection has been reported to serve as a vaccine. Soon after DNA vaccines were reported to induce some form of immunity against a dozen or more infectious agents including bovine herpesvirus, rabiesvirus, human hepatitis C virus, malarial antigens, bovine leukemiavirus (BLV), human T cell leukemia virus type-1 (HTLV-1), simian immunodeficiency virus (SIV), and herpes simplex virus (Reviewed in 5, 6). The utility of DNA vaccines has been extended to noninfectious diseases such as cancer, autoimmunity, and allergy (7, 8).

Plasmid DNA vectors as vaccines have several appealing features. The simplicity of preparation and application is the most attractive as a vaccine. Construction and purification of relevant plasmid is not only simple, but also less expensive than production and purification of the recombinant protein. Virtually any DNA sequences, including large inserts, can be placed into a plasmid. Once constructed and purified in large quantities, plasmid DNA can be lyophilized for storage at room temperature for extended periods of time, making transport of such vaccines easy and inexpensive. One or more genes encoding protein(s) of interest may be inserted to the expression vector. Multiple plasmids can be used at one time, since gene expression from one plasmid is not affected by other plasmid DNA. The lack of a protein component represents another advantage because it makes them unlikely to be affected by pre-

existing immune responses. Neonatal immunity limits the usage of conventional vaccines in newborns because of their lower responsiveness, higher susceptibility to tolerance, and a preexisting immune response like maternal antibodies. DNA immunization of neonates has been shown to induce specific T and B cell immune responses to the plasmid DNA encoding antigen (9 - 11). In addition, the apparent long-term persistence of antigen expression from DNA vaccines may mean that there will be an enduring effector T cell response as well as the establishment of traditional immunological memory

On the down side, the immunologic properties of DNA, especially the induction of anti-DNA antibodies after repeated inoculation, may represent a potential problem. Pathogenic anti-DNA antibody response is well-documented in a disease such as systemic lupus erythematosus (12). In addition, no DNA vaccine currently appears to be superior to the existing vaccines, but this situation may change as the topic becomes more fully investigated. Although there is yet to be evidence that integration into genomic DNA occurs, it remains a possibility.

CHAPTER 2. VACCINES AGAINST HERPES SIMPLEX VIRUSES

Herpes simplex (HSV) virus represents one of the most successful pathogens in humans, with around 80 % of the population is infected with either HSV-1 or HSV-2 (13, 14). Many suffer significant clinical consequences, but so far none of the vaccines developed have passed the security of double blind experiment testing (15).

The first generation of HSV vaccines developed in the 1920 and 1930' consisted of inactivated virus preparation made by formalin treatment of HSV-infected animal tissues such as rabbit brain (16). However, inactivated virion vaccines have several shortcomings, including difficulties in providing consistent concentrations of the important immunogens, problems assuring that all of the virus are inactivated, problems eliminating contaminating viral DNA that may be potentially oncogenic and high production cost. To overcome some of these problems, an approach based on defining and eliminating genes involved in neurovirulence, latency, or reactivation was developed. Such attenuated live vaccines include HSV TK$^-$ and HSVγ34.5$^-$. However, these attenuated vaccines were poorly immunogenic or their virulence was not fully eliminated (16, 17).

Subunit vaccines such as the HSV glycoprotein B and D (gB and gD) were evaluated for their ability to generate humoral and cell mediated immunity. Although many subunit vaccines have been shown to be immunogenic and efficacious in animal model of primary and recurrent HSV infection (17, 18), it is unknown whether vaccines contain multiple viral proteins are more effective than vaccines containing only one or two viral protein(s). In addition, clinical trials by Chron using HSV-2 gB/gD-MF59 proved relatively ineffective as a therapeutic vaccine in human (18).

At least four groups have reported that plasmid DNA encoding HSV proteins is immunogenic and induces protection when used prophylactically in various animal models. Studies using the guinea pig model of genital herpes showed that HSV-2 gD and HSV-2 gB DNA vaccines were immunogenic, protected animals from developing acute disease and reduced

viral replication in the genital tract (19). In our lab we have investigated the immunogenicity of two proteins of HSV-1, a regulatory protein ICP27 and a major glycoprotein B. Both DNA constructs induced a range of immune responses in mice, protecting the animals from low dose challenge of HSV (20, 21). Immunization with gB DNA for example, induced the neutralizing antibody response dominated by the IgG2a isotype and the type 1 cytokine producing CD4+ T cell phenotype (20). With ICP27 DNA immunization, a CTL response was observed but there was no detectable antibody production (21).

Furthermore, DNA immunization via nasal mucosa induced both systemic and mucosal immunity and protected the recipients from vaginal and zosteriform HSV challenge (22). Interestingly, the levels of immunity induced were improved by the use of cholera toxin as an adjuvant along with the DNA. These results indicated that DNA vaccines can not only protect against disease but can provide considerable protection from latent infection. Initial phase I studies with a DNA vaccine for HSV are currently underway (17).

CHAPTER 3. DNA VACCINE : POSSIBLE MECHANISMS FOR IMMUNE INDUCTION

Although the mechanism involved in the immune induction by DNA vaccine has been investigated in depth, it is not clearly understood. It has been well known that live viral vaccines induce strong CD8 T cell responses, because the vaccine organism replicates and synthesizes its proteins intracellularly, and thus its protein can be efficiently introduced into the MHC Class I (endogenous) antigen presentation pathway.

There is no doubt that the encoded proteins are synthesized within the muscle cells, as demonstrated by expression of the reporter proteins such as chloramphenicol acetyl transferase (CAT), β-galactosidase, and green fluorescent protein. This capacity for endogenous protein synthesis led to the prediction that DNA immunization provides an effective means of inducing CTL directed against the encoded protein. However, the mechanism underlying CTL induction is not clear, even though a mechanism by which CTL precursors are activated has been proposed. The expression of encoded antigen by muscle cells provoked suggestions that the muscle cells themselves present antigen and directly induce Class I restricted CTL (12). However, myocytes express only low levels of MHC Class I (although this is upregulated in the presence of interferon), and levels of accessory molecules appear to be extremely low. Therefore, the ability of myocytes to present antigen in a manner capable of inducing CTL remained a question. Recent studies have shown that bone marrow derived APCs play a critical role in the presentation process for intramuscular or intradermal injection of plasmid DNA (23 - 26). Chimeric mice expressing two MHC haplotypes on their muscles cells, but are only on their bone marrow derived APCs, mounted CTL responses restricted to the bone marrow haplotype. Thus, it appears unlikely that muscle cells play a direct role in CTL induction. Furthermore, Robinson group has also reported that removal of injected muscle within a few minutes of DNA inoculation has no effect on the magnitude, suggesting that transfected cells in injected muscle do not play a vital role in DNA initiated antibody and CTL responses (27).

It has been shown that cutaneous injection of plasmid DNA results in direct transfection of skin-derived dendritic cells (28). A recent report also demonstrated that dendritic cells derived from skin injected with plasmid DNA encoding hen egg lysozyme (HEL) are able to induce both primary and secondary immune responses (29). These migratory dendritic cells presented the relevant HEL peptides for the generation of class I-restricted CTL and for class II-restricted CD4+ responses. Furthermore, Akabari (30) reported that although DNA vaccination resulted in transfection of only a small proportion of dendritic cells, it led to general activation of all dendritic cells, providing optimal conditions for effective T cell activation and maintenance for memory. In addition, cross-priming of dendritic cells by antigen expressed in keratinocytes did not occur unless keratinocytes died, indicating that dendritic cells uptake apoptotic bodies released from dying keratinocytes and present antigen in the context of MHC class II (30).

Overall, it is likely that the DNA is primarily expressed in weak APC, such as dermal and muscle cells. Subsequently the expressed antigen may be taken up by professional APC and processed. Dendritic cells isolated from immunized animals could be directly transfected. They process and present the corresponding peptides to the specific T cells and thus can activate both CD4 + and CD8+ T cells.

CHAPTER 4. DNA VACCINE FOR IMMUNE MODULATION

It has become evident that the cytokine and costimulatory microenvironment can qualitatively and quantitatively influence the outcome of immune responsiveness. The use of such molecules to enhance the immune response to a vaccine is an area of growing interest. In 1992 Raz *et al.* (31) reported that coinjection of plasmid DNA encoding cytokines such as IL-2, IL-4, and TGFβ-1 with keyhole limpet hemocyanine (KLH) could modulate the humoral and cellular immune responsiveness to KLH. This finding opened up the potential usage of DNA vaccine encoding immunomodulators. More convincing results were reported by Ertl group (32). In this report, coadministration of plasmid DNA encoding granulocytes macrophage colony-stimulating factor (GM-CSF) markedly enhanced the B cell and T cell activity to plasmid DNA encoding the rabies virus G protein. Interestingly, either cotransfection of individual cells with both plasmid DNAs or close proximity of APC's to GM-CSF secreting cells was crucial for the immune enhancement, presumably reflecting the localized activity of the cytokine.

Soon after modulation or enhancement of immunogenicity of plasmid DNA encoding antigen by coadministration of plasmid DNA encoding cytokine (33, 34), costimulatory molecules (35), chemokines (36), and adhesion molecules (37) were reported. In summary, coadministration of plasmid DNA encoding GM-CSF, IL-5, IL-18 or MIP-1α has been reported to increase the humoral response. For CD8+ CTL, IL-12, IL-15, TNFα, MCP-1, or ICAM-1 were strong enhancers. IL-12 and IFNγ genes promoted Th1 and suppressed Th2 differentiation. Conversely, coinjection of plasmid DNA expressing IL-4 promotes Th2 and inhibited Th1 development. Coadministration of the IL-12 and GM-CSF gene mainly enhanced Th1 cell differentiation, with Th2 being development was unaffected.

The potential value of plasmid DNA expressing immunomodulators has extended to treat inflammatory diseases, autoimmune diseases and cancer. One of the first reports appeared in 1995 by Moldwer and his colleagues. They found that plasmid DNA expressing

tumor necrosis factor receptor (TNFRp55) and IL-10 in cationic liposomes ameliorated local inflammatory responses and decrease mortality to lethal endotoxemia (38).

The modulation of airway hyperreactivity in asthma is another example of treatment with a cytokine gene (39). In this report, the pulmonary eosinophilia and airway hyperreactivity following conalbumin sensitization was greatly reduced by transfer of plasmid DNA encoding IFNγ in liposome to nasal mucosa, suggesting that IFNγ suppresses the production of Th2 cytokines and decreases pulmonary cell infiltration.

The technology using plasmid DNA encoding cytokines has been also applied to cancer treatment. In 1996 Yang and his colleague (40) showed that gene gun mediated transfer of plasmid DNA encoding two subunits of IL-12 protein into skin epidermal cells led to regression of established primary and metastatic murine tumors. The authors found that transfer of IL-12 cDNA into epidermal cells overlaying an implanted intradermal tumor resulted in detectable but low levels of transgenic protein (IL-12) at the skin tissue treatment site. The antitumor effect was CD8+ T cell dependent and led to the generation of tumor-specific immunological memory, suggesting that IL-12 enhances the development of a tumor-specific CTL response.

Recently, it was reported that naked DNA encoding C-C chemokines could induce a long-lasting protective immunity to experimental autoimmune encephalomylelitis (EAE) (41). In this report, the authors showed that MIP-1α or MCP-1 DNA vaccines prevented EAE in an MBP-induced rat model, even if disease was induced 2 month after administration of naked DNA vaccines. In contrast, administration of the MIP-1β naked DNA significantly aggravated the disease. They suggested that immunization with plasmid DNA MIP-1α and MCP-1 induces neutralizing anti-chemokine antibodies, thereby providing subsequent protection from severe EAE.

In addition, recent reports using plasmid DNA encoding regulatory cytokines demonstrated the potential therapeutic role of naked DNA vaccines for the modulation of autoimmune and inflammatory diseases. The report by the Rouse group (42) showed suppression of ongoing ocular inflammatory disease by topical administration of plasmid DNA

encoding IL-10, even though the mechanistic studies have not been completed. The authors, however, demonstrated that gene expression, as determined by β-galactosidase activity, could occur in the eye following topical administration of plasmid DNA encoding β-galactosidase to the scratched eyes.

The Prud'home group (43) also showed that intramuscular injection of plasmid DNA encoding TGFβ-1 led to expression in the muscles and elevation of the plasma level of TGFβ-1. This increased level was sufficient to exert immunosuppresive effects, exhibiting suppression of DTH responses and protection from insulitis. Furthermore, the authors showed that pancreatic IL-12 and IFNγ mRNA expression was depressed and the ratio of IFNγ to IL-4 was decreased, indicating that the protection from inflammatory disease was associated with shift from Th1 to Th2 responses.

In another report the Wahl group (44) stated that plasmid DNA encoding human TGFβ-1 suppressed chronic disease in a streptococcal cell wall induced arthritis model. They showed that a single dose of 300 μg plasmid DNA administered at the peak of the acute phase profoundly suppressed the subsequent evolution of chronic erosive disease typified by disabled joints, swelling, and deformity. Moreover, delivery of the TGFβ-1 DNA, even as the chronic phase commenced, virtually eliminated subsequent inflammation and arthritis.

CHAPTER 5. T HELPER CELL SUBSETS

The discovery by Mosmann and colleagues in 1986 (45) that the CD4+ T cell subset could be distinguished according to their ability to produce contrasting and crossregulating cytokines was enthusiastically taken up in every field of Immunology. This Th1-Th2 paradigm has been exciting a topic in the area of infectious disease, cancer, transplantation, and autoimmunity. Th1 cells secrete IL-2 and IFNγ and promote cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, and IL-10, and are important for humoral immune responses (especially IgE production). To a significant extent the role of Th1 and Th2 has been considered as dogma in which Th1 responses mediate removal of intracellular parasites and Th2 responses eliminate extracellular ones. In addition, it has been well-accepted that Th type I and type II cytokines are mutually counteractive, inhibiting development to either type or effector functions of the differentiated Th cells.

The development of these distinct Th subsets is strongly dictated by the type of antigen/microorganism, the genetic background of invading host, the dose of antigen, and the route of antigen immunization (46 - 48). The most clearly defined factors determining Th1 and Th2 differentiation from the precursor Th cells are cytokines, which are present at the initiation of the immune response. For example, IL-12 or IFNγ produced from dendritic cells, macrophages or natural killer cells drive the development of Th1 cells. IL-12 has been shown to be a dominant factor in Th1 development, and activate signal transducer and activator of transcription (STAT)-3 and STAT-4 (49, 50). Gene deletion of IL-12 or STAT-4 showed that IL-12 signaling through this pathway is required in vivo, since both resulted in markedly reduced Th1 responses (51, 52). Functional receptors (IL-12Rβ1 and IL-12Rβ2) for IL-12 appear to be restricted to recently activated cells and Th1 cells, and they (especially IL-12Rβ2) are lost during differentiation of Th2 cells (53). Recently, the Murphy group identified an Ets family transcription factor, ERM, as an IL-12 induced STAT-4 dependent gene selectively expressed by Th1 cells (54). This factor seems to cooperate with STAT-4 or some STAT-4-induced factor in the regulation of IFNγ gene

transcription since expression of ERM in STAT-4 deficient cells is not sufficient for restoring IFNγ production to normal Th1 cells.

The development of Th2 cells has been attributed to the exposure of naïve CD4+ T cells to IL-4 at the initiation of an immune response, even though initial source of IL-4 is still unclear. The ability of IL-4 in inducing Th2 development is dominant over Th1 polarizing cytokines (55). If IL-4 levels reach a certain threshold at the initiation of immune response, the differentiation to Th2 cells commences, increasing levels of IL-4 progressively. IL-4 binding to its receptor leads to a signal through activation of STAT-6 (56). Gene deletion of the IL-4 gene or STAT-6 results in deficient Th2 responses (57, 58). A consensus STAT-6 binding site is present in the IL-4 promoter, suggesting that STAT-6 may activate IL-4 transcription in response to IL-4 itself and plays a crucial role in IL-4 driven Th2 development.

A transcription factor, GATA-3, was recently found to be selectively expressed in differentiating effector Th2 but not Th1 cells (59). The loss of GATA-3 expression by developing Th1 cells requires IL-12 signaling through STAT-4 and does not simply result from an absence of IL-4 (60). Furthermore, GATA-3 is not only required for the transcription of all Th2 genes, but it also directly depresses IL-12 signaling for Th1 development by repressing IL-12Rβ2 expression. Thus, GATA-3 expression and IL-12 signaling seem to be mutually antagonistic.

To date, at least six transcription factors (STAT-6, c-Maf, NF-IL-6, NF-AT, AP-1, and GATA-3) have been implicated in Th2 cell differentiation even though the precise mechanisms of how these factors interact and lead to Th2 differentiation remain to be clarified (61). However, the differentiation of naïve T cells into mature Th1/Th2 effector cells seems to fix their phenotype by remodeling of the chromatin structure, especially in regarding to cytokine gene loci (62). These epigenetic phenomena render the differentiated and memory T cells to produce cytokines with a much higher level and more rapid kinetics following antigenic stimulation than naïve T helper cells.

The concept of this T helper cell subset has been applied to clinical situation in which cyokines play an important role in the development of disease. It has become evident from the

animal models that Th1 type cytokines are involved in the genesis of organ-specific autoimmmune disease such as experimental autoimmune encephalomyelitits (EAE) or insulin dependent diabetes mellitus (IDDM) (63, 64). Both EAE and IDDM can be induced by CD4+T cells, primarily Th1 cells that produce proinflammatory cytokines such as IFNγ or TNFα. In contrast, administration or upregulation of the Th2-associated cytokine IL-4 or IL-10 is beneficial and can ameliorate the autoimmune disease.

Several findings support that allergies result from Th2-associated responses to single or multiple environmental allergens (65). Th2-like cells are shown to accumulate in target organs of allergic patients. Successful specific immunotherapy is associated with changes from a prevalent Th2 to Th1 profile of allergen-reactive Th cells or with administration of Th1 type cyokines.

Another example in which T cell subsets play a vital role in pathogenesis is the infection model of *Leishmania major (L. major)* (66). Infection of susceptible strains, such as BALB/c mice, with *L. major* mounts Th2 type responses and the animals succumb to death. Resistant strains (C3H, C57BL/6) mice, however, develop Th1 responses characterized by IFNγ production. Susceptible BALB/c mice could become resistant by treatment of anti-IL-4 or IL-12.

Overall, these reports suggest that the influence of Th1/Th2 balance by a variety of immunological manipulations can affect the clinical outcome. Cytokines exert a critical factor in T helper cell differentiation. Therefore, administration of Th1 or Th2 cytokines in vivo can promote or inhibit the development of several diseases. However, direct administration of cytokines to treat such disease may not be feasible because of the short half-life of cytokine in vivo and the difficulty in delivering them to the site of the immune response (67).

CHAPTER 6. SPECIFIC AIMS AND RATIONALE

Chronic lesions are frequently associated with viral infections that lead to inflammatory reactions against viral antigen itself or changes imposed by replicating virus on host tissue antigens. Manipulation of such inflammatory responses has been explored by using several manipulators such as pharmacological anti-inflammatory drugs or immunological materials. However, it is desirable to make use of the body's own anti-inflammatory mechanisms such as regulatory cytokines rather than to use nonspecific and toxic pharmacological drugs. Although cytokine therapy may represent a therapeutic option for manipulating inflammatory responses, its short bioactivity makes the approach impractical and costly. The alternative promising strategy to overcome such a problem is gene delivery. Among gene delivery systems, the naked plasmid DNA approach has been investigated for vaccination and immunomodulation purposes and has proven its effectiveness, but the appropriate means of achieving gene expression requires further clarification.

The present project was designed to evaluate the potential value of plasmid DNA encoding cytokines for modulating virus-induced inflammatory lesions and to understand the mechanisms of cytokine action and its regulatory effect. The prophylactic and therapeutic approaches to inhibit inflammatory responses with cytokine DNAs were examined. The relative efficacy of cytokine gene delivery vectors using viral vectors and plasmid DNA was also compared and evaluated for the practical application. Furthermore, the mechanisms involved in immunomodulation by plasmid DNA encoding cytokines were investigated to understand their modulatory effects. These studies provide the invaluable information about not only the practical means of modulating inflammatory disease, but also the basic mechanism involved in cytokine regulation of the immune system.

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PART 2. MODULATION OF VIRAL IMMUNOINFLAMMATORY RESPONSES WITH CYTOKINE DNA ADMINISTERED BY DIFFERENT ROUTES

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CHAPTER 1. ABSTRACT

The efficacy of plasmid DNA encoding cytokine was evaluated and compared after different routes of administration, systemic or surface exposure, for their modulating effects on subsequent lesions caused by infection with herpes simplex virus (HSV). Systemic or topical administration of both IL-4 and IL-10 DNA, but not IL-2 DNA caused a long lasting suppression of HSV-specific DTH response. IL-4 or IL-10 DNA preadministration also modulated the expression of immunoinflammatory lesions associated with corneal infection of HSV. Suppression of ocular lesions required that the DNA be administered to the nasal mucosa or ocular surfaces and was not evident after intramuscular administration. The modulating effect of IL-10 DNA was most evident after topical ocular administration, whereas in the case of IL-4 DNA, both routes appeared to be equal . Preexposure of IL-4 DNA, but not IL-10 DNA resulted in a significant change in Th subset balance following HSV infection. Our results indicate that the modulating effect of IL-4 or IL-10 DNA may proceed by different mechanism. Furthermore, our results suggest that surface administration of cytokine DNA represent a convenient means of modulating immunoinflammatory lesions.

CHAPTER 2. INTRODUCTION

The realization that plasmid DNA eukaryotic expression vectors could be used to induce immunity against the encoding protein following systemic or even mucosal administration, opened up a novel means of vaccination (1- 5). Many harbor the hope that DNA vaccines might replace some existing preparations and may even be successful against infectious agents which currently lack effective vaccines (6). The naked DNA approach also holds promise as a convenient means of achieving gene transfer since the vehicle contains no host recognizable protein and even the existence of specific antibody to the encoded protein appears not to block gene expression (7). Consequently, DNA vaccines represent a potential method of boosting or modulating the nature of immunity in previously primed animals.

Previous studies from this and other laboratories have shown that the plasmid DNA approach can be used to express natural molecules such as cytokines which can influence the nature of immune responses (8). The administration of DNA encoding a cytokine may affect the extent and type of immune reaction to coadministered antigens (9). Furthermore, recently it became evident that plasmid DNA encoding cytokines such as IL-10 can influence the severity of immunoinflammatory lesions, even when administered during the disease process (8). In our previous study, in which DNA encoding IL-10 was shown to attenuate herpes simplex virus (HSV) induced ocular immunoinflammatory lesions, it was necessary to administer the plasmid directly to the ocular tissue. Intramuscular administration was without beneficial effect (8). Such results indicated that the route of plasmid DNA exposure may critically influence efficacy.

In the present report, we have further investigated the influence of the administration route using 3 cytokine DNAs for their ability to modulate the expression of both ocular and cutaneous inflammatory responses caused by HSV. Our results show that prophylactic treatment by either systemic or surface exposure with IL-4 or IL-10 DNA, but not IL-2 DNA, markedly suppressed cutaneous HSV-specific DTH reactions. Ocular lesions, in contrast, were inhibited by both IL-4 and IL-10 DNA pretreatment, but only when given via the intranasal or

ocular route and not when administered systemically. Since only IL-4 DNA, but not IL-10 DNA preexposure, resulted in a significant change in the subsequent Th1: Th2 HSV specific T cell response, the inhibition observed was assumed to proceed by different mechanisms. Suppression caused by IL-10 DNA may depend on local cytokine expression at the inflammatory site itself, whereas the effect of IL-4 DNA may result mainly from central immune modulation. The implication of our observations as regards to the use of cytokine DNA to modulate immunoinflammatory disease is discussed.

CHAPTER 3. MATERIALS AND METHODS

Mice

Female BALB/c mice ($H2^d$), 3 to 4 weeks old, were purchased from Harlan Sprague Dawley (Indianapolis, IN), and acclimated for 1 week prior to beginning experimentation. All experimental procedures were followed with Association of Research in Vision and Ophthalmology (ARVO) resolution on the use and care of laboratory animals. The animal facility of the University of Tennessee is fully accredited by AAALAC.

Virus

HSV-1 strain RE and KOS were grown on vero cells (#CCL70, ATCC). The virus were maintained in DMEM containing 2 % heat-inactivated FCS (Life Technologies, Grand Island, NY), and titrated following standard protocol (10). Virus stocks were aliquoted and stored at -80°C.

Plasmid preparation

Plasmid DNA encoding murine IL-2 with the CMV promoter was a gift of Dr. H. Ertl (PcDNAIII IL-2) (Philadelphia, PA). Plasmid DNA encoding murine IL-10 containing the SV40 promoter was provided by Dr. T. Mosmann (Alberta, Canada). Plasmid DNA expressing murine IL-4 was generated in our laboratory, using IL-4 cDNA from ATCC (No. 37561) (Rockville, MD). All plasmids were purified by polyethylene glycol precipitation according to Sambrook, et al (11), with some modifications as previously described (8). The expression of each plasmid DNA was identified by reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA) (for IL-10) or bioassay (for IL-2 and IL-4). PcDNAIII was used as a control vector.

Plasmid DNA administration

To administer DNA, mice were deeply anesthetized with methoxyflurane (Metophane; Pittman-Moore, IL). For intramuscular administration (i/m) mice were injected into the tibialis or biceps muscles of both legs with 100 μg of plasmid DNA in 25 μl of PBS three times at weekly intervals. Intranasal immunization (i/nas) was performed three times at weekly intervals with 200 μg of plasmid DNA in 25 μl of PBS. For intraocular administration (i/oc), corneas were slightly scarified with a 27-g needle, and 100 μg of plasmid DNA in 4 μl of HBSS was applied to the corneas three times at weekly interval.

Corneal infection and clinical observation

On the day after the last DNA administration, mice were anesthetized and the scarified corneas were infected with 1 x 10^6 PFU of HSV-1 RE in 4 μl of sterile PBS. The corneas were gently massaged with the eyelids. The animals were examined daily after infection and severity of stromal keratitis was graded 0 to 5 using slit lamp biomicroscopy (Keelen Instrument, Biomeg, PH) as followed : 0 : clear eye, 1 : local or mild limbal neovascularization, 2 : abundant neovascularization , mild corneal haze, 3 : opaque cornea, iris vessel engorgement, 4 : severe corneal opacity, iris not visible, 5 : complete corneal rupture and necrotizing stromal keratitis.

Cytokine detection in tissues

Three days after intramuscular (i/m), intranasal (i/nas), or intraocular (i/oc) administration of plasmid DNA encoding either IL-2, IL-4 or IL-10 and control vector, the corneas and cervical LN were collected and transferred to DMEM (10% serum). Additionally, skeletal muscles and popliteal LN are also obtained from intramuscularly treated group. The samples were frozen at -80°C, thawed at 37°C, homogenized for 45 *s* (ProScientific, Pro 0200, Monroe, CT), and centrifuged 2 min. at 10,000 x *g* at 4°C. The supernatants were analyzed for IL-2, IL-4 or IL-10 production by ELISA. The plates were coated with 2 μg of rat antimouse IL-2, IL-4 or IL-10 antibody (Pharmingen, catalog No. 18161D, 18191D, 18141D, respectively) at 4 °C

overnight. The plates were blocked with 3 % dry milk for 1 hr at 37 °C. The samples and rIL-2, rIL-4 or rIL-10 (Pharmingen, catalog No. 19211T, 19231V, 19281V, respectively) at a concentration of 1 ng/ml were added and serially diluted. The standard and samples were incubated overnight at 4 °C. After washing, 1 μg/ml of biotinylated anti IL-2, IL-4 or IL-10 antibodies (Pharmingen, catalog No. 18172D, 18042D, 18152D, respectively, was added and incubated at 37 °C for 2 hrs. After washing, peroxidase conjugated streptavidin (Jackson Immunoresearch) was added and incubated at 37 °C for 1 hr. The ELISA was developed as described previously (6).

HSV-specific lymphoproliferation assay

To test whether HSV specific T cell responses were affected by plasmid DNA encoding cytokines, the animals were sacrificed approximately 21 days following infection. Two spleens were pooled and used as the responder population. Details for this method have been described elsewhere (6). Briefly, these responders were restimulated in vitro with irradiated syngenic splenocytes infected with UV-HSV (1.5 m.o.i. prior to UV inactivation) or irradiated naive splenocytes, and incubated for 5 days at 37°C. Eighteen hours before harvesting [^{3}H]-thymidine was added to all culture wells. Harvested cells were assayed for radioactivity and results expressed as cpm ± SD of 5 replicates per sample.

Delayed-type hypersensitivity (DTH)

Eighteen days after infection, test antigens in 20 μl of PBS were injected in the ear pinna of anesthetized mice and the ear thickness was measured 48 hrs postinjection with a screw gauge meter (Oditest, H.C. Kroeplin GHBH, Germany) as described elsewhere (12). Test antigens used were UV HSV-1 KOS (1 x 10^5 PFU prior to UV-inactivation) or vero cell extract in the right and left ear, respectively. The mean increase between the thickness of right and left ear was calculated. In the separate experiments, 20 μl of IL-10 protein and HSV-1 KOS (10^5 PFU prior to UV inactivation) were given to right ear and 20 μl of HSV-1 KOS was injected to left ear

and footpads. For the control, 20 µl of HSV-1 KOS and vero extract were injected to right and left ear/footpad, respectively.

Virus isolation and titration

To collect ocular virus samples eyes were swabbed at different time points after HSV infection and samples resuspended in 500 µl of serum free DMEM. The samples were stored at - 80°C until tested. Individual samples (125µl) were further diluted and viral titers were obtained using a plaque assay performed on vero cells as described elsewhere (10).

Antibody analysis

Serum samples from each mouse were collected at day 21 post infection, and analyzed individually for HSV-specific antibody (IgG) using a standard quantitative enzyme-linked immunosorbent assay (ELISA) as described in detail elsewhere (5). Briefly, serum was tested for IgG_{2a} , IgG_1 , and total IgG using ELISA plates coated with HSV antigen and isotype specific antibody. The system was quantified by generating standard curves using Spectra Max ELISA reader softmax version1.2 (Molecular devises, Sunnyvvale, CA)

Quantification of cytokine producing cells by ELISPOT

The method used was described in detail previously (5). Approximately 21 days following infection 8 mice from each group were sacrificed and two spleens were pooled. The resulting four samples were analyzed for IL-4, IL-5, IL-10 and IFNγ spot-forming cells by ELISPOT. To generate cytokines, the splenocytes were stimulated with enriched dendritic cell (DC) populations obtained as described by Nair *et al.* (13), that had been pulsed 3 hr before adding to responder splenocytes with UV-inactivated HSV (m.o.i of 5 before UV-inactivation). The responder (R) splenocytes and stimulator (S) DC (naive or pulsed) were added at R to S ratio of 50:1, 25:1, 12.5:1, and 6.25:1 in 200µl RPMI 10% FBS per well into ELISPOT plates which were coated with various anti-cytokine antibodies. After 72 hrs incubation, the plates were

washed and biotinylated anti-cytokine antibodies were added. After 1 hr incubation at 37 °C, 1 µg /100µl of alkaline phosphatase-conjugated streptavidin in PBS was added and the plates were incubated for another hour at 37 °C. The spots were developed using the substrate NBT/BCIP, and counted 24 hr later using a dissecting microscope

Statistical analysis

Student's *t* test was used where applicable.

CHAPTER 4. RESULTS

Cytokine Expression

To determine whether the three cytokine DNA constructs employed were expressed, two approaches were used. Firstly human embryonic kidney cells (293 cells) were transfected in vitro with cytokine DNA. After 3 days of culture, supernatants were harvested and tested, without concentration, for the presence of cytokines. All 3 cytokines (IL-2, IL-4 and IL-10) were detectable, the former two measured by bioassay, and IL-10 by ELISA (data not shown). More importantly, in vivo expression of cytokine proteins was measured 3 days following DNA administration by various routes. As is evident in Figure 1, cytokine DNAs were expressed, but the route of administration markedly affected the outcome. In ocular tissue, all 3 cytokine proteins were demonstrable following ocular exposure (i/oc) to cytokine DNA. However, the cytokine proteins were undetectable in ocular tissue following intranasal (i/nas) or intramuscular (i/m) DNA administration. Cervical lymph node (a draining lymph node for both ocular and nasal tissues) extracts were positive for all 3 cytokine proteins following i/oc or i/nas administration, but proteins were undetectable in the cervical LN following i/m injection. The latter, however, resulted in cytokine expression in muscle and popliteal LN (data not shown).

Effect of prophylactic cytokine DNA on the subsequent expression of HSV induced immunoinflammation

Groups of mice were treated on 3 occasions by different routes with cytokine or vector control DNA, after which animals were infected oculary with HSV. Animals were evaluated clinically at intervals for the development of herpetic ocular lesions, sampled periodically for viral secretion in tears as well as antibodies in serum. Tests for DTH reactions were also performed. Around 21 days post treatment, most animals were terminated and tissues collected for immunological evaluation. As is apparent in Figure 2, preadministration of both IL-4 and IL-10 DNA by either the ocular or i/nas routes led to significant suppression in the severity of ocular

disease. The level of IL-10 mediated suppression was greater following i/oc administration (60% of eyes had score less than 2) than after i/nas treatment (36%). Both routes appeared equally effective when IL-4 DNA was administered (i/oc:55%, i/nas:47%). In contrast, i/m administration of either IL-4 or IL-10 DNA had no apparent effect on the severity of HSK lesions. Similarly, IL-2 DNA given by any of three routes failed to reduce the severity of HSK but instead appeared to exacerbate severity. In vector control treated animals lesions were approximately equal to those occurring in untreated mice (data not shown). The influence of cytokine DNA administration on the expression of subsequent HSV specific cutaneous DTH reactions was also measured (Figure 3a). As with HSK, suppression resulted from preadministration of either IL-4 or IL-10 DNA, but not IL-2 DNA. All 3 routes of DNA exposure proved efficacious in suppression of DTH reactions and inhibitory effects appeared prolonged. Suppressed DTH reactions were still present at 7 and 8 weeks p.i., respectively (Figure 3b). Interestingly, in the separate experiment, where IL-10 protein was injected along with antigen during the elicitation of DTH reaction in sensitized animals, suppressed responses were evident in the IL-10 injected ear but not the noninjected ear (Figure 4). Furthermore, DTH responses in distal sites such as footpad from the IL-10 injected site were unaffected. In addition, i/m injection of IL-10 protein by the same protocol had no effect on DTH responses (data not shown). Thus although the IL-10 DNA had widespread suppressive effect, the effect of IL-10 protein was confined to the injection site

Effect of cytokine DNA pretreatment on HSV-specific immune responses.

Samples taken at intervals from mice revealed little effect of cytokine DNA pretreatment on the duration or level of ocular viral secretion following infection (data not shown). However, blood samples examined on day 21 p.i. revealed changes in the IgG isotype ratio in mice which received IL-4 DNA by each of 3 routes of administration (Figure 4). The isotype pattern was consistent with a shift towards the Th2 profile. Such a shift was not evident in recipients of IL-10 or IL-2 DNA. As regards to T cell function measured in vitro in animals sacrificed around 21 days p.i., once again recipients of IL-4 DNA showed a shift in antigen induced cytokine production

towards the Th2 pattern (Figure 5). In such mice, numbers of SFC for IFNγ were reduced and SFC for IL-4, IL-5 and IL-10 increased (the latter between 10-20 fold). IL-10 DNA recipients did have diminished numbers of IFNγ SFC, but there was no significant elevation of Th2 cytokine producing SFC. However, both IL-4 DNA and IL-10 DNA inhibited HSV specific lymphoproliferation (Figure 6). Taken together, our results indicate that IL-4 DNA administration shifts the T cell reactivity pattern toward a Th2 profile while IL-10 DNA exposure induces the downregulation of HSV induced Th1 response rather than a shift. This suggest that the suppressed inflammatory response which resulted from both IL-4 and IL-10 DNA administration may proceed by different mechanisms.

CHAPTER 5. DISCUSSION

This report addresses the issue of whether viral induced inflammatory responses can be modulated by the preadministration of naked plasmid DNA (eukaryotic expression vectors) encoding cytokines. Our results show that cytokine DNAs encoding IL-10 or IL-4 administered topically to the cornea or nasal surfaces do modulate the severity of ocular and cutaneous lesions associated with HSV infection. Both of the HSV lesions are considered to represent immunoinflammatory responses resulting primarily from antigen recognition by $CD4^+$ T cells of the type 1 cytokine producing profile (14 - 16). Not unexpectedly, control experiments with DNA encoding IL-2 or IFNγ (data not shown) failed to modulate the severity of lesion expression. Although ocular inflammatory lesions were modulated by topical administration of IL-10 or IL-4 DNA, the same preparations given intramusculary had no inhibitory effects. In contrast, however, both of the cytokine DNAs did cause suppression of cutaneous DTH lesions following i/m administration, and this suppression persisted for at least 7 weeks. It is notable that nonreplicating plasmid DNA can affect the immune responses for a prolonged period following herpes virus infection.

The essential mission of the present research was to evaluate the route of cytokine DNA exposure for their modulatory effects since most previous studies using plasmid DNA either for vaccination or modulatory effects used systemic administration (9, 17). We have shown that plasmid DNA encoding certain HSV proteins given mucosally or to the ocular surface resulted in immune induction (5, 18). Others have also shown that cytokine DNA given in a liposome formulation to the nasal mucosa may modulate the subsequent expression of allergic disease (19). Other studies, however, have not simultaneously compared routes for modulatory effects or studied numerous cytokine DNAs in parallel. The present observation that cytokine DNAs given topically, especially to readily accessible sites such as the nasal mucosa, provides a novel and convenient means of managing unwanted inflammatory lesions. Although our present

report deals with prophylactic cytokine DNA administration, at least with IL-10 DNA topical application to early immunoinflammatory ocular lesions can have beneficial effects (8).

Our observation that the effect of cytokine DNA on ocular and cutaneous reactions associated with HSV infection differed according to the route of cytokine DNA administration was unexpected since both lesions were assumed to involve similar cellular mechanisms of expression. As mentioned earlier, both lesions are assumed to largely represent CD4 T cell orchestrated events with type 1 cytokines principally involved. However, our data showed that both IL-10 and IL-4 DNA diminished DTH reactions regardless of the route of cytokine DNA administration. Moreover, suppression persisted for a surprisingly long time (at least 7 weeks). By way of contrast, ocular lesion modulation did not occur following i/m DNA administration. Modulation of these lesions was most evident following topical application of cytokine DNA directly to the corneal surface. This was especially true for IL-10 DNA application. As was evident from in vitro measures of immunity in cytokine DNA treated animals, the results of IL-4 DNA administration was to affect the nature of the subsequent Ag specific T cell immune response. In fact, even though certain motifs of DNA (CpG sequence) may cause Th1 differentiation nonspecifically, there was a shift toward the Th2 pattern in IL-4 pretreated groups and this was reflected by results of both T cell cytokine measurement and Ig isotype ratios. Thus the effect of IL-4 DNA on DTH reactions could be explained by a central effect on the balance of the immune response. Why such an effect failed to diminish HSK expression following i/m administration is difficult to explain but it may be that multiple mechanisms are at play in HSK, including CD8 T cell reaction (20) or CD4 Th2 responses as some other investigators have proclaimed (21).

Since IL-10 DNA led to little influence on T cell subset balance, it could be that the effect of IL-10 is largely dependent on local action at sites of inflammation. Indeed modulation of ocular lesions by IL-10 DNA was most efficient on ocular lesions when administered topically to the eye itself. Furthermore, following ocular administration of IL-10 DNA, IL-10 protein expression could be directly demonstrated (Figure 1). IL-10 is well known to reduce the capacity

of antigen presentation, and inhibit the production of proinflammatory cytokines such as IFNγ, IL-1 and TNFα (22). In fact, we have found that topical ocular IL-10 DNA administration led to reduce TNFα production in the ocular tissue (unpublished data). Our results did show, however, that i/nas administration of IL-10 DNA had some modulatory effects on HSK expression. This could be due to some IL-10 protein gaining access to the eye following i/nas administration although we could not formally demonstrate this fact. It is known, however, that lymphoid tissue draining the eye and nasal cavity includes some of the same nodes, and IL-10 protein expression was demonstrated in cervical LN following i/nas administration. If IL-10 DNA operates by causing local expression in actual lesions themselves, then the most difficult observation to explain was that IL-10 DNA given topically or systemically suppressed DTH reactions and this effect persisted for some weeks. Recently, it was reported that repeated IL-10 protein exposure induced a particular T cell population (Tr1) which produces mainly IL-10 but not IL-4 (23). It may be possible that IL-10 DNA administration induces such a T cell population and that such cells gain access to cutaneous sites during DTH reactions.

Alternatively, following administration, DNA may gain access to cutaneous sites and persist there for weeks. The traffic pattern which follows DNA administration to various sites has not been elucidated. Surprisingly, DNA can be observed at remote locations from the point of deposition as detected by PCR or protein expression of markers such as β-Gal. Using β-Gal DNA, we have also found signals (expression) in the DTH inflamed ears following systemic or topical administration (unpublished observation). Indeed, we have shown that surface exposure of β-Gal DNA can induce gene expression in the distal tissues such as cervical LN and spleen (18). Therefore, locally expressed IL-10 might serve to suppress DTH responses. In support of this idea, we showed that IL-10 protein administration caused inhibition of the DTH responses in the protein injected ear, but not at the distal sites.

How the DNA is transported to cutaneous locations and whether the process can cause therapeutic effects to become magnified is an intriguing topic currently under investigation in our laboratory. Whatever the mechanism involved, our results serve to demonstrate that plasmid

DNA encoding cytokines administered to readily accessible surface sites represents a convenient means of modulating immunoinflammatory lesions.

Figure 1. Expression of cytokine in vivo. To assess cytokine protein expression in vivo, groups of BALB/c mice were administered either i/m, i/nas or i/oc with 100 μg of plasmid DNA encoding either IL-2, IL-4 or IL-10. Three days later the corneas and the cervical LN were pooled separately in DMEM 10 % FBS and frozen at -80°C. Following thawing, tissues were homogenized and sonicated. After centrifugation, the supernates were analyzed for either IL-2, IL-4, or IL-10 by ELISA.

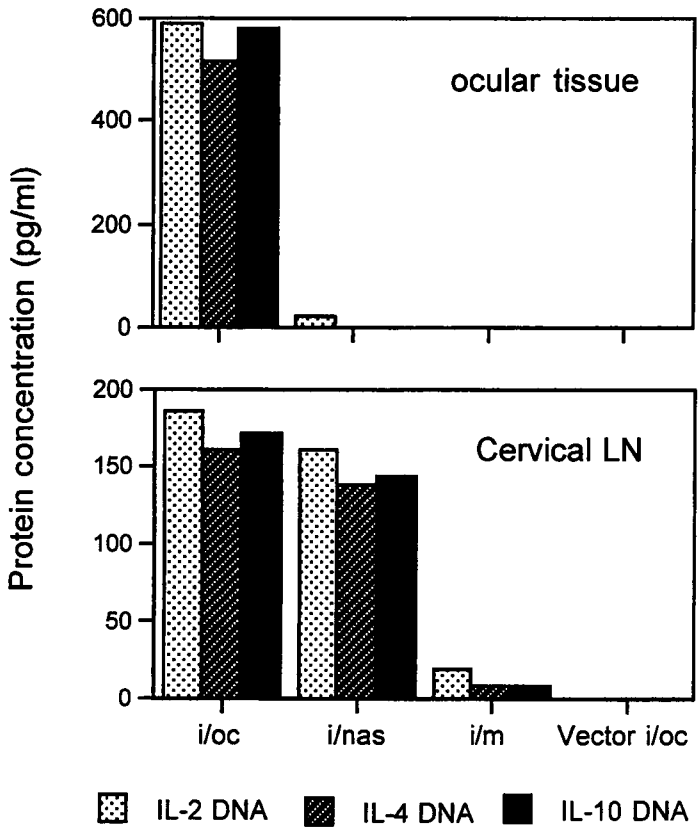

Protein concentration (pg/ml)
600
400
200
0
ocular tissue
200
150
100
50
0
Cervical LN
i/oc
i/nas
i/m
Vector i/oc
IL-2 DNA
IL-4 DNA
IL-10 DNA

Figure 2. The effect of prophylactic cytokine DNA administration on the severity of ocular inflammation (HSK). Groups of animals were administered either i/m, i/nas, or i/oc with plasmid DNAs encoding either IL-2, IL-4 or IL-10, or vector on three occasions at weekly intervals. On the day after the last treatment, the corneas were challenged with 1 x 10^6 PFU of HSV-1 RE. The animals were clinically observed at various time points p.i. for development of herpectic stromal keratitis. Scores of individual eyes are shown on the graph. The total number (n) of mice used: IL-2 DNA i/m (n=38), IL-2 DNA i/nas and i/oc (n=40), IL-4 DNA i/m (n=34), IL-4 DNA i/nas and i/oc (n=52), IL-10 DNAi/m and i/nas (n=38), IL-10 DNA i/oc (n=34). *, **, #, ## Differences were statistically significant ($p < 0.05$) compared with those of vector or IL-2 DNA treated group. * vs **, not statistically significant between groups ($p = 0.068$). # vs ## statistically significant between groups ($p < 0.041$).

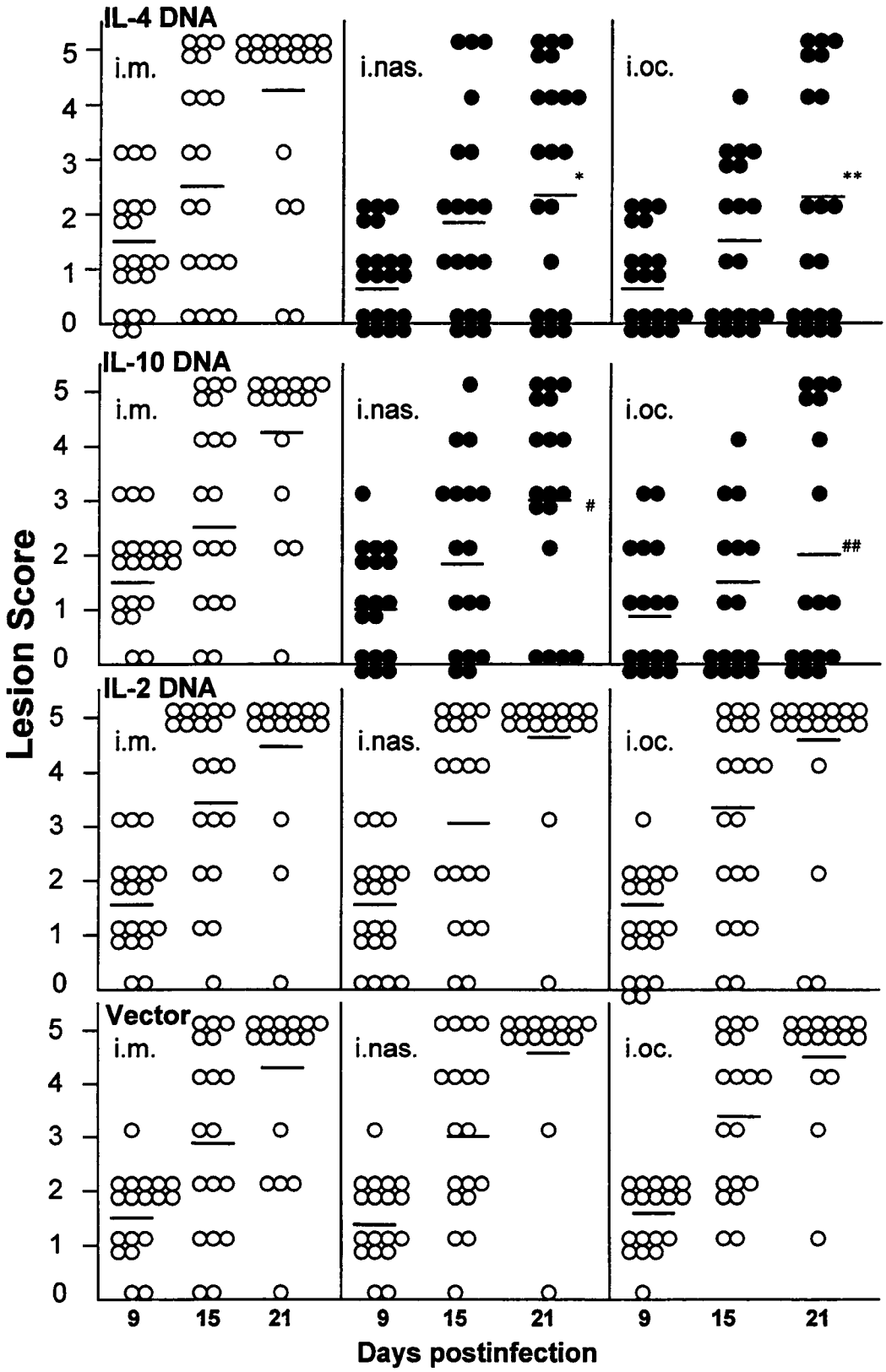
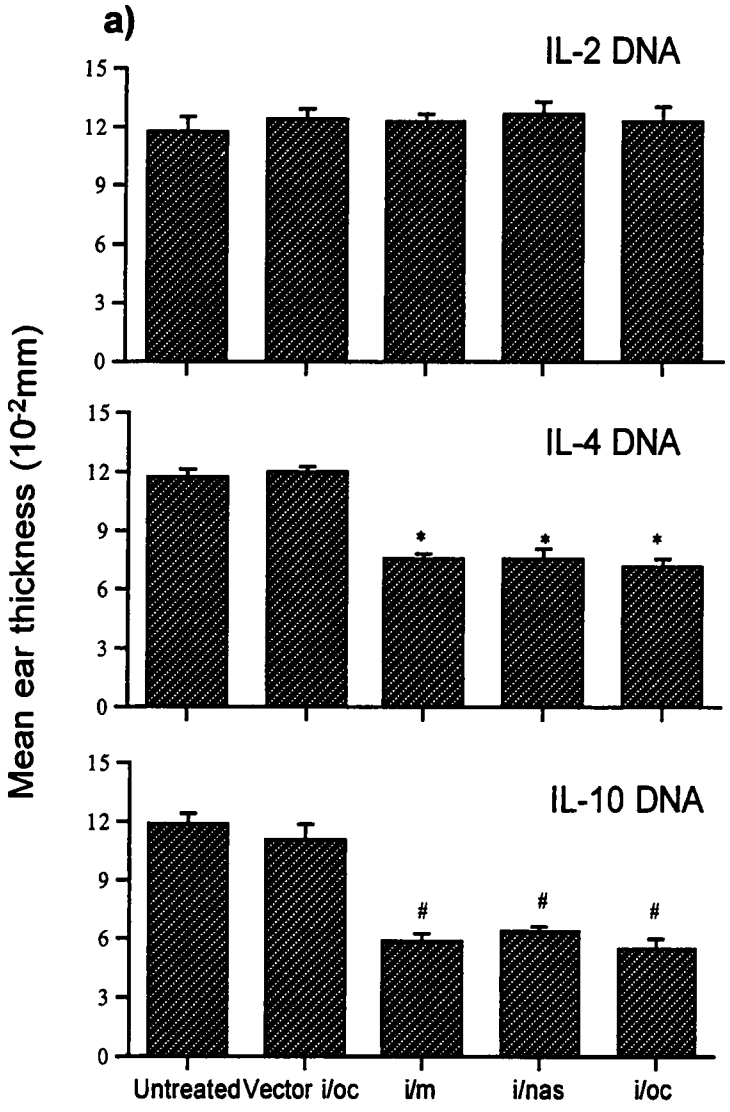

IL-4 DNA
IL-10 DNA
IL-2 DNA
Vector
i.m.
i.nas.
i.oc.
Lesion Score
0
1
2
3
4
5
*
**
#
##
9
15
21
Days postinfection

Figure 3. Suppression of HSV-specific DTH response after prophylactic administration of plasmid DNA encoding cytokines. Groups of mice were treated with plasmid DNA encoding IL-2, IL-4, or IL-10 either i/m, i/nas or i/oc, or vector on three occasions at weekly intervals, and infected with 1 x 10^6 PFU of HSV-1 RE ocularly at the day after the last DNA administration. On day 18 following HSV infection, these mice were challenged with 20 µl of HSV-1 KOS (1 x 10^5 prior to UV inactivation) or vero extract in the right or left ear pinna, respectively. 48 hrs later, the increase in ear thickness was measured. (a). On day 38, 48 and 58, the mice were challenged and DTH responses were measured 48 hrs later (b). Similar results were found in i/m or i/nas administered groups. The figures show the mean difference between the thickness of left and right ear pinna at 48 hrs after challenge. Each groups contains at least 10 mice.* and # ($p <$ 0.01), ## IL-10 DNA versus vector ($p < 0.01$), ** IL-4 DNA versus vector ($p > 0.05$ at day 60).


a)
IL-2 DNA
IL-4 DNA
IL-10 DNA
Mean ear thickness (10-2mm)
15
12
9
6
3
0
*
#
Untreated
Vector
i/oc
i/m
i/nas
i/oc

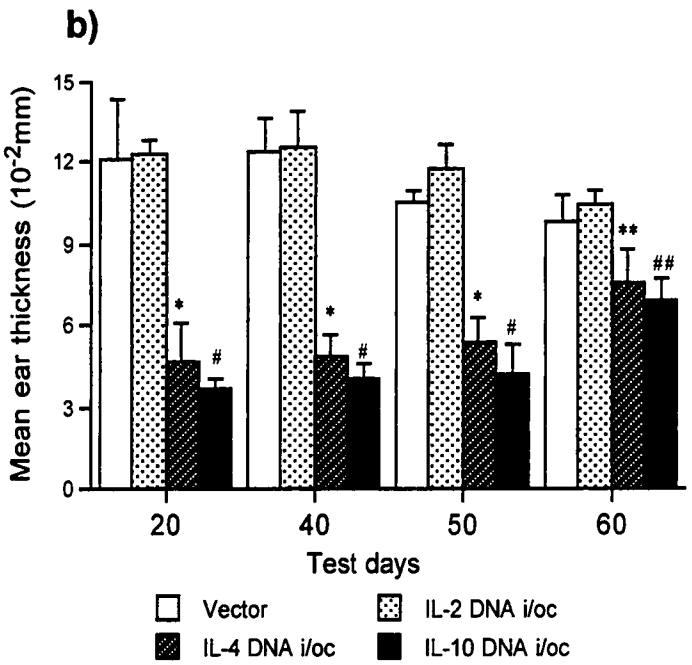

b)
Mean ear thickness (10^{-2}mm)
15
12
9
6
3
0
20
40
50
60
Test days
Vector
IL-2 DNA i/oc
IL-4 DNA i/oc
IL-10 DNA i/oc
*
#
**
##

Figure 4. The effect of IL-10 protein on cataneous DTH responses. Groups of mice were immunized with HSV-1, and twenty five days later the animals were challenged with 20 µl of HSV-1 KOS and IL-10 protein and with 20 µl of HSV-1 in the right ear and left ear, respectively. For the control group, mice were injected with 20 µl of HSV-1 and vero cell extract in the right and left ear, respectively. For the footpad swelling, the same mice were challenged with 20 µl of HSV-1 and vero cell extract in the right and left footpad, respectively. 48 hrs later, the increase in ear or footpad thickness was measured as described in Materials and Methods. The graphs show the mean increase of thickness at 48 hrs after challenge.

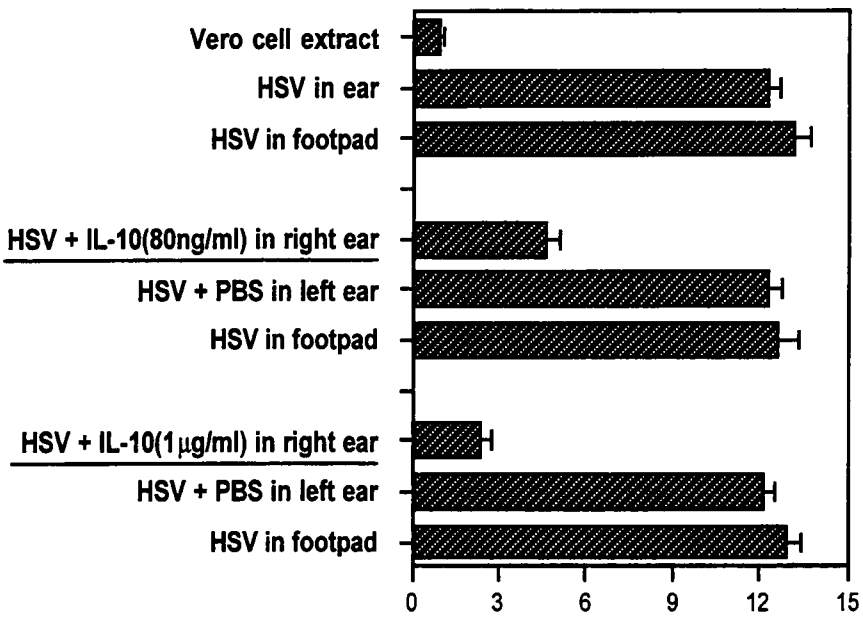

Vero cell extract
HSV in ear
HSV in footpad
HSV + IL-10(80ng/ml) in right ear
HSV + PBS in left ear
HSV in footpad
HSV + IL-10(1μg/ml) in right ear
HSV + PBS in left ear
HSV in footpad
0
3
6
9
12
15

Figure 5. The effect of prophylactic administration of cytokine DNAs on humoral immune responses. Serum samples from mice given plasmid DNA encoding cytokines were collected at day 21 post infection, and individually analyzed for HSV specific antibody responses as described in Materials and Methods. Each group consisted of 10 to 14 mice. *, ** Statistically significant (IL-4 DNA vs IL2 DNA or vector treated group, $p < 0.05$), # Statistically not significant (IL-10 DNA vs IL-2 DNA or vector treated group, $0.05 < p < 0.1$)

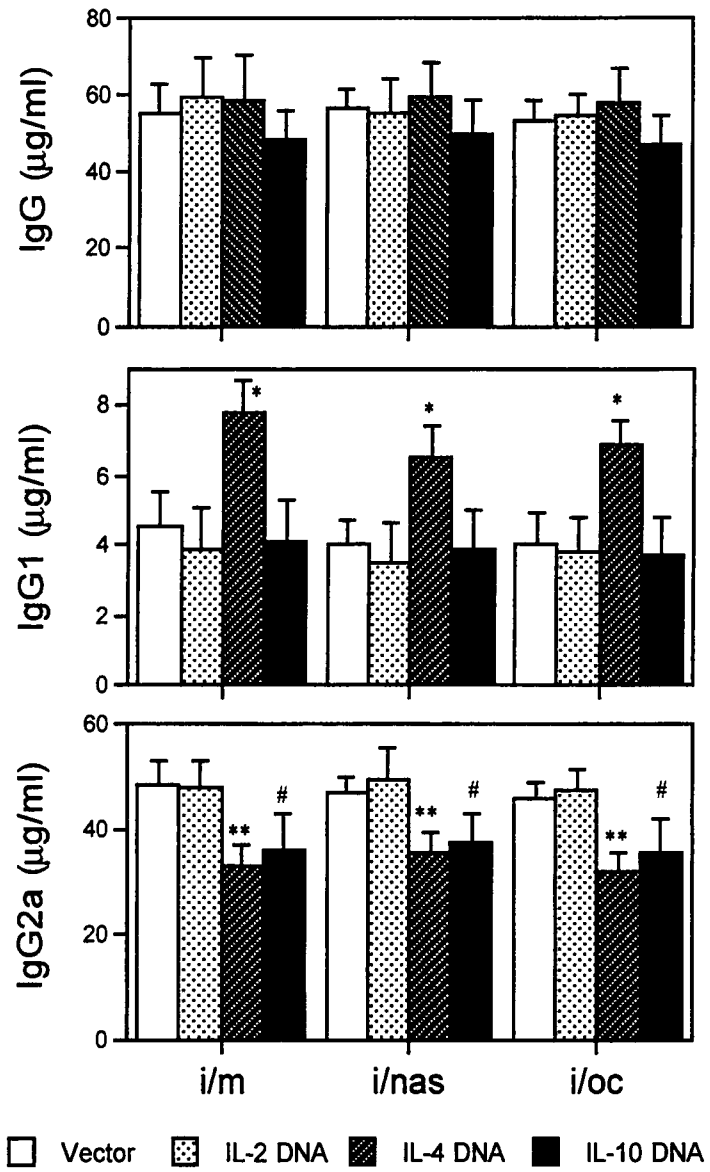

IgG (µg/ml)
80
60
40
20
0
IgG1 (µg/ml)
8
6
4
2
0
*
IgG2a (µg/ml)
60
40
20
0
**
#
i/m
i/nas
i/oc
Vector
IL-2 DNA
IL-4 DNA
IL-10 DNA

Figure 6. The effect of prophylactic administration of cytokine DNAs on splenic cytokine forming cells. Approximately 21 days p.i., splenocytes from 2 mice of each group were pooled and restimulated in vitro for 4 days with enriched DC population either naive or pulsed with UV-HSV (multiplicity of infection = 5 before UV inactivation). Frequencies of cytokine producing cells were measured with ELISPOT assay. The number of SFC after naive DC restimulation are subtracted from the values of UV-HSV pulsed DC restimulation. The graph shows mean and standard deviation of four independent experiments. *, # IL-4 DNA or IL-10 DNA versus vector, $p < 0.01$, **, *** IL-4 DNA versus vector, $p < 0.05$, **** IL-4 DNA versus vector, $p < 0.01$, ## IL-10 DNA versus vector, $0.05 < p < 0.01$

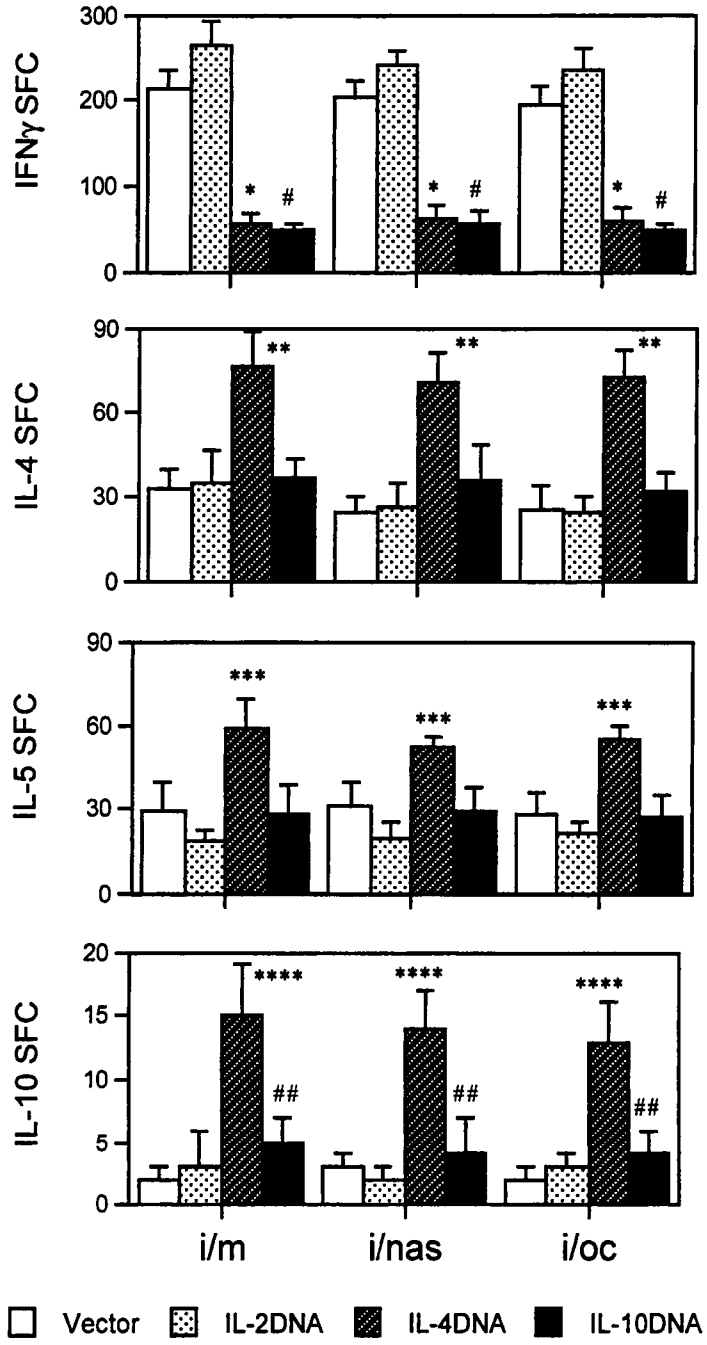
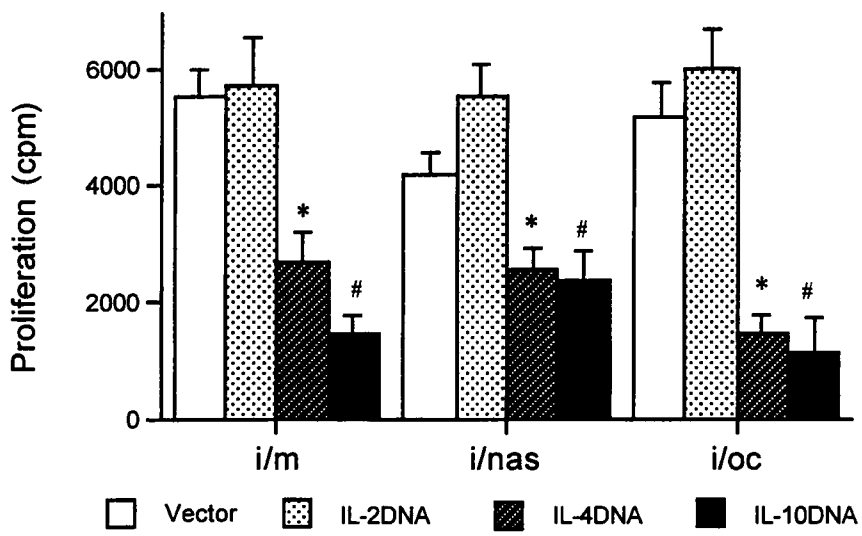

IFNγ SFC
300
200
100
0
*
#
IL-4 SFC
90
60
30
0
**
IL-5 SFC
90
60
30
0
***
IL-10 SFC
20
15
10
5
0
****
##
i/m
i/nas
i/oc
Vector
IL-2DNA
IL-4DNA
IL-10DNA

Figure 7. The effect of prophylactic cytokine DNA administration on HSV-specific lymphoproliferation. Approximately 21 days p.i., splenocytes from 2 mice of each group were pooled and used as responders for proliferation. These responders were mixed with irradiated syngenic spleen cells infected with 1.5 m.o.i. UV-HSV or irradiated naive splenocytes, and incubated for 5 days as described in Materials and Methods. The graph shows responder plus irradiated UV-HSV pulsed syngenic splenocytes. The graph represents one of five independent experiments which showed similar results. *, # IL-4 DNA or IL-10 DNA versus vector, $p < 0.01$. Proliferation index (PI) was also calculated. IL-2 DNA i/m, PI = 11.5; i/nas, PI = 14.3 ; i/oc, PI = 16.5, IL-4 DNA i/m, PI = 5.1; i/nas, PI = 6.1; i/oc, PI = 3.9, IL-10 DNA i/m, PI = 3.2 ; i/nas, PI = 5.7 ; i/oc, PI = 2.8, Vector i/m, PI = 10.4 ; i/nas, PI = 10.2 ; i/oc, PI = 13.5.


Proliferation (cpm)
6000
4000
2000
0
*
#
*
#
*
#
i/m
i/nas
i/oc
Vector
IL-2DNA
IL-4DNA
IL-10DNA

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PART 3. IMMUNE MODULATION BY IL-10 GENE TRANSFER VIA VIRAL VECTOR AND PLASMID DNA : IMPLICATION FOR GENE THERAPY

(Cellular immunology, 1999, 194 : 194)

CHAPTER 1. ABSTRACT

In the present report, we have evaluated and compared the modulatory effect of the cytokine interleukin (IL)-10 expressed via viral vector or plasmid DNA on viral antigen induced cutaneous inflammatory lesions. Our data demonstrate the superior potency of both recombinant vaccinia virus and herpes simplex virus IL-10 expression vectors after single intramuscular administration, but the effects were only short term and only functioned in animal lacking immunity to the viral vectors used for modulation. In contrast, modulatory effects achieved by plasmid DNA expressing IL-10 were delayed in onset and milder in effect but were far more persistent than those achieved by viral vectors. Moreover, plasmid DNA expressing IL-10 provided effective modulation when given repeatedly to animals. Our data also showed that IL-10 gene delivery resulted in a systemic and durable modulatory effect while the effect caused by a single IL-10 protein treatment was transient and confined to the injected site. Our results imply that the viral vector system is superior for obtaining short-term effect, whereas the plasmid DNA approach represents a better strategy to achieve gene therapy to modulate chronic inflammatory lesions.

CHAPTER 2. INTRODUCTION

Modulating lesions by protein or gene transfer is likely to be a practical outcome of the cellular and molecular understanding of inflammatory reactions. For example, in many instances chronic lesions caused by viral infections result from immunoinflammatory reactions against persisting viral antigen or changes imposed by replicating virus on host tissue antigens (1). Often times, the inflammatory reaction is orchestrated by T cells whose induced principal products are cytokine molecules such as IFNγ, IL-2 and TNFα, so called the Th1 cytokines (2, 3). Moreover, counteracting the production or activity of such molecules results in lesion resolution (2, 4). Currently, the main therapeutic option for achieving this result is to use nonspecific and often toxic anti-inflammatory drugs, but more selective procedures should be feasible. One of such approaches may be to employ one of the body's own means of curtailing immunoinflammation. For example, cytokines such as TGFβ and IL-10 may oppose some of the proinflammatory actions of activated Th1 T lymphocytes (5, 6).

The production of IL-10 by antigen stimulated CD4+ T lymphocytes may exert a variety of antiinflammatory effects (6). These include the shut down of stimulated Th1 T cells, the inhibition of inflammatory cell migration as well as the production of proinflammatory molecules by recruited macrophages (7-14). Consequently, expressing IL-10 in inflammatory lesion represents an attractive option for therapy. Experimentally, this has been tested by injecting IL-10 protein, but the effect is short lived and impractical for therapeutic use since usually multiple injection is necessary to achieve modulatory effect (12, 15-17). The alternative strategy of gene delivery represents the most promising approach, but practical means of achieving this are yet to be perfected. In a previous report, we demonstrated the feasibility of using naked plasmid DNA encoding cytokines as a means of modulating viral induced inflammatory lesions (4, 18, 19). Other studies have focused on viral vectors encoding regulatory molecules such as IL-12 or viral IL-10 as a way of controlling inflammatory reactions (20, 21). Few, if any, reports have compared the efficacy of multiple approaches against a single inflammatory lesion.

In this communication, we have compared the activity of two viral vector systems which express IL-10 with naked plasmid DNA encoding IL-10 for their suppressive effects against the effector phase of a viral antigen induced inflammatory reaction. Our data demonstrate that a single administration of IL-10 protein had a local and transient effect while IL-10 gene transfer had a systemic modulatory effect. In addition, our results show the superiority of both recombinant vaccinia and herpes simplex viruses encoding IL-10 over IL-10 plasmid DNA in terms of short-term efficacy. However, lesion control resulting from plasmid DNA therapy was far more persistent. Furthermore, plasmid DNA could be administered repeatedly and remain effective whereas neither viral vector could induce lesion suppression in animals immune to the homologous virus. The implications of our data regarding the use of gene delivery systems to control immunoinflammatory diseases are discussed.

CHAPTER 3. MATERIALS AND METHODS

Mice

Female BALB/c mice (H-2^d), 3-4 weeks old, were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed in the animal facilities at the University of Tennessee. All investigations follow the guidelines of the Committee on the care of Laboratory Animals Resources, commission on Life Science, National Research Council. The animal facilities were fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Viruses

Herpes simplex virus type-1 (HSV-1) KOS strain, HSV-1 γ34.5 deletion mutant (HSVγ34.5-) and recombinant HSV-1 expressing murine IL-10 (HSV-IL-10) were grown on vero cell monolayers (Cat. NO. CCL70, American Type Culture Collection (ATCC), Rockville, MD). HSV-IL-10 was kindly provided by Dr. Bernard Roizman (University of Chicago, Chicago, IL). Both copies of the γ34.5 gene in HSV-1 were replaced by cDNA encoding IL-10 (22). The production of IL-10 in HSV-IL-10 was derived from of early growth response (egr)-1 promoter that maximizes the expression of the inserted gene in dividing cells (22). Vaccinia virus tk- (Vvtk-) and recombinant vaccinia virus expressing murine IL-10 (VV-IL-10) were grown on CV-1 cells (Cat. NO. CCL81, ATCC). VV-IL-10 was a gift from Dr. Michael Kurilla (University of Virginia, Charlottesville, VA). The expression of IL-10 in VV-IL-10 is initiated from VV early-late promoter P7.5, providing constitutive expression throughout the viral multiplication cycle (23). The viruses were concentrated, titrated and stored at - 80°C until use.

Plasmid DNA preparation

Plasmid DNA encoding murine IL-10 (IL-10 plasmid DNA) containing simian virus 40 promoter was provided from Dr. T. Mossman (Edmonton, Alberta, Canada). For the construction of control vector DNA, IL-10 plasmid DNA was digested with *EcoR*I followed by ligation of

agarose gel purified vector fragment. All plasmid DNAs were purified by polyethylene glycol precipitation by the method of Sambrook et al. (24), with some modification as described previously (4). The expression of IL-10 was identified by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA).

Immunization.

Groups of mice were anesthetized with methoxyfurane (Metofane; Pittman-Moore, Mundelein, IL) and infected intramuscularly with 10^6 plaque forming unit (PFU) of HSV-1 KOS strain or 10^6 PFU of VVtk-.

IL-10 gene transfer into virus-immunized animals.

Three weeks post infection, groups of HSV-immune mice were anesthetized and given into biceps or tibialis muscle of both legs with the followings : 25 or 100 μg of IL-10 plasmid DNA or vector, 2.5 x 10^5 or 5 x 10^6 PFU of HSV-IL-10 or HSVγ34.5- , or 2.5 x 10^5 or 5 x 10^6 of VV-IL-10 or VVtk- into biceps or tibialis muscle of both legs (total either 50 or 200 μg of plasmid DNA, or 5 x 10^5 or 10^7 of Viruses per mouse). Similarly, groups of VV-infected mice were injected intramuscularly with either IL-10 plasmid DNA or vector, or HSV-IL-10 or • SVγ34.5- as described above. The optimized doses (200 μg of IL-10 plasmid DNA and 10^7 PFU of Viral vector expressing IL-10) for the gene transfer studies were chosen by measuring antigen specific DTH responses and used for further experiments.

Antigen specific delayed type hypersensitivity (DTH)

At different time points following the administration of IL-10 plasmid DNA or VV-IL-10, HSV-specific DTH was tested by injecting ultraviolet (UV) -inactivated HSV-1 KOS (10^5 before UV-inactivation) and vero cell extract into the right and left ear pinnae of HSV-immunized animals, respectively. Similarly, to measure VV-specific DTH responses UV-inactivated VV (10^5 prior to UV-inactivation) and CV-1 cell extract were injected into the right and left ears of VV-

infected mice which received either IL-10 plasmid DNA or vector, or HSV-IL-10 or HSVγ34.5-. Test antigens were injected in 20 μl volumes in the ear pinnae and the increase in ear thickness was measured with a screw gauge meter (Oditest; H. C. Kroeplin GHBH, Schluechtern, Germany) as described elsewhere (25). The mean difference between the thickness of right and left ears at 48 hours after challenge was calculated.

In a separate experiment, 0.1 or 1 μg of IL-10 protein and phosphate buffered saline (PBS) were injected once into either right and left ear or muscle before and after the elicitation of HSV-specific DTH responses and the increase in thickness was measured as described above.

Lymphoproliferation assay

To evaluate the effect on antigen specific lymphoproliferation, the mice were sacrificed on day 7 or 25 after IL-10 gene transfer, and spleens were isolated. These immune splenocytes (responders) were stimulated *in vitro* with irradiated syngeneic splenocytes (stimulators) pulsed with UV-inactivated HSV for the HSV-specific proliferation (or UV-inactivated VV for the VV-specific proliferation) or irradiated naive splenocytes for 5 days as described previously (26). Eighteen hours before harvest, [^{3}H]-thymidine (1 μCi/well) was added to all the cultures. Harvested cells were measured for radioactivity using a beta scintillation counter (Inotech, Lancing, MI). Responders cultured in media containing 1 μg/ml of concanavalin A (Con A) were used as a polyclonal stimulator positive control for the lymphoproliferation assay. The results are expressed as the cpm of responders plus antigen pulsed stimulators from six independent experiments. Proliferation Index (cpm in UV-HSV pulsed stimulator / cpm in naïve stimulator) was also calculated and expressed on each bar graph. In a separate experiment, forty eight hours after a single IL-10 treatment into the ear, spleens were isolated individually, and draining lymph node (cervical LN) cells were pooled from 2 mice. HSV-specific proliferative responses of these responder cells were measured as described above.

Quantification of Cytokine-producing cells by enzyme-linked immunoSPOT (ELISPOT).

The detailed methods of ELISPOT assay for the quantification of cytokine producing cells were described previously (27). Seven or twenty-five days later following IL-10 gene transfer, the mice from each group were sacrificed and splenocytes were obtained. The responder splenocytes were analyzed for IL-4 and IFNγ spot-forming cells by ELISPOT. To generate cytokines, the splenocytes were stimulated in vitro with enriched dendritic cell (DC) populations obtained by the method of Nair *et al.* (28). The enriched DC were pulsed with UV-inactivated HSV (m.o.i. of 5 before UV-inactivation) for the HSV-specific cytokine production and UV-inactivated VV (m.o.i. of 5) for VV-specific cytokine production. The responder splenocytes and stimulator DC (naive or pulsed) were added at a responder-to-stimulator ratio of 10:1, 5:1, 2.5:1 and 1.25:1 in 200 µl of RPMI with 10 % fetal bovine serum per well into ELISPOT plates which were coated with anti-mouse IL-4 (Cat. NO. 18191D, Pharmingen) or anti-mouse IFNγ (Cat. NO. 18181D, Pharmingen). After 96 hours incubation, the plates were washed and biotinylated anti-mouse IL-4 (Cat. NO. 18042D, Pharmingen) or biotinylated anti-mouse IFNγ (Cat. NO. 18112D, Pharmingen) was added. After 1 hour of incubation at 37 °C, 100 µl of alkaline phophatase-conjugated streptavidin in PBS (1 µg/ml) was added and the plates were incubated for another one hour. The spots were developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as a substrate and counted 24 hours later under a dissecting microscope. Mean cytokine spot forming cells (SFC) ± standard deviation from 6 independent experiment was expressed as results.

Antibody assay by enzyme linked immunosorbent assay (ELISA)

Serum samples were collected from each mouse at day 15 following IL-10 gene transfer and analyzed individually for HSV-specific or VV-specific antibodies in a standard quantitative ELISA, as described previously (27). Briefly, sera were tested for total IgG, IgG2a and IgG1 using ELISA plates coated with HSV-antigen or VV-antigen and isotype-specific antibodies. The

results were quantified by generating a standard curve, using Spectra Max ELISA reader Softmax Version 1.2 (Molecular devices, Sunnyvale, CA).

IL-10 detection in serum after IL-10 gene transfer.

Groups of mice received intramuscularly either 200 μg of IL-10 plasmid DNA or vector, 10^7 PFU of VV-IL-10 or VVtk-, or 10^7 PFU of HSV-IL-10 or HSVγ34.5. Every other day, serum samples were collected and analyzed for the presence of IL-10 in the serum by ELISA assay. ELISA plates (Dynatech, Chantilly, VA) were coated overnight at 4 °C with 2 μg of rat antimouse IL-10 (Cat. No. 18141D). The wells in the plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 3 % milk for 2 hours at 37 °C. The sera samples and the recombinant IL-10 (Cat. No. 19281V) at a concentration of 1 ng/ml) were added and serially diluted. The standard and samples were incubated overnight at 4°C. After washing with PBS-T, 1 μg of biotinlyated anti IL-10 antibody (Cat. No. 18152D, Pharmingen) per ml was added and incubated at 37 °C for 2 hrs. After the wells were washed, peroxidase-conjugated streptavidin (Jackson Immunoresearch) was added and incubated at 37 °C for 2 hrs. ABTS solution (11 mg of 2, 2"-azino-bis-3 ethylbenzthiazoline-6- sulfonic acid in 25 ml of 0.1 M citrate acid, 25 ml of 0.1 M sodium phosphate and 10 μl of hydrogen peroxide) was used for color development. IL-10 concentration was measured by automated ELISA reader (Spectra MAX 340, Molecular devices, Sunnyville, CA). Each time point contains sera from at least 4 mice.

Statistical analysis

Significant differences between groups were evaluated using the Student's *t* -test.

CHAPTER 4. RESULTS

Comparison of Modulatory effects of IL-10 expressing vectors on antigen induced cutaneous inflammation

Mice were primed by infection with either HSV or vaccinia viruses and three weeks later were injected once with one of various IL-10 expressing vectors, or IL-10 protein. At intervals following such treatment, the inhibitory effects on viral antigen induced delayed type hypersensitivity (DTH) responses were evaluated using optimal doses of each gene delivery system as described in the Materials and Methods. The results of sample experiments are shown in Figure 1 - 3. It is readily apparent that the cutaneous inflammatory reaction can be suppressed by exposure to IL-10 protein. However, as shown in Figure 1, the suppressive effect achieved by a single IL-10 protein administration was of short duration and limited to the site of injection. Intramuscular injection of IL-10 protein did not have any effect on DTH responses. Thus suppression of the DTH reaction was only evident if skin testing was begun 2 hours before or after single IL-10 administration, and then was only evident at the site which received the protein injection (Figure 1). By way of contrast, suppression of DTH reactions occurring after administration of vectors encoding IL-10 was systemic and of far greater duration. (Figures 2 and 3).

Our investigations compared the immunomodulatory effect of IL-10 expressed by a single intramuscular injection of plasmid DNA encoding IL-10, or of two recombinant viruses which encoded the mouse IL-10 gene. As shown in Figures 2 and 3, which evaluated the immunomodulatory effects of the various vectors in HSV and vaccinia virus primed mice respectively, there was a time lag before suppressive effects occurred. After IL-10 DNA administration, the modulatory effects took around 5 days to become evident, but suppression continued in some animals for up to 7 weeks post administration.

The modulatory effects that resulted from viral vector administration occurred rapidly being evident by 2 days p.i.. Moreover, the level of suppression induced by either HSV-IL-10 or

VV-IL-10 was greater than that observed in IL-10 DNA administration. However, whereas suppression after plasmid DNA persisted for 6-7 weeks, the inhibitory effects of recombinant virus encoding IL-10 lasted for less than 3 weeks. The suppressive effect on DTH reaction by empty vector plus viral vector encoding IL-10 or empty viral vector plus IL-10 plasmid DNA treatment did not significantly differ from that of groups treated with IL-10 viral vector alone or IL-10 plasmid DNA alone (Figure 2, 3).

Finally and potentially of greatest significance with regard to practical immunotherapy, the modulatory effects of viral vectors were only evident in animals not previously exposed to the virus used for immunotherapy. Thus, whereas HSV-IL-10 functioned effectively in vaccinia virus primed mice, this was not the case in HSV-primed animals. The obverse situation pertained in vaccinia virus primed animals. In this instance, HSV-IL-10, but not VV-IL-10, effectively suppressed the DTH reaction. In other experiments, we have observed that a second administration of the same viral vector in animals which responded previously (since unprimed) was usually unsuccessful (Figure 4). However, multiple exposures to plasmid DNA encoding IL-10 invariably resulted in effective suppression of inflammation (Figure 4).

Effects of IL-10 administration on immune responsiveness measured in vitro

To measure the relative efficacy of IL-10 expression for therapeutic immunomodulation, sample animals were killed either at day 2 after IL-10 protein treatment or at day 7 or 25 following IL-10 gene transfer, and selected humoral and splenic T cell responses were quantified. None of the treatments had any significant effects on the titer or IgG isotype profile to viral antigens (data not shown). Significant T cell changes in the recipients were not detectable after a single IL-10 protein injection (Figure 5). However, effects on T cell responsiveness were evident at certain times in groups of animals given IL-10 DNA or viral vectors expressing IL-10.

At 7 days post treatment with viral vectors expressing IL-10, splenocytes from all animals showed diminished antigen specific proliferative responses (Figure 6). However, Con A responsiveness by such cells were usually unaffected. Furthermore, in day 25 samples, antigen

specific proliferative responses were in most cases comparable to those in control animals.

Similarly, using the ELISPOT assay to quantify antigen stimulated IFNγ and IL-4 spot forming cells (SFC), splenocytes from viral vector IL-10 treated animals showed diminished IFNγ SFC responses in day 7 samples (Figure 7). However, by day 25 post treatment, all IFNγ SFC responses were normal. The magnitude of the IL-4 SFC response appeared unaffected by viral vector IL-10 treatment at both day 7 and day 25. Accordingly, our results indicate that exposure of heterologous virus immune animals to either HSV-IL-10 or VV-IL-10 results in a temporary T cell suppression affecting primarily cells of the Th1 cytokine producing phenotype. However, T cell suppression resulting from viral vector IL-10 was only evident in mice not previously immune to the virus used (data not shown). In addition, antigen stimulated proliferation and cytokine production in empty vector plus viral vector encoding IL-10 or empty viral vector plus IL-10 plasmid DNA treated group were not significantly changed compared to those in groups treated with IL-10 viral vector alone or IL-10 plasmid DNA alone (Figures 6 and 7).

In the same series of experiments, the splenic T cell responses of mice given plasmid DNA encoding IL-10 were measured both at day 7 and day 25 post exposure. The pattern of results observed differed to those from animals given recombinant virus expressing IL-10. Thus at day 7, most animals (6/8 in HSV-specific proliferation, 5/8 in VV-specific proliferation) showed reduced antigen specific T cell proliferation, although the level of suppression was usually less than that detectable in viral vector recipients (Figure 6). Nevertheless, suppressed proliferative responses were present in all animals analyzed at day 25 post treatment. The results of assays to measure IFNγ and IL-4 SFC responses also revealed suppressed IFNγ (but not IL-4) SFC which were evident in both day 7 and day 25 samples (Figure 7). Accordingly, modulation of T cell responses resulting from plasmid DNA encoded IL-10 administration were more persistent than those occurring after treatment with viral vectors expressing IL-10.

Circulating IL-10 protein detection after gene transfer

To detect the presence of IL-10 protein in blood from mice exposed to IL-10 DNA or viral vector IL-10, samples were collected at intervals from 4-5 animals and the presence of IL-10 in serum was measured by ELISA. The results are expressed in Figure 8. Two points were worth noting. Firstly, levels of IL-10 following both IL-10 viral vector injection were higher and peaked earlier than those after IL-10 DNA administration. Secondly, whereas IL-10 levels following viral vector administration were minimal or undetectable by day 8 p.i., low but significant serum IL-10 was still evident at 2 weeks p.i. in IL-10 DNA treated mice. Moreover, 2 out of 5 animals showed detectable serum IL-10 protein in IL-10 DNA recipients at day 25 serum samples, but by day 40 serum IL-10 was unmeasuralble. The difference observed in IL-10 production between VV-IL-10 and HSV-IL-10 treatment might be explained by difference in production levels associated with promoters and replication efficiency of the viral vectors. Since it was not possible to use the same promoter in all contructs, this issue of promoter effect could not be evaluated.

Muscle tissues from the recipients were taken at day 3 and day 14 after IL-10 gene transfer to measure IL-10 protein expression. From day 3 samples, the expression of IL-10 in the muscles was found in all recipients of IL-10 gene transfer. However, by day 14 p.i., IL-10 protein was no longer detectable in IL-10 viral vector treated group, whereas minimal amount of IL-10 protein was still detectable after the administration of IL-10 plasmid DNA (data not shown).

CHAPTER 5. DISCUSSION

This report evaluates the efficacy of different delivery systems expressing cytokine IL-10 to modulate viral antigen induced cutaneous inflammation. This inflammatory response could be modulated by a single IL-10 protein administration but direct injection into lesions close to the time of elicitation was required. Thus the inhibitory effect by a single IL-10 protein treatment was transient and confined at the site of injection. More prolonged modulation was achieved if an IL-10 gene expression system was given to primed recipients. We show that a single intramuscular injection of either recombinant vaccinia or herpes simplex virus expressing IL-10 modulated the response, the effect being evident 2 days later but inapparent by 2-3 weeks p.i.. The modulation that resulted from viral vector encoded IL-10 only occurred in animals lacking immunity to the specific virus. Modulation also occurred after administration of plasmid DNA encoding IL-10. The effect was slower in onset and less effective, but modulation was far more persistent than that observed after virus vector IL-10 administration. Moreover, whereas viral vector IL-10 was only effective in mice lacking immunity to the specific virus, plasmid DNA encoding IL-10 modulated effectively if used a second time in the same animal.

One valuable outcome from the increased understanding of molecular mechanisms at play during inflammatory reactions has been the identification of novel means of control which may replace in part the use of nonspecific anti-inflammatory drugs (29). At least two cytokines, TGFβ and IL-10, have been recognized as physiological mediators of anti-inflammatory effects (5, 6). In this report, we focused on IL-10 since this cytokine modulates Th1 T cell mediated inflammatory reactions, common in viral associated immunoinflammatroy disease (4, 18, 19). In several Th1 mediated inflammatory reactions, administration of IL-10 protein, oftentimes into the lesion itself, may also achieve therapeutic modulation (12, 14, 15, 17). However, continual administration is required for successful therapy since cytokines such as IL-10 have a short half-life in vivo, making the approach impractical for clinical application (12, 15, 17). Endogenous production following gene delivery represents the desirable option and numerous systems for

expressing cytokines have been described (4, 20, 22, 23). Seldom if any, however, have multiple systems been compared for their value and efficacy against a single inflammatory lesion. In the present report, we have compared and evaluated their efficacy against viral antigen induced inflammtory lesions that have finite duration and easy to quantify.

The viral antigen induced inflammatory reaction was modulated in severity if IL-10 protein was injected at lesion sites. However, the duration of efficacy was short and only evident if administered early during lesion induction. Both viral vector systems, in contrast, provided a durable modulatory response, but two days were required following a single intramuscular injection before effects became evident. The latent period was presumably the time that needed to synthesize sufficient IL-10 protein. Indeed serum IL-10 was detectable by day 2 and was at peak levels with both viral vectors at day 4 p.i. By around one week p.i., however, serum IL-10 was no longer detectable, likely correlating with the time when viral vectors were removed by the immune response. The differences in level of IL-10 resulting from VV-IL-10 or HSV-IL-10 might be explained by the different replication efficiency of the viral vectors and different promoters for IL-10 expression. Nevertheless suppression of inflammatory reaction following the administration of either viral vector showed similar pattern of results, and persisted for at least 1 week even though IL-10 was no longer detectable in serum. This may mean that IL-10 induced suppression proceeds by a mechanism other than by serum transported local inhibitory effects of IL-10 protein at lesion sites. More likely in fact, the presence of IL-10 for a certain period leads to suppression of Th1 T cell function and this silences, at least temporarily, the effector cells which orchestrate the inflammatory lesions. Recently, it was reported that IL-10 treatment induces *de novo* repressor-like protein(s) synthesis, leading to inhibition of cytokine gene expression (30). In a such case, newly synthesized repressor(s) exerted suppression of proinflammatory cytokine synthesis, which could be blocked by the protein synthesis inhibitor cycloheximide. Also longer exposure to IL-10 had greater effect on the suppression of cytokine production (30). Conceivably, IL-10 gene transfer may generate such proteins in T cells, affecting their functions beyond the time when IL-10 was no longer present or detectable in the

treated animals. With IL-10 DNA administration, suppression beyond the time when IL-10 protein was detectable also occurred. Alternatively, it was recently reported that repeated IL-10 exposure generates a particular T cell population (Tr1) which produces mainly IL-10 but not IL-4 (31). It might be possible that IL-10 DNA induces such a T cell population and the cells can gain access to cutaneous sites during DTH reaction, exerting modulatory effect. However since plasmid DNA may transport to the site of lesions, it is also possible that modulation in this case was additionally achieved by local cytokine production. We are currently attempting to determine if ultimate breakdown of plasmid induced modulation corresponds with the disappearance of the plasmid or represents the decline in activities of silenced Th cells.

Whereas significant modulation of inflammatory reactions occurred with both viral vectors, their effect was temporary and in fact only occurred in nonimmune animals. Recent report also showed that host immune responses to viral vector infected cells can be generated even in the absence of viral gene expression, complicating the use of viral vectors (29). Therefore, other strategies that can bypass such problems are necessary if gene expression and consequent therapy is to be achieved (32, 33). In this report, modulation with plasmid DNA encoding IL-10 represents such a promising approach. Thus modulation following injection of plasmid IL-10 DNA was unaffected by previous plasmid exposure and, although slower to become effective, was far more persistent in activity than was in either viral vector system. Presumably, the longer term efficacy of the plasmid vector approach was because the plasmids were not rapidly discarded by the immune system and in addition failed to induce an immune response. Thus it may be that in clinical situations although viral vectors expressing recombinant genes may be most valuable for short term effects, plasmid DNA vehicles may be suitable when durable expression is required. For example, such a plasmid DNA approach might be a practical and cost-effective strategy for the long-term production of proteins following gene delivery to treat diseases that involve a deficiency in circulating proteins such as clotting factors or enzymes. Alternatively, combinatory strategy of viral vector and plasmid DNA may maximize the therapeutic potentials. As currently used, however, even the plasmid DNA effects terminate

within weeks. We are currently evaluating other routes of administration as well as accessory vehicles for delivery in an attempt to improve gene expression following plasmid DNA administration.

Figure 1. The short-lived and local effect after single IL-10 protein administration on DTH response. Mice were immunized with HSV and three weeks later injected once into right and left ear pinnae with 0.1 or 1 μg IL-10 protein in 20 μl of PBS or the same volume of PBS, respectively before or after DTH elicitation by injecting UV-inactivated HSV-1 KOS into both ear pinnae. Similarly 1 μg IL-10 protein in 20 μl of PBS was injected once into muscles. The increase in ear thickness was measured 48 hours later. The graph shows mean increase in thickness of right and left ear ± standard deviation. Each time point consists of at least 4 mice. * statistically significant between PBS treated and IL-10 treated ear ($p < 0.05$ at test time -2 and +2 hour, $p < 0.01$ at time 0).

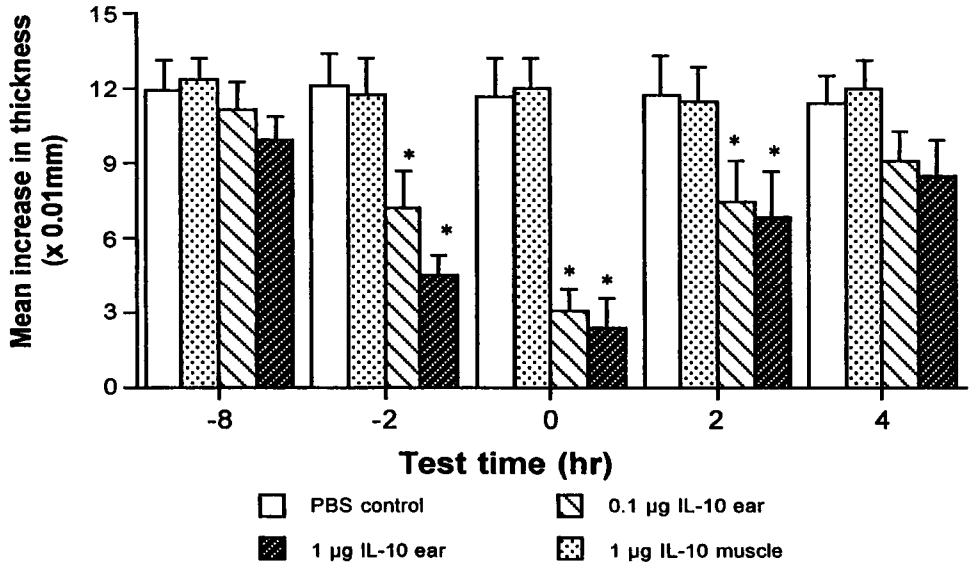

Mean increase in thickness (x 0.01mm)
15
12
9
6
3
0
-8
-2
0
2
4
Test time (hr)
PBS control
0.1 µg IL-10 ear
1 µg IL-10 ear
1 µg IL-10 muscle

Figure 2. The inhibitory effect of IL-10 gene transfer on HSV-induced delayed-type hypersensitivity (DTH). Recipient mice were all immunized with HSV and three weeks later given i.m. one of the following ; 200 μg of IL-10 DNA or vector, 10^7 PFU of HSV-IL-10 or HSVγ34.5-, 10^7 PFU of VV-IL-10 or VVtk-, 200 μg of IL-10 DNA plus 10^7 PFU of VVtk-, or 10^7 PFU of VV-IL-10 plus 200 μg of empty vector . At different time points after IL-10 gene transfer, HSV-specific DTH responses were elicited. Each group consisted of at least 7 mice. The graph represents mean differences between the thickness of right and left ear pinnae ± standard deviation 48 hours after challenge. * statistically significant between vector DNA- and IL-10 DNA-treated group ($p < 0.05$), ** statistically significant between VVtk- and IL-10 DNA plus VV-tk- treated group ($p < 0.05$) ; # statistically significant between VVtk- and VV-IL-10-treated group ($p < 0.05$ at day 2 and 15, $p < 0.01$ at day 5), ## statistically significant between vector and VV-IL-10 plus vector treated group ($p < 0.05$ at day 2 and 15, $p < 0.01$ at day 5) ; at day 5, statistically significant between * and # ($p < 0.05$).

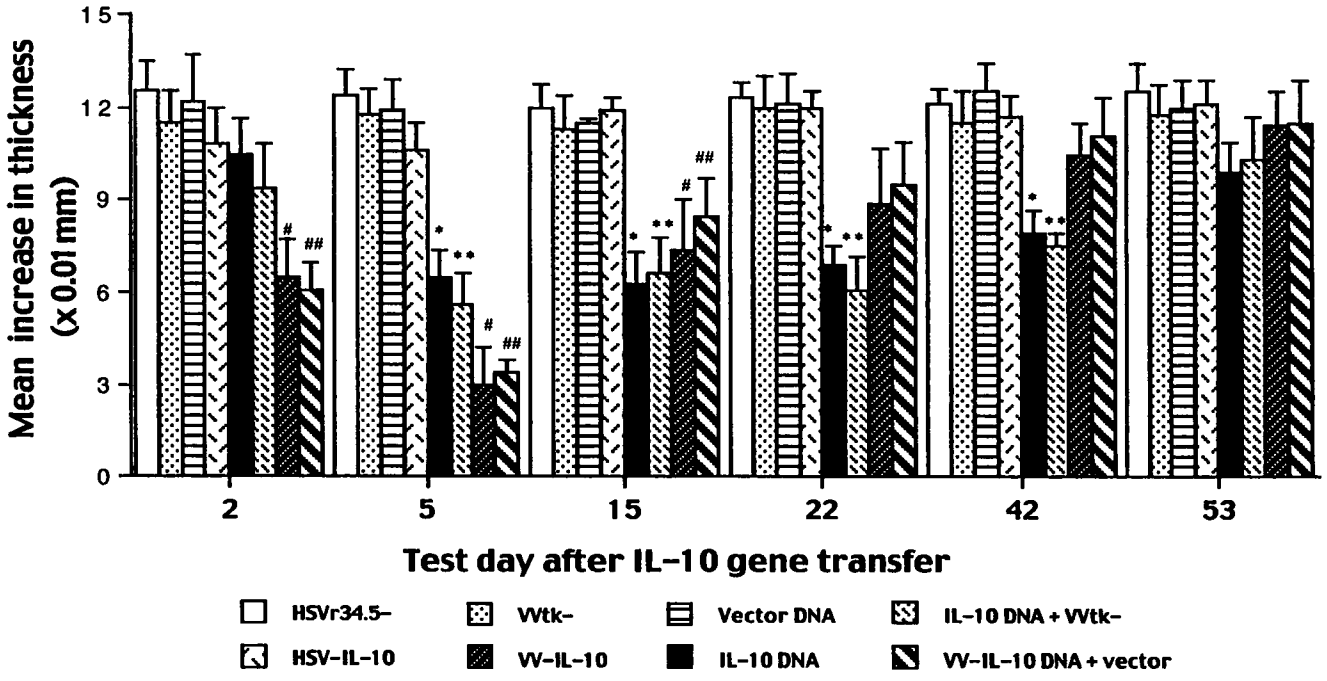

Mean increase in thickness (x 0.01 mm)
15
12
9
6
3
0
2
5
15
22
42
53
Test day after IL-10 gene transfer
HSVr34.5-
VVtk-
Vector DNA
IL-10 DNA + VVtk-
HSV-IL-10
VV-IL-10
IL-10 DNA
VV-IL-10 DNA + vector

Figure 3. The suppressive effect of IL-10 gene transfer on VV-specific DTH responses. Groups of mice were immunized with VVtk- and 3 weeks later received i.m. injections of either 200 μg of IL-10 DNA or vector, 10^7 PFU of HSV-IL-10 or HSVγ34.5-, 200 μg of IL-10 DNA plus 10^7 PFU of HSVγ34.5-, or 10^7 PFU of HSV-IL-10 plus 200 μg of empty vector. At different time points after administration, the mice were tested for VV-specific DTH responses by injecting UV-inactivated VVtk- and CV-1 cell extract into right and left ears, respectively. The increase of ear thickness was measured 48 hours later. Each group consisted of at least 7 mice. The graph represents mean difference between the thickness of right and left ear pinnae ± standard deviation 48 hours after challenge. * statistically significant between vector DNA- and IL-10 DNA-treated group ($p < 0.05$) ; ** statistically significant between HSVγ34.5- and IL-10 DNA plus HSVγ34.5- treated group ($p < 0.05$), # statistically significant between HSVγ34.5- and HSV-IL-10-treated group ($p < 0.05$ at day 2 and 15, $p < 0.01$ at day 5), ## statistically significant between vector and HSV-IL-10 plus vector treated group ($p < 0.05$) ; At day 5, statistically significant between * and # ($p < 0.05$).

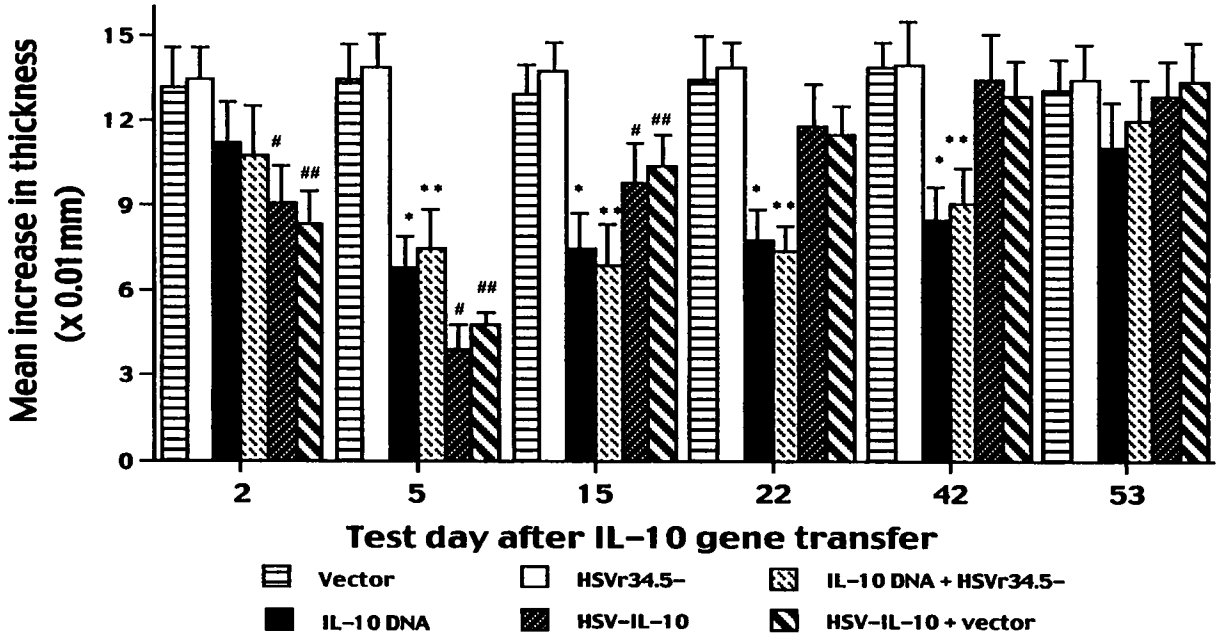

Mean increase in thickness
(x 0.01 mm)
15
12
9
6
3
0
2
5
15
22
42
53
Test day after IL-10 gene transfer
Vector
HSVr34.5-
IL-10 DNA + HSVr34.5-
IL-10 DNA
HSV-IL-10
HSV-IL-10 + vector

Figure 4. The modulation of DTH responses following readministration of IL-10 plasmid DNA and viral vectors expressing IL-10. Groups of HSV-immune mice were injected with IL-10 DNA, VV-IL-10 or HSV-IL-10. After 75 days post treatment, the mice were received the second i.m. injection of either 200 μg of IL-10 DNA, 10^7 PFU of VV-IL-10 or 10^7 PFU of HSV-IL-10 and their corresponding controls. At different time points after the second administration, HSV-specific DTH responses were elicited as described earlier and measured 48 hours later. The graph shows mean differences between thickness of right and left ear ± standard deviation forty-eight hours after challenge. Each group consisted of at least 6 mice. * statistically significant between the second VV-IL-10- and IL-10 DNA-treated group ($p < 0.05$), ** Statistically significant between vector DNA and IL-10 DNA treated group ($p < 0.05$).

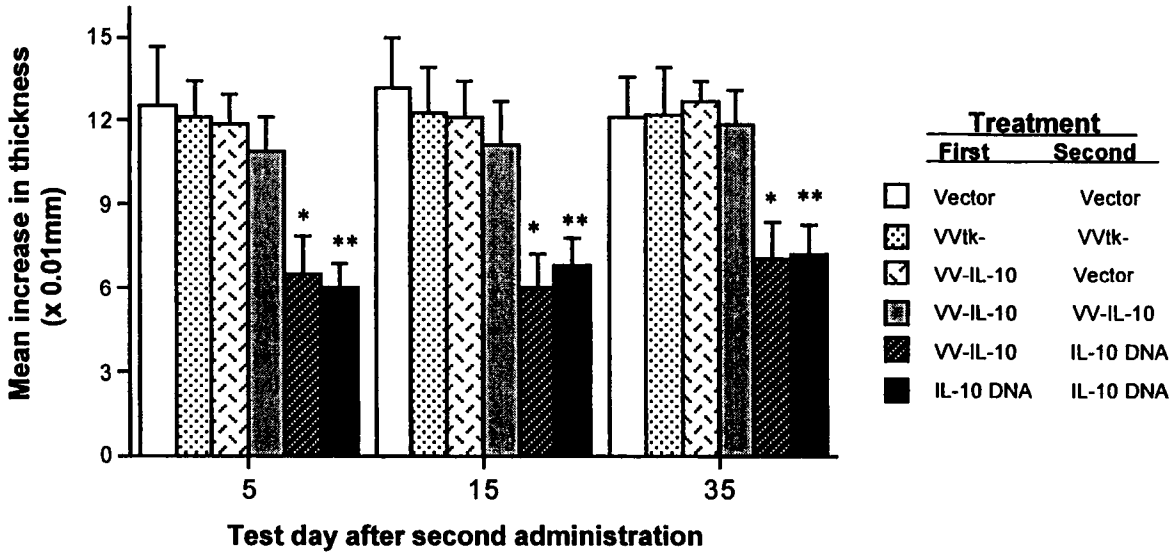

Mean increase in thickness (x 0.01mm)
15
12
9
6
3
0
*
**
*
**
*
**
5
15
35
Test day after second administration
Treatment
First
Second
Vector
Vector
VVtk-
VVtk-
VV-IL-10
Vector
VV-IL-10
VV-IL-10
VV-IL-10
IL-10 DNA
IL-10 DNA
IL-10 DNA

Figure 5. A single IL-10 protein injection does not affect HSV-induced lymphoproliferation. Mice were infected with HSV-1 KOS and three weeks later injected once with 1 μg of IL-10 protein into ear. Forty eight hours later p.i., draining lymph nodes (D.L.N., cervical L.N.) were isolated from 2 mice and pooled. Spleen cells were obtained individually. Those D.L.N. and splenocytes were used as responders for HSV specific proliferation. The responders were mixed with either irradiated syngeneic splenocytes exposed to UV-inactivated HSV-1 KOS (m.o.i. of 1.5 before UV-inactivation) or irradiated syngeneic naive splenocytes, and incubated for 5 days as described in Materials and Methods. 1 μg/ml of Con A was added in the culture of responders as a polyclonal stimulator positive control and incubated for 3 days. The test was done in quadruplicated wells. Each bar represents mean cpm of responders plus irradiated syngeneic splenocytes pulsed with UV-inactivated HSV or Con A stimulated responders from one of two independent experiments which showed similar results.

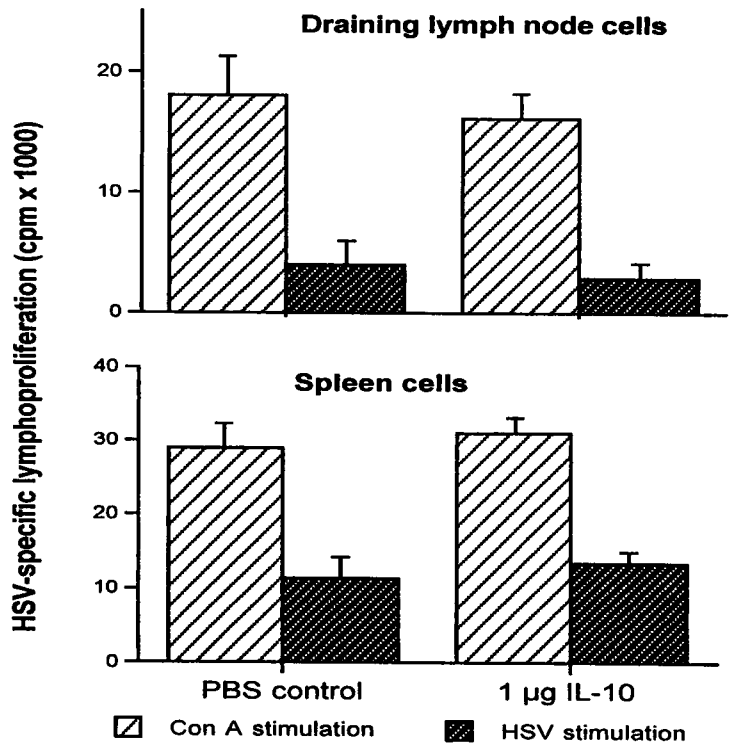
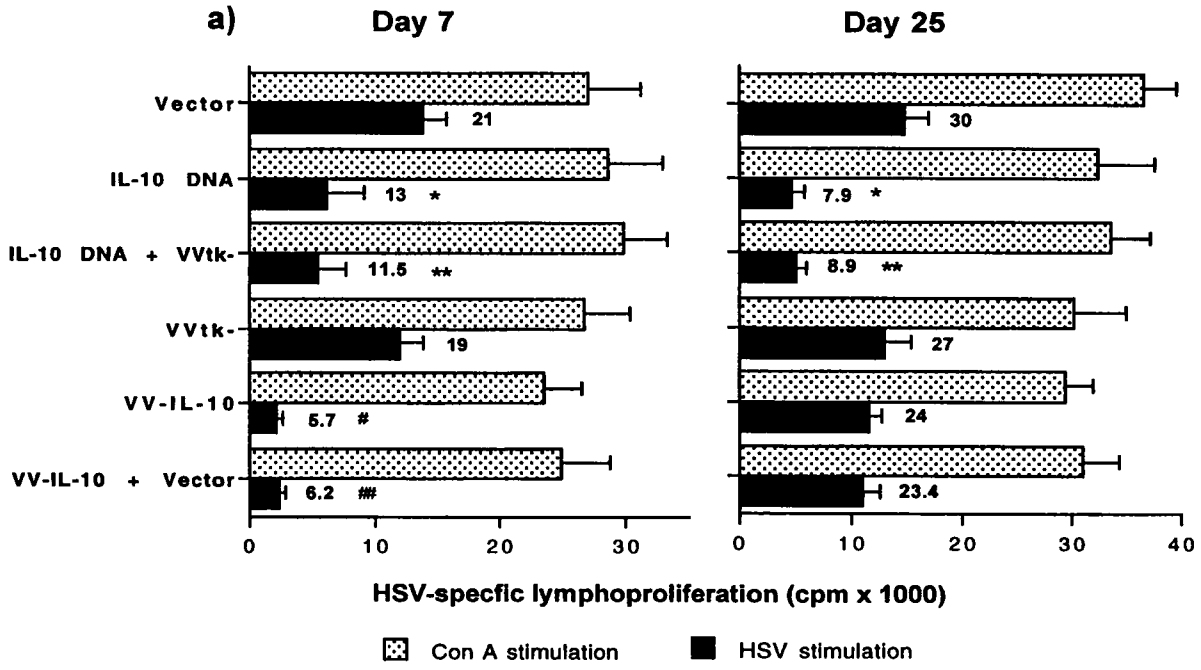

Draining lymph node cells
HSV-specific lymphoproliferation (cpm x 1000)
20
10
0
Spleen cells
40
30
20
10
0
PBS control
1 µg IL-10
Con A stimulation
HSV stimulation

Figure 6. Antigen specific proliferation following IL-10 gene transfer. (a) HSV-specific proliferation : Groups of mice were immunized with HSV and three weeks later injected i.m. with either 200 μg of IL-10 DNA or vector, 10^7 PFU of VV-IL-10 or VVtk-, 200 μg of IL-10 DNA plus 10^7 PFU of VVtk-, or 10^7 PFU of VV-IL-10 plus 200 μg of empty vector. (b) VV-specific lymphoproliferation : Groups of mice were infected with VVtk- and three weeks later injected i.m. with either 200 μg of IL-10 DNA or vector, 10^7 PFU of HSV-IL-10 or HSVγ34.5-, either 200 μg of IL-10 DNA plus 10^7 PFU of HSVγ34.5-, or 10^7 PFU of HSV-IL-10 plus 200 μg of empty vector. Seven or 25 days later, splenocytes were obtained from the mice and used as responders for HSV (or VV) -specifc proliferation. 1 μg/ml of Con A was used as a polyclonal stimulator positive control and incubated for 3 days. Each bar represents mean cpm of antigen specific proliferation ± standard deviation from six independent experiments. The numbers of the bar graph represent mean proliferation index of each group. * statistically significant between vector and IL-10 DNA treated group ($p < 0.05$ at day 7, $p < 0.01$ at day 25), ** statistically significant between VVtk- and IL-10 DNA plus VVtk- (or HSVγ34.5-) treated group ($p < 0.05$ at day 7, $p < 0.01$ at day 25), # statistically significant both between VVtk- and VV-IL-10 received group and between HSVγ34.5- and HSV-IL-10 treated group ($p < 0.01$). ## statistically significant between vector and VV-IL-10 (or HSV-IL-10) plus vector received group ($p < 0.01$).


a)
Day 7
Day 25
Vector
IL-10 DNA
IL-10 DNA + VVtk-
VVtk-
VV-IL-10
VV-IL-10 + Vector
21
13 *
11.5 **
19
5.7 #
6.2 ##
30
7.9 *
8.9 **
27
24
23.4
0
10
20
30
40
HSV-specfic lymphoproliferation (cpm x 1000)
Con A stimulation
HSV stimulation

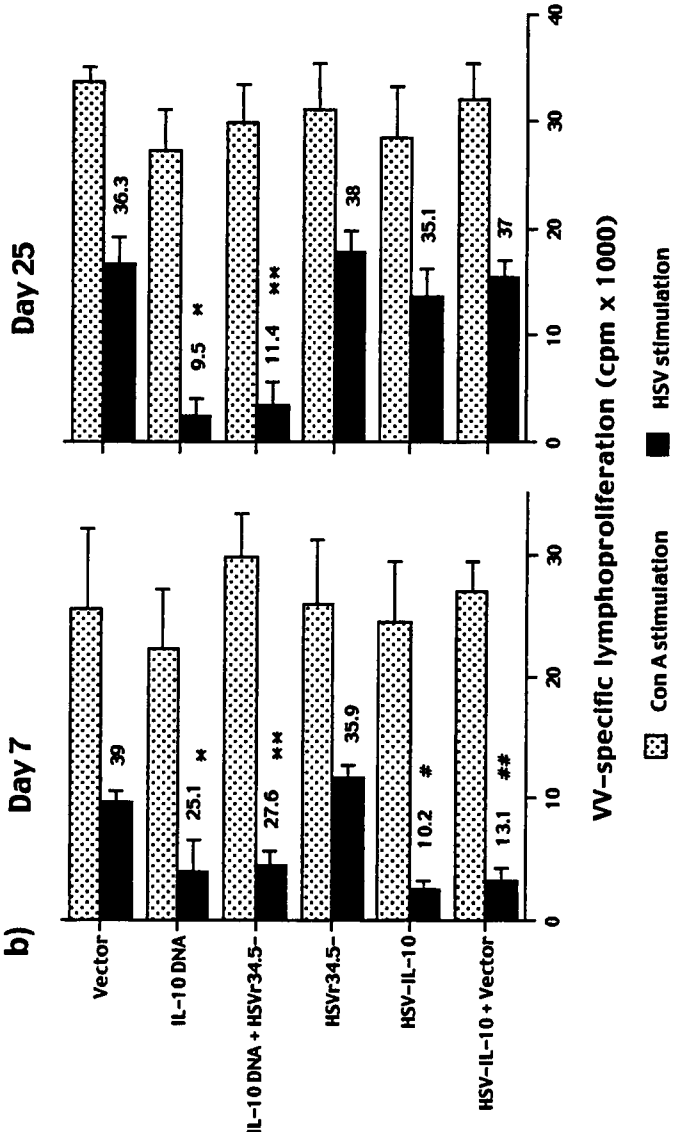
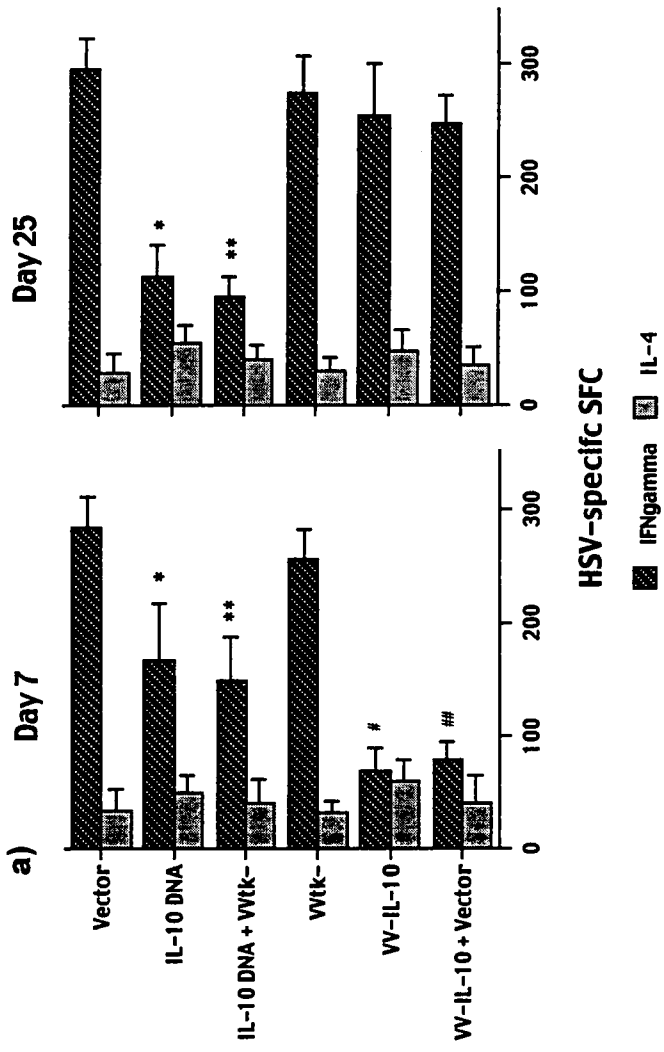

b)
Day 7
Day 25
Vector
IL-10 DNA
IL-10 DNA + HSVr34.5
HSVr34.5
HSV-IL-10
HSV-IL-10 + Vector
39
25.1
27.6
35.9
10.2
13.1
36.3
9.5
11.4
38
35.1
37
*
**
*
**
#
0
10
20
30
40
VV-specific lymphoproliferation (cpm x 1000)
Con A stimulation
HSV stimulation

Figure 7. The effect of IL-10 gene transfer via plasmid or viral vector on splenic cytokine spot forming cell (SFC) responses. Seven and 25 days later following IL-10 gene transfer, splenocytes from each group were obtained and restimulated in vitro for 4 days with either irradiated enriched DC cells that were pulsed with UV-inactivated HSV(a) or UV-inactivated VVtk-(b), or irradiated naive enriched DC. Frequencies of cytokine-producing cells were measured by the ELISPOT assay. The number of SFC after naive DC stimulation was subtracted from the values of UV-inactivated HSV-pulsed DC stimulation (a) or UV-inactivated VVtk- pulsed DC stimulation (b). The graph shows means SFC ± standard deviation from six independent experiments. * statistically significant between vector and IL-10 DNA treated group ($p < 0.05$ at day 7, $p < 0.01$ at day 25), ** statistically significant between VVtk- and IL-10 DNA plus VVtk- (or HSVγ34.5-) treated group ($p < 0.05$ at day 7, $p < 0.01$ at day 25), # statistically significant both between VVtk- and VV-IL-10 received group and between HSVγ34.5- and HSV-IL-10 treated group, ($p < 0.01$), ## statistically significant between vector and VV-IL-10 (or HSV-IL-10) plus vector received group ($p < 0.01$).


a)
Day 7
Day 25
Vector
IL-10 DNA
IL-10 DNA + Vtk-
Vtk-
VV-IL-10
VV-IL-10 + Vector
0
100
200
300
*
**
#
##
HSV-specifc SFC
IFNgamma
IL-4

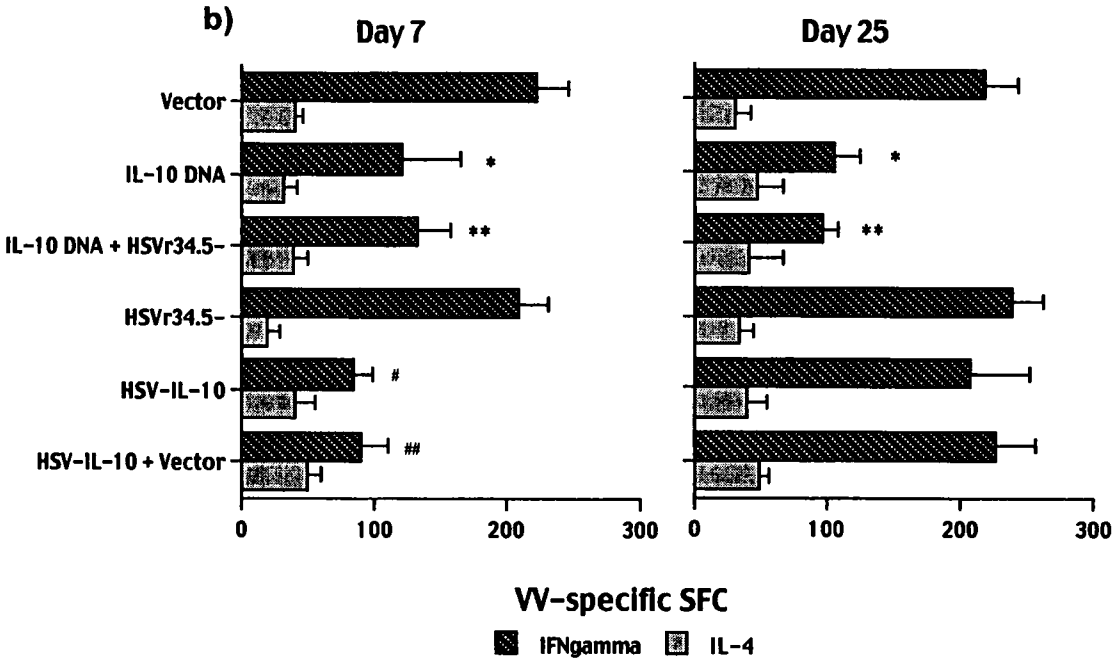
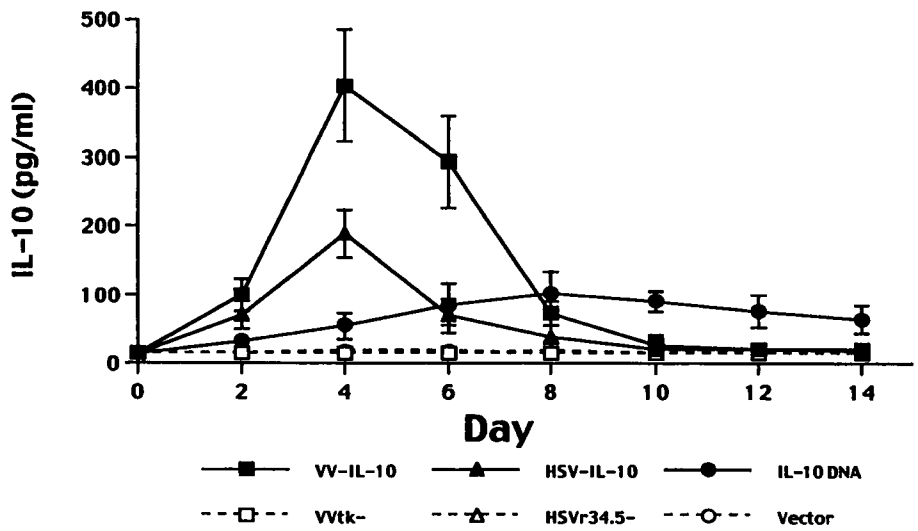

b)
Day 7
Day 25
Vector
IL-10 DNA
IL-10 DNA + HSVr34.5-
HSVr34.5-
HSV-IL-10
HSV-IL-10 + Vector
*
**
#
##
0
100
200
300
VV-specific SFC
IFNgamma
IL-4

Figure 8. Circulating IL-10 level after IL-10 gene transfer.

Groups of mice were given intramuscularly 200 μg of plasmid DNA, 10^7 PFU of VV-IL-10, or 10^7 PFU of HSV-IL-10 and their corresponding controls. At different time points, serum samples were collected from the mice and individually analyzed for the presence of IL-10 protein by a standard ELISA assay. The line graph shows mean concentration ± standard deviation. Each time point consists of sera from at least 4 mice.


IL-10 (pg/ml)
500
400
300
200
100
0
0
2
4
6
8
10
12
14
Day
VV-IL-10
HSV-IL-10
IL-10 DNA
VVtk-
HSVr34.5-
Vector

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PART 4. DISTRIBUTION FATE AND MECHANISM OF IMMUNE MODULATION FOLLOWING MUCOSAL DELIVERY OF PLASMID DNA ENCODING IL-10

(Journal of Immunology, 1999, In press)

CHAPTER 1. ABSTRACT

DNA vaccination has been widely studied in the several models of vaccination and treating inflammatory diseases even though the mechanism involved is still unclear. This report demonstrates that mucosal administration of plasmid DNA leads to rapid and widespread distribution around body. Dissemination likely occurred via the bloodstream since plasmid DNA was present in blood plasma. The plasmid DNA was also detectable in several tissues including D.L.N., spleen, liver, bone marrow and even the dermis of ear pinnae. Except for the site of administration, plasmid DNA was no longer detectable in tissues after 3 weeks post administration. RNA and protein expression was also found in the tissues and bloodstream. Animals previously primed by HSV infection and subsequently given IL-10 DNA via the nasal mucosa, showed diminished Ag-induced DTH reactions for upto 5 weeks post treatment. The mechanism of modulation involved diminished the Ag-specific proliferation and produciton of Th1 cytokines. The Ag-specific silencing effects persisted beyond the duration of detectable plasmid encoded protein and was maintained upon adoptive transfer of T cells into a plasmid free environment. The silenced T cells were not a source of IL-10, and their anergic state was reversible by exposure to Ag in the presence of exogenous IL-2.

CHAPTER 2. INTRODUCTION

Chronic inflammatory reactions may be driven by sets of proinflammatory cytokines produced by stimulated T cells. This situation can be controlled by the application of inhibitory cytokines such as TGFβ and IL-10 which act in some way to silence the production or activity of the proinflammatory molecules (1,2). Thus cytokine therapy represents a therapeutic option in clinical medicine although administering molecules with short bioactivity as occurs with cytokine proteins is inconvenient and costly. The alternative approach of gene delivery is being explored although the relevant means of achieving cytokine gene expression requires definition. Recently, using a viral inflammatory lesion model, we compared the efficacy of IL-10 gene expression achieved by recombinant vectors or eukaryotic expression plasmid DNA (3). The latter approach provided the most durable efficacy and unlike viral vectors was not subject to interference by the immune response. The naked plasmid DNA idea is under consideration for both vaccination and immunomodulation purposes, but the mechanisms of action and long term effects of such an approach require further study (4, 5). Accordingly, long-term gene expression does occur especially at the intramuscular injection site (6). These myocytes act as an enduring source of plasmid encoded protein, but it appears that the cells may not be the primary inducer of immune responses (7). Thus removal of the injected sites after inoculation has little effect on the level or nature of immunity (7). Indeed, it seems that dendritic cells transfected at the injection site and migrating subsequently to draining lymph nodes are the principal source of immunogenic materials (7 - 12).

Most investigations on the fate of plasmid DNA and the origin of immunogenic material have focused on systemic administration especially the i.m. route (13). Our laboratory has focused on mucosal delivery and has observed that this form of plasmid administration results in good immune induction as well as immune modulation (14 - 16). Following mucosal inoculation, protein expression was observed in the lung and draining LN, but it is not clear how plasmid

disseminates to such sites or how mucosal plasmid DNA delivery results in the systemic immune induction and the immunomodulatory effects observed.

In the present report, we probe the fate of eukaryotic expression plasmid DNA after mucosal delivery, and evaluate possible mechanisms by which plasmid DNA expressing IL-10 achieves immunomodulation. Our results indicate that plasmid DNA disseminates widely in the body following mucosal delivery. Distal tissues such as the dermis, bone marrow and lymphoid tissues remote from the deposition site become seeded with plasmid DNA and in addition become accessible to cells presumably transfected at the inoculation site or draining lymphoid tissue. Whereas IL-10 protein can be demonstrated in the blood stream and can additionally be produced by transfected cells at local sites, the modulatory effects of IL-10 plasmid DNA appears mainly to result from central effects in T cell function. Thus T cells appear to be silenced for a period that extends beyond the duration of plasmid DNA gene expression. However, the silenced T cell reactivity was readly recovered by exogenous IL-2 treatment, indicating that the mechanism involved in long-term modulation is induction of an anergic-like state.

CHAPTER 3. MATERIALS AND METHODS

Animals

Five to six weeks old female BALB/c (Harlan Sprague-Dawley, Indianapolis, IN) and 6 to 8 weeks old CB-17 SCID mice (Taconic, Germantown, NY) were used for the study. BALB/c mice were housed conventionally, and SCID mice were housed in sterile microisolator cages in the animal facility. All food, water, bedding, and instruments were autoclaved or disinfected. All manipulations were performed in a laminar flow hood. To prevent bacterial superinfection, all SCID mice received treatment of sulfamethoxazole/trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5 ml per 200 ml of sterile drinking water. The investigators adhered to the guidelines set by the Committee on the Care of Laboratory Animal Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee are fully accredited by AALAC.

Viruses

HSV-1 KOS and HSV-1.17 strains were grown on a Vero cell monolayer (ATCC, Cat. No. CCL81), titrated, and stored in aliquots at -80 °C until used.

Plasmid DNA preparation

Plasmid DNA encoding IL-10 under SV40 promoter was a gift of Dr. T. Mosmann (Edmonton, Alberta, Canada). A vector DNA without IL-10 gene was made by digestion of IL-10 plasmid DNA with EcoRI followed by ligation of the purified vector fragment. Plasmid DNA encoding β-galactosidase (pCMVβ ; Cat. No. 6177-1) and plasmid DNA encoding a red-shift variant of green fluorescent protein (pGFP ; Cat. No. 6084-1) were purchased from Clontech laboratory (Palo Alto, CA). Plasmid DNA was purified by polyethylene glycol precipitation as described previously (17). Protein expression of the different plasmids in eukaryotic cells (human embryonic kidney cells, 293) was determined as described elsewhere (15).

Immunization with HSV

BALB/c mice were anesthetized with methoxyflurane (Metophane ; Pittman-Moore, Mundelein, IL) and received footpad injection of 10^6 PFU of HSV-1 KOS.

Plasmid DNA administration

For intranasal administration (i.n.), three weeks post infection, 200 µg of plasmid DNA was suspended in 25 µl of sterile PBS and dropped onto the nasal mucosa. The mice inhaled the DNA as they breathed. Intramuscular administration (i.m.) was performed by injecting 100 µg of plasmid DNA in 50 µl of sterile PBS to each biceps muscles.

Adoptive transfer protocol

HSV-infected BALB/c mice received i.n. administration of 200 µg of IL-10 DNA or vector DNA. Around four weeks later, the splenocytes were obtained from the mice and were enriched for T cells by passing though a nylon-wool column. These T cells were adoptively transferred intravenously to untreated SCID mice by tail injection. In some experiments, 500 unit of recombinant IL-2 (Cat. No. 906011, Hemagen, Maryland) was i.p injected into SCID mice at day 0, 1, 2, and 3 following adoptive transfer. The enriched populations were analyzed by flow-cytometric analysis that usually consists of more than 85 % of $CD3^+$ T cells (> 65 % of CD4+ T cells and > 25 of CD8+ T cells). The enriched cells were also analyzed for the presence of administered plasmid DNA by PCR that showed no amplified bands for β-gal or IL-10 plasmid DNA.

Delayed type hypersensitivity (DTH)

At different time points after IL-10 DNA treatment, test antigens in 30 µl of sterile PBS were injected into the ear pinnae of the HSV infected BALB/c mice. The increase in ear thickness was measured 48 hours later with a screw gauge meter (Oditest; H. C Kroeplin

GHBH, Schluechtern, Germany) as described previously (18). Test antigens used were UV-inactivated HSV-1 KOS (10^5 PFU prior to UV inactivation) or Vero cell extract. In a separate experiment, the ear of vector DNA treated mice received the injection of 80 ng of IL-10 protein along with HSV antigen. At the same time or three hours after HSV + IL-10 protein injection, the ear pinnae were injected with 1 µg of anti-IL-10 antibody (Pharmingen; Cat. No. 18140D) or 1 µg of rat IgG1 isotype antibody (Pharmingen; Cat. No.20610D). The DTH response of the IL-10 DNA treated mice was tested as described above. In some animals, at day 28 after i.n.treatment of IL-10 DNA, 1 µg of anti-IL-10 antibody or 1 µg of rat IgG1 isotype antibody were injected into ear pinnae along with HSV antigen. Similarly, SCID mice were tested for the DTH responses on the next day of adoptive transfer.

Virus challenge

On day three following adoptive transfer, the SCID mice were challenged with HSV as described elsewhere (19). Briefly, before challenge the left flank of mice was depilated by using a hair clipper (Oster Animal Hair Clipper, Milwaukee, WI) and a chemical depilator, Nair (Cater-Wallace, NY). After depilation, the mice were anesthetized with metophane and a total of 7 scarifications were made on an approximate 5 mm^2 area using a 27 gauge needle. 10 µl of 10^6 PFU (100 LD_{50}) of HSV-1 strain 17 was added to the scarifications and gently massaged. Animals were inspected daily for the development of zosteriform ipsilateral lesions, general behavior changes, encephalitis and mortality. The severity of the lesions was scored as follows : 1 = vesicle formation; 2 = local erosion and ulceration; 3 = mild to moderate ulceration; 4 = severe ulceration, hind limb paralysis, and encephalitis, 5 = death.

HSV-specific lymphoproliferation

This assay has been described in detail elsewhere (19). Briefly, at day 28 or 65 after plasmid DNA treatment, the splenocytes of vector or IL-10 DNA treated mice were enriched for T cells by a nylon-wool column and used as responder populations. These T cells were

restimulated in vitro with irradiated syngeneic splenocytes infected with UV-inactivated HSV (multiplicity of infection of 1.5 prior to UV inactivation) or irradiated naive splenocytes, and incubated for 5 days at 37 °C. In some experiments, rIL-2 (10 Unit/well) was added. 5 μg/ml of Con A was used as a polyclonal positive control and incubated for 3 days. Eighteen hours before harvesting, 3[H]-thymidine was added to the cultures. In at least five independent experiments, proliferative responses were tested in quadruplicated wells and the results expressed as mean counts per minute ± standard deviation.

IL-2 assay by ELISA

On day 28 after IL-10 DNA treatment, splenocytes from the IL-10 DNA or vector DNA treated BALB/c mice were obtained. The enriched T cells (5×10^6 cell/ml) were restimulated *in vitro* with 5×10^5 of irradiated syngeneic splenocytes infected with HSV-1 KOS or irradiated naive splenocytes for three days at 37 °C. Similar number of cells were stimulated with 5 μg of Con A as a polyclonal positive stimulator for 48 hours. The culture supernatants were screened for the presence of IL-2 by ELISA assay. ELISA plates were coated with anti-IL-2 antibody (Pharmingen, Cat No. 18161D) and incubated overnight at 4 °C. The plates were washed three times with PBS containing 0.5% Tween-20 and blocked with 3% of non-fat dry milk for 2 hours at room temperature. After washing, serially diluted samples and standard were added to the plates in duplicate, and incubated overnight at 4 °C. After being washed, biotinylated anti-IL-2 antibody (Pharmingen, Cat. No. 18000D) was added and incubated 2 hours at 37 °C. The plates were washed and peroxidase conjugated streptavidin (Jackson ImmunoResearch, Cat. No. 016-030-084) was added. The color was developed by adding the substrate solution (11 mg of 2,2"-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid in 25 ml of 0.1M citric acid, 25 ml of 0.1M sodium phosphate, and 10 μl of hydrogen peroxide). IL-2 concentration was calculated with an automated ELISA reader (SpectraMAX 340, Molecular devices, Sunnyville, CA).

Eenzyme-linked immunoSPOT (ELISPOT) forcytokine producing cells.

The detailed methods of ELISPOT assay for the quantification of cytokine producing cells were described previously (14). Twenty-eight days later following IL-10 gene transfer, the splenocytes of vector or IL-10 DNA treated mice were enriched for T cells by a nylon-wool column. The T cells were analyzed for IL-4 and IFNγ spot-forming cells by ELISPOT. To generate cytokines, the T cells were stimulated in vitro with enriched dendritic cell (DC) populations obtained by the method of Nair *et al.* (20). The enriched DCs were pulsed with UV-inactivated HSV (m.o.i. of 5 before UV-inactivation) for the HSV-specific cytokine production. The T cells and stimulator DC (naive or pulsed) were added at a responder-to-stimulator ratio of 10:1, 5:1, 2.5:1 and 1.25:1 in 200 µl of RPMI with 10 % fetal bovine serum per well into ELISPOT plates which were coated with anti-mouse IL-4 (Cat. NO. 18191D, Pharmingen) or anti-mouse IFNγ (Cat. No. 18181D, Pharmingen). After 96 hours incubation, the plates were washed and biotinylated anti-mouse IL-4 (Cat. No. 18042D, Pharmingen) or biotinylated anti-mouse IFNγ (Cat. No. 18112D, Pharmingen) was added. After 1 hour of incubation at 37 °C, 100 µl of alkaline phosphatase-conjugated streptavidin in PBS (1 µg/ml) was added and the plates were incubated for another one hour. The spots were developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as a substrate and counted 24 hours later under a dissecting microscope. Mean cytokine spot forming cells (SFC) ± standard deviation from five independent experiment was expressed as results. In some experiments, rIL-2 (10 unit) was added to culture wells.

IL-10 detection in serum after IL-10 gene transfer.

Groups of mice received i.m. or i.n. treatment of either 200 µg of IL-10 plasmid DNA or vector. At different days, serum samples were collected from the mice and analyzed for the presence of IL-10 in the serum by ELISA assay. ELISA plates (Dynatech, Chantilly, VA) were coated overnight at 4 °C with 2 µg of rat antimouse IL-10 (Cat. No. 18141D). The wells in the plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 3% milk for 2

hours at 37 °C. The sera samples and the recombinant IL-10 (Cat. No. 19281V) at a concentration of 1 ng/ml) were added and serially diluted. The standard and samples were incubated overnight at 4 °C. After washing with PBS-T, 1 μg of biotinylated anti IL-10 antibody (Cat. No. 18152D, Pharmingen) per ml was added and incubated at 37 °C for 2 hrs. After the wells were washed, peroxidase-conjugated streptavidin (Jackson Immunoresearch) was added and incubated at 37 °C for 2 hrs. Substrate was added for color development. IL-10 concentration was measured by automated ELISA reader (Spectra MAX 340, Molecular devices, Sunnyville, CA). Each time point contains sera from at least four mice.

Isolation of DNA and RNA

At different time points following administration of pCMVβ, pGFP or vector DNA (not shown), several tissues including muscle, D.L.Ns, spleen, lung, liver, bone marrow, and ear were obtained. The tissue samples were minced into small pieces (or sometimes, single cell suspensions of D.L.N. and spleen were made). The preparations were treated with TRI Reagent (Molecular Research Center, Cincinnati, OH). Total RNA and DNA were isolated by manufacturer's protocol. Briefly, RNA was extracted with chloroform and precipitated with isopropyl alcohol. The contaminating plasmid DNA was removed with RQ-1 RNase-free DNase (Promega Corp, Madison, WI). The absence of plasmid DNA in the RNA preparations was verified by performing PCR on the total cellular RNA without reverse transcription. DNA was isolated by precipitation of interphase and phenol phase with ethanol. The precipitated DNA was washed with 0.1M sodium citrate followed by 75% ethanol. Anticoagulated bloods were also obtained at different time points after i.n. administration of pCMVβ or pGFP. The blood samples were centrifuged and plasma was carefully separated from the blood cell pellet. DNA from 0.5 ml of plasma were extracted twice with phenol/chloroform and precipitated with ethanol. The extracted DNA was dissolved with sterile nuclease-free water. Peripheral blood cells were washed with DMEM containing 10 % FBS and incubated for 90 min to separate adherent and nonadherent cell populations. Each population was treated with TRI reagent, and total DNA and

RNA were extracted as described above. All the precedures including PCR were performed in a separate larminar flow hood to avoid cross-contamination.

Reverse transcription and polymerase chain reaction

Total cellular RNA (10 μg/ml) was reverse transcribed using oligo(dT) primers and reverse transcriptase (Promega Corp, Madison, WI) according to protocol described previously (21). The contaminating plasmid DNA was removed by treatment with RQ1-RNase free DNase and verified by PCR using both CMV-1/β-gal-2 (or GFP-1) primers and β-gal-1/ β-gal-2 (or GFP-1/GFP-2) primers. The cDNA was made by the reverse transcription reaction incubated at 42 °C for 90 min. The cDNA (5 μl) was subject to 35 cycles of amplification as described earlier (22) using β-gal-1 and β-gal-2 (or GFP-1 and GFP-2), and β-actin-1 and β-actin-2 primers. DNA isolated from the plasma was also amplified by 35 cycles of amplification using CMV-1 and β-gal-2 (or GFP-2) primers. The primers used follow : CMV-1 : 5'-CCATTGACGTCAATGGGAG-3', β-gal-1 : 5'-ACTTTGACCAACAAGAACGTG-3', β-gal-2 : 5'-AAATGTGAGCGATAACAACC-3', GFP-1 : 5'-TCGTGACCACCCTGACCTAC-3', GFP-2 : 5'-CCTAGGTGGCCTAGATCTATTG-3', β-actin-1 : 5'-GTGGGGCGCCCCAGGCACCA-3', β-actin-2 : 5'-CTCCTTAATGTCACGCACGAT-3'.

β-galactosidase staining

At different days after pCMVβ DNA administration, D.L.N., spleen, lung and ear tissues were isolated from the mice and fixed in 4% paraformaldehyde in PBS for 1 hr at 4°C. The tissues were washed and stained for β-galactosidase activity as described previously (22). Briefly, the tissues were incubated overnight with the substrate solution containing 2 mg/ml of 5 bromo-4-chloro-3-indolyl-β-D-galactosidase, 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM $MgCl_2$, and 120 μl of 10% Nonidet and 100 μl of 1% sodium deoxycholate in 20 ml of PBS. After the reaction, the tissues were washed with PBS, and fixed with 4% of

formaldehyde. The tissues were embedded in paraffin, sectioned at 5 μm, and counter-stained with nuclear fast red.

FACS analysis

At day 10 following i.n. pGFP administration, peripheral blood cells (PBC) were isolated from anticoagulated blood. Following lysis of RBC, the PBCs were incubated in the culture flask for 90 min at 37 °C. The adherent cell populations were carefully separated from the nonadherent cells. After washing with DMEM containing 10% FBS, the cells (2×10^6 cells/ml) were immediately fixed with 4% paraformaldehyde. The green fluorescence signals were analyzed by flow cytometry analysis using FL-1 channel. As a positive control, human kidney embryonic cells (293) were transfected with pGFP and 60 hours later the harvested 293 cells were fixed. The fluorescent signals from the cells were detected as described above.

Statistical analysis

Significant differences between groups were evaluated using the Student's *t* -test.

PART 4. RESULTS

IL-10 plasmid DNA suppresses antigen specific immune responses in primed animals.

Mice were infected systemically with HSV, and 3 weeks later received via the intranasal (i.n.) or intramuscular (i.m.) routes 200 μg of IL-10 plasmid DNA either encoding IL-10 or vector DNA. At various days after this exposure, mice were tested for their cutaneous DTH responses to HSV antigen injected into the ear pinna. Suppressed responses in IL-10 DNA treated mice first became evident around 5 days post treatment (Table 1). However, the duration of suppression differed from i.m. or i.n. treatment. Such suppression by i.m. injection of IL-10 DNA lasted for 7 weeks as reported earlier (3), whereas suppression had declined by day 45 in i.n. recipients of IL-10 DNA (Table 1). Thus DNA encoding IL-10 administered mucosally as well as systemically is capable of suppressing antigen induced inflammatory responses even when administered after sensitization has occurred.

Sample mice in the group that showed suppressed DTH reactions were sacrificed 28 days post IL-10 DNA administration to evaluate the extent of T cell responsiveness to HSV antigen of enriched splenic T cells. Cells from IL-10 treated animals showed reduced proliferative responses to HSV and in addition showed marked reduction in IL-2 production (Table 2). Both responses are usually measures of $CD4^+$ T cell function (19). The responsiveness of the cells to Con A (polyclonal activator) was equal between IL-10 DNA or Vector DNA treated group. Cells were also tested by ELISPOT for the number of IFNγ and IL-4 HSV specific spot forming (SFC) cells (Table 2). The cell population from IL-10 treated mice had reduced IFNγ SFC but IL-4 SFC were similar to controls. Thus the data indicate that IL-10 DNA administration suppresses the reactivity of CD4+ Th1 T cells.

Mechanism of modulation

The observations above indicate that mucosal delivery of IL-10 DNA results in systemic suppression of T cell reactivity. Several possibilities were considered to explain how mucosally

delivered plasmid could result in such suppression. These included the passage of plasmid DNA to remote sites followed by cell transfection and expression at such sites. The plasmid DNA could be transported within cells that had been transfected at the mucosal delivery site or draining lymph nodes. Alternatively, the IL-10 protein could be expressed locally and enter the blood stream to distribute to distal sites and actively suppress inflammatory responses. Further explanations could be that T cells were silenced in the local or distal lymphoid tissues by exposure to the IL-10 protein or that regulatory cells were induced that inhibited the responses of the antigen stimulated immune T cells.

Detection of plasmid DNA and transfected cells in blood and peripheral sites

To investigate if plasmid DNA and the expression of encoded protein was present at the remote sites or in the blood stream, plasmids encoding β-galactosidase or green fluorescent protein (GFP) were used to avoid the complication of detecting endogenous cytokine gene expression. Animals were given the plasmid DNAs or vector control mucosally, and then killed at intervals and multiple tissues, including the separated plasma and peripheral blood cells, were collected to detect DNA, RNA, or protein. The results recorded in Table 3 indicate that plasmid DNA received i.n. was present in blood by 5 hours as detected by PCR using primer sets that can amplify only the introduced gene and its promoter (Figure 1, Table 3). This DNA persisted for at least 3 days in plasma and was evident in all tissues analyzed at day 1 and 7. These tissues included the ear pinna and spleen and in the latter plasmid DNA was detectable until at least day 14. Furthermore, in a separate experiment, the IL-10 plasmid DNA also disappeared from most tissues by day 20. The presence of DNA in the plasma means that early on plasmid DNA can transport via blood as cell free plasmid DNA to multiple tissues, but since the technique used did not permit quantification whether or not the plasma route is more important than the intracellular route could not be established. Thus plasmid DNA was also present in separated and washed leukocytes.

Furthermore, semiquantitative RT-PCR revealed that GFP (not shown) and β-gal RNA were present in separated blood cells (Figure 1). The RNA was detectable from day 1 to day 7 and some RNA was still present at day 14. Even though RNA expression was not detected, the β-gal and IL-10 DNA could be still found in nasal tissues at day 21, but had disappeared from by day 28 (data not shown). Regarding protein expression 230 to 580 cells per 10^5 adherent blood cells were positive for the GFP signal at day 10 but by 21 days protein expressing cells in the blood were undetectable (see Figure 2). These data indicate that plasmid DNA transfection likely occurs at the sites of inoculation and that some cells enter the blood stream and circulate for prolonged but not indefinite periods. Additionally, peripheral blood cells might take up cell-free plasmid DNA within circulation.

RNA expression was also found at multiple peripheral sites (Figure 1, Table 3). In addition, protein-expressing cells were evident in lungs, draining LN and spleen. Furthermore, some cells were also demonstrable at cutaneous sites (Figure. 2). Regarding the latter, a few protein-expressing cells could be also demonstrated in the inflammatory reactions induced at 7 days post plasmid DNA inoculation but not in reactions induced at day 21 or beyond. Such data may indicate that the source of plasmid expression may be recruited cells from the blood stream as well as cells transfected at cutaneous sites by plasmid DNA arriving soon after mucosal inoculation. Our data could not distinguish if the presence of RNA and protein expression at remote sites results from direct transfection by plasmid DNA at such sites or the consequence of seeding by cells transfected by plasmid at inoculation sites. These issues are under further investigation. However, these results indicate that mucosal IL-10 DNA delivery leads to wide dissemination of plasmid DNA to several tissues, and these sites could serve as a source of IL-10 expression.

Active suppression is not involved in long term modulation of inflammtory response

The initial mechanism considered was that suppression resulted from the effects of IL-10 protein produced at the sites of plasmid DNA transfection and this protein actively inhibited

the response at the tissue site of inflammation. For this mechanism to occur, IL-10 protein should be present in the bloodstream and tissues. To show that IL-10 protein could inhibit DTH, purified IL-10 was injected into the ear pinna of HSV primed mice at the same time as antigen. A single injection of IL-10 protein to the local inflammatory site was sufficient to mediate inhibition as observed previously by others (23). Furthermore, the subsequent injection (3 hrs later) of anti-IL-10 abrogated the inhibitory effect of the IL-10 protein (Figure 3). Abrogation also occurred if the anti-IL-10 was injected at the same time as IL-10 protein and Ag. Following mucosal administration of IL-10 plasmid DNA, IL-10 protein was evident in serum in minimal amounts in some animals by day 2 and peaked around 8 days p.i. (Figure 4). Most animals still had measurable IL-10 on day 14 but by day 20 IL-10 protein was undetectable. Therefore, the increase of serum IL-10 level might explain the inhibition of DTH responses. However, the inhibitory effect of IL-10 DNA administration persisted for at least 2-3 weeks beyond the time of detection of serum IL-10. Furthermore, in other experiments, anti-IL-10 antibody was injected along with Ag into the ears of HSV primed mice which 10 or 28 days previously were given IL-10 DNA intranasally. The anti-IL-10 treatment failed to affect the extent of the DTH reaction even though anti IL-10 could reverse the inhibitory effects by rIL-10 treatment (Figure 3). Furthermore, only very few cells expressing proteins could be found at inflammatory sites at day 14, and the effects of plasmid DNA modulation persisted beyond the time of plasmid DNA, RNA or protein demonstration at peripheral sites (or even the central lymphoid tissue). Such observations likely mean that the long-term inhibitory effect on the cutaneous inflammatory reaction was not the consequence of IL-10 protein expression at the local site. This observation argues against active suppression as an explanation for long-term modulation by IL-10 DNA.

T cell silencing and regulatory cells

An alternative mechanism of modulation by IL-10 DNA could involve silencing of antigen reactive T lymphocytes or the induction of regulatory cells in the draining LN or spleen by exposure to IL-10 protein. The regulatory effect could result from the action of other sets of T

cells (such as Tr1 cells described by others [24]). A series of experiments were designed to evaluate these issues. Firstly, it was supposed that if T cells were silenced as a consequence of exposure to IL-10, their inhibitory activity should be maintained upon transfer to a neutral environment lacking IL-10 protein. This issue was evaluated by adoptively transferring enriched T cells from HSV primed and IL-10 DNA treated mice (4 wks post IL-10 DNA treatment) into recipient SCID mice. The transferred cell population lacked adherent cells and was taken at a time when protein and RNA expressing cells were no longer present as detected in parallel experiments in mice given β-galactosidase marker plasmid DNA. Furthermore, IL-10 DNA could not be demonstrated by PCR in the adoptively transferred cell populations (data not shown). Thus apparently plasmid DNA free lymphocytes were transferred into the SCID mice.

One day after treatment, the SCID mice were tested if the adoptive transfers could support a DTH response. SCID mice, which received transfers from HSV-primed mice treated with vector DNA, served as positive controls for DTH reactions. As shown in Table 4, the recipients of T cells from IL-10 DNA treated mice showed inhibited inflammatory responses. After testing animals for DTH reactions, the groups of SCIDs used for adoptive transfer were challenged on the scratched skin with HSV virus. Animals were followed for the development of cutaneous zosterification lesions as described previously (19). As shown in Table 4, whereas SCIDs which received HSV immune lymphocytes from vector treated mice, were protected from zoster lesions, those given cells from IL-10 DNA treated animals showed severe lesions and died. Some level of immunity was still evident, however, since animals did survive longer than control SCIDs that received no adoptive transfers. The above observations provide evidence of silencing of effector cells or the presence of inhibitory cells in the transfer population. Thus in some systems T cells exposed to IL-10 protein can become inhibitory and also become themselves a source of IL-10 (24). Such cells have been termed Tr 1 cells (24).

To analyze for the presence of regulatory cells, splenocytes were taken from the HSV-infected mice at day 28 following i.n. administration of IL-10 DNA. Such splenocytes, enriched for T cells by fractionation on nylon wool columns, were mixed at a 1:1 ratio with nylon wool

nonadherent cells from HSV primed mice that were not subsequently exposed to modulatory IL-10 plasmid DNA. The cell mixtures were transferred into syngeneic SCID mice and the animals tested for DTH responses one day later. No evidence for inhibition by the IL-10 exposed population was observed (data not shown). Furthermore, no evidence for an increase in the number of IL-10 producing cells was observed by intracelluar IL-10 cytokine staining in the enriched cell populations taken at day 28 post IL-10 DNA treatment (data not shown). These observations provide no support for the operation of an inhibitory cell population such as Tr 1 cells.

rIL-2 treatment reverses silenced T cell reactivity.

Some reports have indicated that inhibitory effect of IL-10 on T cell functions is reversible by antigenic stimulation in the presence of exogenous IL-2 (25). To evaluate if the T cell silence was affected by IL-2, *in vitro* and *in vivo* experiments were performed on T cells collected 4 weeks after mucosal administration of IL-10 DNA. As shown in Table 5, *in vitro* stimulation of silenced T cells with antigen in the presence of exogenous rIL-2 supported both proliferation and IFNγ SFC responses.

To measure the effects of rIL-2 on T cell silencing *in vivo*, SCID mice were given rIL-2 at same time as adoptive transfer of T cells from IL-10 DNA treated mice. Recipient mice were given additional IL-2 injection on day 1, 2, and 3. The animals were tested both for the magnitude of HSV specific DTH reactions as well as their resistance to systemic challenge by HSV virus. The results shown in Table 6 indicates that the IL-2 treatment reversed the silencing effects in that animals both developed DTH reaction and showed resistance levels to HSV comparable to that mediated by immune T cells from vector treated animals. Our data indicate that the T cell silencing effect mediated by IL-10 appears reversible by antigenic stimulation in the presence of exogenous IL-2.

CHAPTER 5. DISCUSSION

This report shows that mucosal administration of plasmid DNA leads to rapid and widespread plasmid DNA distribution around the body. The inoculation route represents a useful method of modulating immune responsiveness with cytokine DNA. Dissemination likely occurred via the blood stream since plasmid DNA was present in plasma from day I until day 3 after nasal administration. The plasmid DNA was detectable in all tissues analyzed including lungs, draining lymph node, spleen, liver, bone marrow and even the non-inflamed dermis of the ear pinnae. Except for the site of administration, plasmid DNA was no longer detectable in tissues after 3 weeks post inoculation. RNA expression was also detected in the tissues. In addition to plasmid DNA, the bloodstream also contained protein-expressing cells and secreted protein. Accordingly, animals given plasmid encoding GFP had detectable protein expressing cells until at least day 10 following i.n. administration. Secreted protein was present between day 6 and 14 following i.n. inoculation of IL-10 DNA. Animals previously primed by HSV infection and subsequently given IL-10 DNA intranasally, showed modulated antigen induced cutaneous inflammatory reactions for up to 5 weeks post administration. The mechanism of modulation involved diminished antigen induced proliferation and the production of Th1 cytokines such as IL-2 and IFNγ. The modulatory effect likely proceeded by multiple mechanisms, but since suppressed antigen specific T cell functions persisted beyond the duration of detectable plasmid encoded protein and was maintained upon adoptive transfer of T cells into a plasmid free environment, one of the modulatory effects was assumed to involve silencing of T cell reactivity. Such silenced T cells were not themselves transfected by plasmid DNA and not a source of IL-10. Moreover, their anergic state was reversible by exposure to antigen in the presence of exogenous IL-2.

The observation that plasmid DNA was evident in plasma following intranasal inoculation was surprising, although such has been reported after systemic injection (26 - 28). How plasmid DNA gains entrance from a mucosal surface into the blood stream was not

resolved. Likely routes could be through the highly vascularized nasal cavity or lung alveoli (29). In fact, protein-expressing cells were readily detectable in the lung. Others have reported that the circulation half-life of plasmid DNA is brief and of the order of 5 min to 16 min (26, 27, 30, 31). However, in our studies plasmid DNA was detectable for at least 3 days after a single mucosal administration. This may imply that seeding occurs over a lengthy period or that some plasmid DNA may be protected from breakdown by binding to cells or by being bound to some serum protein. Some support for latter idea has been reported by others (27, 32, 33). The presence of free plasmid DNA in the plasma could mean that this is the primary means by which plasmid DNA disseminates widely to distal tissues.

An additional transport mechanism could involve transfected cells, which were detectable in the blood stream for a longer period than was free plasmid DNA. Thus by RT-PCR using RNA from washed leukocytes, signals were detectable for 2 weeks and using the non-secreted protein GFP, transfected cells were evident for at least 10 days. The nature of the transfected cells was not established but were likely mainly macrophages and dendritic cells. In other studies, macrophages were shown to rapidly take up plasmid DNA via their scavenger receptor following intravenous plasmid DNA administration (27, 34). A recent report also showed macrophages containing GFP were evident in blood following intramuscular injection of plasmid DNA encoding GFP (35). Other reports have indicated that plasmid DNA transport, at least from the site of muscle or skin inoculation to the draining lymph node, proceeds via transfected dendritic cells (8 - 12). It will be of interest to further identify the nature of transfected blood cells as well as define the localization of their transfection. Possibilities include the bloodstream itself, the inoculated nasal mucosa, or the draining lymph node. We are currently attempting to verify the latter idea by comparing the number of transfected cells in the circulation of normal mice with LTα k/o animals that lack lymph nodes (36, 37). It seems likely, however, that not all cells were transfected at the local lymph node or blood stream since cells of nonmyeloid type were evident in peripheral tissues. Thus occasional cells with fibroblast morphology were evident in non-inflamed skin sections in animals inoculated mucosally with β-

gal plasmid DNA. These observations may mean that plasmid DNA may exit the blood stream and also transfect cells at tissue sites. Experiments are underway to further investigate these issues.

Our studies demonstrate that mucosal delivery of plasmid DNA encoding IL-10 provided long term suppression of an antigen-induced inflammatory reaction orchestrated by CD4+ Th1 T lymphocytes. Accordingly, mice infected with HSV and subsequently given IL-10 DNA had suppressed cutaneous DTH reactions to viral antigens. Suppression took a few days for onset but persisted for 5 weeks. Many investigations have demonstrated that IL-10 can modulate Th1 mediated functions with multiple mechanisms likely occurring (2). These include inhibition of cytokine production and action on target cells, as well as inhibition of recruitment of nonspecific cells to inflammatory sites (2, 38). IL-10 protein in lesion sites can mediate such effects as was demonstrated in this communication as well as by others (23). However, whereas direct active suppressive effects of IL-10 protein in lesions might account for part of the suppression at least in the early phase, this mechanism could not provide the complete explanation for our observations. Thus, the suppression persisted well beyond the duration of detectable IL-10 protein in serum (6-14 days) or even protein producing cells (up to 3 weeeks) at least as measured by detection of a marker protein. In addition, whereas the modulatory effect of exogenous IL-10 protein could be neutralized by anti IL-10 injected into inflammatory sites, similar injections were without effect on suppression induced by mucosal plasmid DNA administration. Furthermore, the IL-10 DNA suppressed activity of T cells was retained when such cells were adoptively transferred to an environment lacking IL-10 plasmid DNA.

We interpret such observations to mean that some form of central suppression of lymphoid function had occurred. This notion was further supported by the observation that antigen specific lymphocyte responses measured in vitro were suppressed especially as regards Th1 cytokine production. We have referred to the inhibited effect as T cell silencing and favor this mechanism over one of inhibition by a separate set of regulatory cells. Thus, in some systems investigators have demonstrated that long term stimulation of $CD4^+$ T cells in the

presence of IL-10 leads to the production of a subset of T cell (Trl cells) which secret IL-10 and inhibit the action of Th1 cells (24). Using adoptive transfers of cell mixtures into recipient mice and measuring their DTH reactions, no evidence for regulatory effects was forthcoming. Furthermore, in T cell populations taken from suppressed mice, no evidence for an increase in the number of IL-10 producing cells was observed. Although we lacked positive evidence for the existence of regulatory Trl-like cells after mucosal IL-10 DNA administration, this issue is being further investigated.

The nature of T cell silencing requires further study, but this is difficult to accomplish in the system we described since the viral antigen specific CD4+ T cell frequency is low. However, the silenced cells would appear to be in a reversible state of anergy, since in addition to recovering their former function with time, their antigen specific function could be recalled by stimulating cells in the presence of exogenous IL-2. This IL-2 reversible effect was evident both in vitro as well when the suppressed cells were adoptively transferred to SCID mouse recipients given IL-2 and antigen. Interestingly, whereas the adoptive transfer of silenced cells to SCIDs failed to confer protection against HSV challenge, the effect was reversed in recipient of IL-2. In other system involving IL-10 protein mediated unreponsiveness of CD4+ T cells, the effect was reversible by culture of the cells in vitro with exogenous IL-2 (25). However, in another system unresponsiveness induced by IL-10 appears to be irreversible by IL-2 (39). It could be that the IL-10 treatment downregulates IL-2 receptor α chain on the T cells, inhibiting IL-2 signalling. In our system, IL-2 could functionally reverse the antigen unresposiveness state, indicating that the signalling pathway through IL-2 receptor was not inhibited by IL-10 treatment. Infact, we could detected no change on the expression of IL-2 receptor α chain on the T following IL-10 DNA administration (unpublished data).

In conclusion, mucosal delivery of plasmid DNA results in widespread distribution in the body. This distribution may occur by transport of free plasmid DNA as well as by transfected cells. However, persistence of the plasmid DNA is not indefinite and was far shorter than up to 9 months reported by some to occur following i.m. plasmid DNA administration (40). Nevertheless,

this shorter duration represents a potential advantage of mucosal delivery_in some clinical situations. One such instance may be when using gene delivery to express immunomodulators. Thus modulating an unwanted inflammatory immune reaction may concomitantly render recipients more susceptible to infection by agents where defense depends on the function of the type of immune response being suppressed. In fact, in the case of HSV infection, the $CD4^+$ Th1 mediated inflammatory reaction, whilst undesirable and tissue damaging in certain locations such as the eye (41), is actually the principal means of immune control following HSV infection (19, 41). Suppressing the response renders animals more susceptible to infection and virus can spread to critical tissues such as the brain (42). Indeed, in our studies using adoptive transfer experiments we showed that T cells from HSV primed IL-10 DNA treated mice were less immunoprotective against HSV challenge than were T cells from untreated animals. Accordingly, modulating immunity using cytokine gene delivery somewhat resembles a Faustian dèlemma!

Table 1. Administration of IL-10 DNA inhibits cutaneous inflammatory responses.

Treatment	Test days after IL-10 DNA treatment							
	day 2	day 3	day5	day 14	day 35	day 42	day 49	day 63
i.n. IL-10 DNA	11.8 ± 2.1	9.8 ± 1.7	6.9 ± 1.6*	5.9 ± 1.1*	8.2 ± 1.5*	10.1 ± 1.4	11.4 ± 1.7	12.9 ± 2.1
i.n. Vector	12.1 ± 1.7	12.8 ± 1.8	13.9 ± 1.8	15.1 ± 1.3	12.3 ± 1.9	13.0 ± 1.0	12.7 ± 1.5	14.1 ± 1.6
i.m. IL-10 DNA	10.1 ± 1.8	9.0 ± 1.9	5.8 ± 1.4‡	6.1 ± 1.1‡	6.5 ± 2.2‡	7.5 ± 1.1‡	8.1 ± 2.1‡	12.0 ± 1.8
i.m. Vector	12.3 ± 2.1	12.9 ± 1.1	13.1 ± 1.9	13.8 ± 1.7	13.5 ± 1.1	12.5 ± 1.4	12.9 ± 1.8	13.2 ± 2.2

Footnote to Table 1.

Groups of HSV immune mice received i.n. or i.m. treatment of 200 μg of IL-10 DNA or vector DNA. At different days following IL-10 DNA treatment, the mice were tested for DTH responses by injecting 20 μl of UV-inactivated HSV-1 KOS (10^5 PFU prior to UV inactivation) and vero cell extract into the right and left ear pinnae, respectively. The increase in ear thickness was measured 48 hours later. The values were expressed as 10^{-2} mm. The data represent mean difference between the thickness of right and left ear ± standard deviation. Each time point consists of at least 6 mice. * Statistically significant between i.n. vector DNA and i.n. IL-10 DNA treated group ($p < 0.05$), ‡ statistically significant between i.m. vector DNA and i.m. IL-10 DNA treated group ($p < 0.05$), at day 42 and 49, statistically significant between i.n. IL-10 DNA and i.m. IL-10 DNA treated group ($p < 0.05$).

Table 2. IL-10 DNA treatment suppresses HSV-specific T cell immune responses of primed animals .

Treatment	proliferation(cpm)*		Proliferation	IL-2 production (pg/ml)‡			SFC/5x10^5§ cells	
	Con A stimul.	HSV stimul.	Index	Con A stimul.	HSV stimul.	unstimul.	IFNγ	IL4
i.n. IL-10 DNA	29104 ± 2148.3	7432.1 ± 2310.1¶	12.7	3192.5 ± 394.5	311.4 ± 269¶	< 40	101 ± 44¶	45 ± 21
i.n. Vector DNA	27283 ± 3174.5	18390.8 ± 3839.1	26.6	3399.8 ± 184.5	1189.7 ± 355	< 40	254 ± 39	31 ± 17
i.m. IL-10 DNA	28284 ± 3998.3	5983.4 ± 1438.9**	10.1	2953.2 ± 223	281.5 ± 994**	< 40	89 ± 25**	49 ± 14
i.m. vector DNA	30148 ± 2749.6	21983.1 ± 4145.3	29.9	3143.8 ± 389	1383.4 ± 283	< 40	248 ± 27	42 ± 11

Footnote to Table 2.

Groups of HSV immune mice received i.n. or i.m. administration of 200 μg of IL-10 DNA or vector DNA. At day 25 after IL-10 DNA treatment, spleen cells were obtained and enriched for T cells using a nylon-wool column. * These responder T cells were mixed with irradiated syngeneic naive splenocytes (unstimulation) or irradiated splenocytes infected with UV inactivated HSV-1 KOS (1.5 multiplicity of infection (m.o.i.) before inactivation) or irradiated naive splenocytes, and incubated for 5 days. 3[H]-thymidine was added to each well 18 hours before harvest. The results show mean cpm ± standard deviation for 5 independent experiments. Proliferation Index was calculated by cpm of HSV stimulation/cpm of unstimulation. ‡ For the measurement of IL-2, the T cells were restimulated in vitro with irradiated syngeneic naive splenocytes or irradiated splenocytes infected with UV inactivated HSV-1 KOS (1.5 m.o.i. before inactivation). Seventy two hours later, culture sups were collected and analyzed for IL-2 by ELISA. The data represent mean concentration ± standard deviation for 5 independent experiments. Con A (5 μg/ml) was used as polyclonal stumulator for prolifertion and IL-2 production. § To analyze the number of IFNγ and IL-4 producing cells, ELISPOT assay was performed. The results represent mean SFC ± standard deviation for 5 independent experiments. ¶, **, Statistically significant between vector and IL-10 DNA treated group ($p < 0.05$).

Table 3. Distribution of plasmid DNA encoding β-gal and RNA expression in tissues following mucosal administration.

	Test time after administration													
	5 hr		10 hr		day 1		day 3		day 7		day 14		day 21	
Tissues	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Plasma	+	-	+	-	+	-	+	-	-	-	-	-	-	-
Blood (Ad.) cells	-	-	+/-	-	+	+	+	+	+	+	+	+	-	-
D.L.N.	-	-	+	-	+	+	+	+	+	+	+	+	-	-
Spleen	-	-	+/-	-	+	+	+	+	+	+	+	+	-	-
Liver	-	-	+/-	-	+	+	+	+	+	+	+/-	+/-	-	-
Bone marrow	-	-	+/-	-	+	+	+	+	+	+	+/-	+/-	-	-
Ear	-	-	+/-	-	+	+	+	+	+	+	+/-	+/-	-	-
Lung	+	-	+	+/-	+	+	+	+	+	+	+	+	-	-

Footnote to Table 3.

Groups of mice received 200 μg of β-gal DNA, pGFP (not shown) or vector via nasal mucosa. At different time points, various tissues were obtained from some animals, put into TRI reagent and immediately frozen at -70 C. Anticoagulated blood was also collected, and separated into plasma and peripheral blood cells (PBC). Phenol/chloroform extraction was performed to isolate DNA from the plasma. Isolated PBC were washed with DMEM media containing 10% FBS, and incubated in the culture flask for 90 min at 37 C to separate adherent and nonadherent cells. After incubation, nonadherent cells were carefully obtained and adherent (Ad.) cells were harvested. The two cell populations were put into TRI reagent and immediately frozen at -70 C. DNA and total RNA were isolated from the tissues per manufacturer's protocol, and analyzed for the presence of DNA by PCR analysis using CMV-1 and β-gal-2 primers (or CMV-1 and GFP-2 primers). To detect RNA expression, the isolated total RNA was treated with DNase, and PCR without reverse transcription performed using both CMV-1/β-gal-2 primers and β-gal-1/ β-gal-2 primers to exclude the presence of DNA. The RNA samples were reverse transcribed and amplified with β-gal-1 and β-gal-2 (or GFP-1 and GFP-2 primers). The intensity of amplified band was analyzed by the comparison of the band intensity of β-gal (or GFP) to β-actin. The experiment was performed 4 times independently. No amplified bands for β-gal (or GFP) DNA and RNA were detected in the samples of vector DNA treated mice. Similarly, the multiple tissues were analyzed following i.m. injection of plasmid DNA. The data showed similar results except the plasmid DNA was present in plasma at 5 min later. The DNA in muscle tissues after i.m. injection was found at the end of experiment (day 49), whereas RNA expression was occasionally detected at day 28. In separate experiments, multilple tissues were examined for the presence of plasmid DNA following IL-10 DNA administration. The results showed similar pattern as β-gal experiment. + : positive, - : negative, +/- : occasionally positive.

Table 4. Transfer of suppressed T cell immunity by IL-10 DNA to SCID.

	DTH responses		Zosteriform Lesions		
	Mean increase (x 0.01mm)		No. of mice with lesions		
Adoptive transfer	HSV-1 KOS	Vero cell extract	No. of mice used	Ave. Score	Ave. day of death
Untransferred	1.2 ± 0.4	0.4 ± 0.3	6 / 6	5	8 ± 1
T cells from vector treated mice	13.1 ± 0.6	0.4 ± 0.2	0 / 8	0.4	-
T cells from i.n. IL-10 DNA treated mice	7.3 ± 1.5*	0.3 ± 0.2	5 / 8	2.8	23 ± 5

Footnote to Table 4.

Groups of HSV infected BALB/c mice were given 200 μg of IL-10 DNA or vector DNA via nasal mucosa. At day 28 after IL-10 DNA administration, 10^7 cells of an enriched T cell population from the mice was adoptively transferred into SCID mice via tail vein. The next day, DTH reaction was elicited in the SCID mice and measured 48 hours later. The results show mean increase of ear thickness ± standard deviation. Challenge for the development of zosteriform lesions was performed by infecting 10^6 PFU of HSV into the scratched skin of the SCID mice at day 3 following adoptive transfer. At day 20 post challenge, the number of mice that showed lesions and average scores were determined. The presence of IL-10 DNA in the enriched population was tested by PCR, but no amplified signals were demonstrated. To analyze the number of IL-10 producing cells, intracellular cytokine staining for IL-10 was performed in the enriched cells from IL-10 DNA or vector treated mice, but no significant change in the number of IL-10 producing cells was identified (IL-10 positive cells in enriched population from vector treated mice: < 0.4 %, IL-10 positive cells in enriched population from IL-10 DNA treated mice : < 0.5 %).

* Statistically significant between SCID mice received T cells from vector treated mice and from IL-10 DNA treated mice ($p < 0.05$).

Table 5. rIL-2 restores the suppressed T cell reactivity by IL-10 DNA.

		Proliferation (cpm)		Proliferation	IL-2 production (pg/ml)			SFC/5x10^5 cells	
Responder T cells	Treatment	Con A stimul.	HSV stimul.	Index	Con A stimul.	HSV stimul.	unstimul.	IFNγ	IL4
T cells from vector treated mice	PBS	32181 ± 3833	22181 ± 3761	31.7	3192 ± 384	1683 ± 299	< 40	276 ± 42	42 ± 24
Day 28 T cells from i.n. IL-10 DNA treated mice	PBS	30169 ± 2723	3522 ± 258 *	7.5	2994 ± 293	408 ± 109 *	< 40	92 ± 29*	51 ± 11
Day 28 T cells from i.n. IL-10 DNA treated mice	IL-2	N.D.	23296 ± 3138 ‡	38.6	N.D.	N.D.	N.D.	284 ± 48‡	60 ± 23
Day 65 T cells from i.n. IL-10 DNA	PBS	35184 ± 2026	20925 ± 2111	30.6	2823 ± 166	1315 ± 301	< 40	245 ± 37	50 ± 18

Footnote to Table 5.

Groups of HSV immune mice were given 200 μg of IL-10 DNA or vector DNA via i.n. administration. At day 28 and 65 after IL-10 DNA treatment, spleen cells were obtained and enriched for T cells using a nylon-wool column. HSV specific proliferation, cytokine assay were performed as described earlier. rIL-2 was added at 10 unit/well. *, §, Statistically significant between vector and IL-10 DNA treated group ($p < 0.05$), *, ‡, Statistically significant between rIL-2 treated and PBS treated group ($p < 0.05$). N. D., not done.

Table 6. rIL-2 treatment restores the silenced T cell immunity by IL-10 DNA *in vivo*.

Adoptive transfer	Treatment	DTH responses Mean increase (x 0.01mm) HSV-1 KOS	DTH responses Mean increase (x 0.01mm) Vero cell extract	Zosteriform Lesions No. of mice with lesions No. of mice used	Zosteriform Lesions Ave. Score
T cells from vector treated mice	PBS	14.9 ± 1.2	0.3 ± 0.2	0 / 6	0.2
T cells from i.n. IL-10 DNA treated mice	PBS	6.4 ± 1.1*	0.2 ± 0.1	4 / 6	2.9
T cells from i.n. IL-10 DNA treated mice	IL-2	12.8 ± 1.6 ‡	0.5 ± 0.2	0 / 6	0.4

Footnote to Table 6.

Groups of HSV infected BALB/c mice received i.n. administration of 200 μg of IL-10 DNA or vector DNA. At day 28 after IL-10 DNA administration, 10^7 cells of an enriched T cell population from the mice was adoptively transferred into SCID mice via tail vein. DTH responses and HSV skin challenge were performed as described earlier. rIL-2 (500 unit) and PBS were i.p. injected at day 0, 1, 2 , and 3 following adoptive transfer. At day 20 post challenge, the number of mice that showed lesions and average scores were determined. The presence of IL-10 DNA in the enriched population was tested by PCR, but no amplified signals were demonstrated. To analyze the number of IL-10 producing cells, intracellular cytokine staining for IL-10 was performed in the enriched populations from IL-10 DNA or vector treated mice, but no significant number of IL-10 positive cells was identified. *, Statistically significant between SCID mice received T cells from vector treated mice and from IL-10 DNA treated mice ($p < 0.05$), ‡ Statistically significant between rIL-2 and PBS treated SCID mice which were received T cells from IL-10 DNA treated group.

Figure 1. The presence of β-gal DNA (pCMVβ) and RNA expression at different tissues. Groups of BALB/c mice received i.n. administration of 200 μg of plasmid DNA encoding β-galactosidase (pCMVβ), plasmid DNA encoding green fluorescence protein (pGFP, not shown) or vector DNA (not shown). At different time points, several tissues were isolated from the mice and treated with TRI reagent. Anticoagulated blood samples were also obtained and separated into plasma and blood cells. The blood cell samples were further separated into adherent and nonadherent cells. These two populations were treated with TRI reagent, and DNA was extracted from the plasma samples. Total RNA and DNA were extracted from the tissues as described in Materials and Methods. Plasmid DNA was amplified with CMV-1 and β-gal-2 primers (or CMV-1 and GFP-2 primers). To detect RNA expression, plasmid DNA was removed by treatment with RQ-1 RNase free DNase and the absence of plasmid DNA was verified by PCR analysis without reverse transcription. Total RNA was reverse transcribed and amplified with β-gal-1 and β-gal-2 (or GFP-1 and GFP-2 primers). The figures represent β-gal DNA (~870 bp) and RNA (~400 bp) expression. Multiple samples from vector DNA received mice did not show positive signals for β-gal DNA and RNA expression. Similar results were obtained after i. n. administration of pGFP (not shown). 1: D.L.N., 2: Spleen, 3: ear, 4: lung, 5: Liver, 6: Bone marrow, 7: adherent blood cells, 8: plasma.

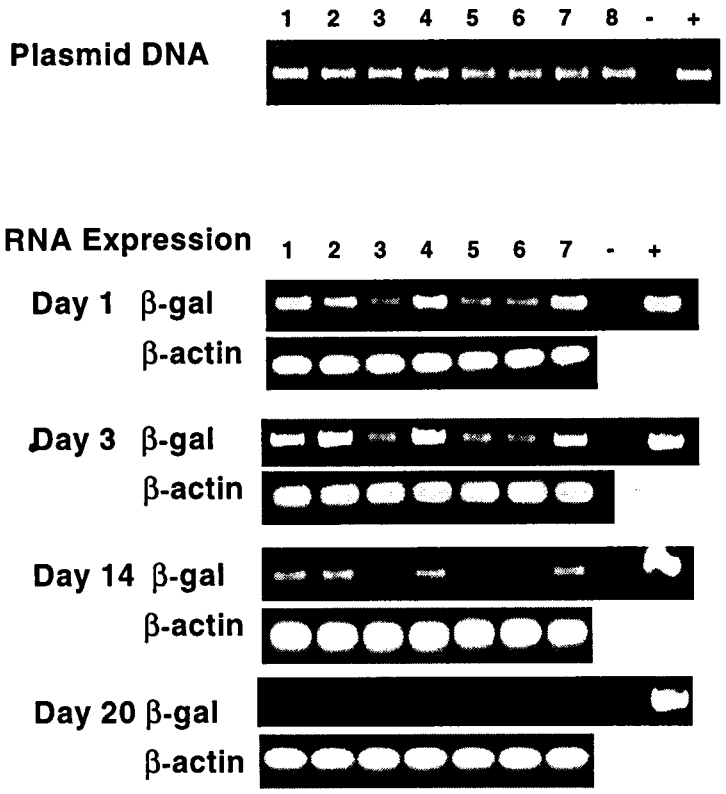

1 2 3 4 5 6 7 8 - +
Plasmid DNA
RNA Expression
1 2 3 4 5 6 7 - +
Day 1 β-gal
β-actin
Day 3 β-gal
β-actin
Day 14 β-gal
β-actin
Day 20 β-gal
β-actin

Figure 2. Plasmid encoded protein expression in the tissues. Groups of mice received pCMVβ , pGFP, or vector DNA (not shown) via the nasal cavity. Seven days later ear tissues (A, X200; B, X400), lung (C, X100), DLN (D, X200), and spleen (E, X200) were obtained from the pCMVβ treated mice, and stained for β-galactosidase activity as described in Materials and Methods. Adherent blood cells (F, X600) were isolated from the vector DNA- (not shown) or pGFP-treated mice and fixed with 4 % paraformaldehyde. The cells expressing green fluorescence protein were identified by fluorescence microscopy. Tissues or cells from vector DNA treated mice were negative for β-galactosidase activity and green fluorescence. Also the number of cells expressing green fluorescence protein was analyzed by flow cytometry at day 10 post DNA treatment. The results follow : i.n. treatment of pGFP; 280 - 580 cells / 10^5 adherent blood cells, i.m. injection of pGFP; 350 - 790 / 10^5 adherent blood cells.

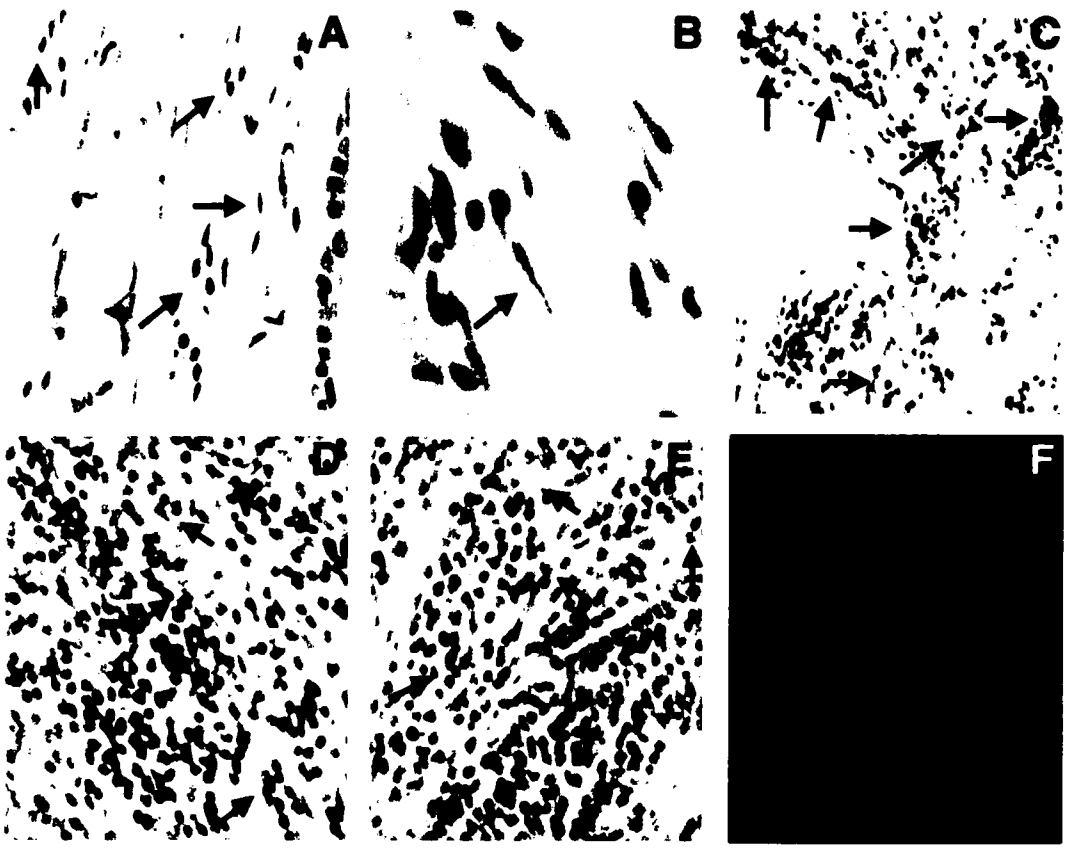
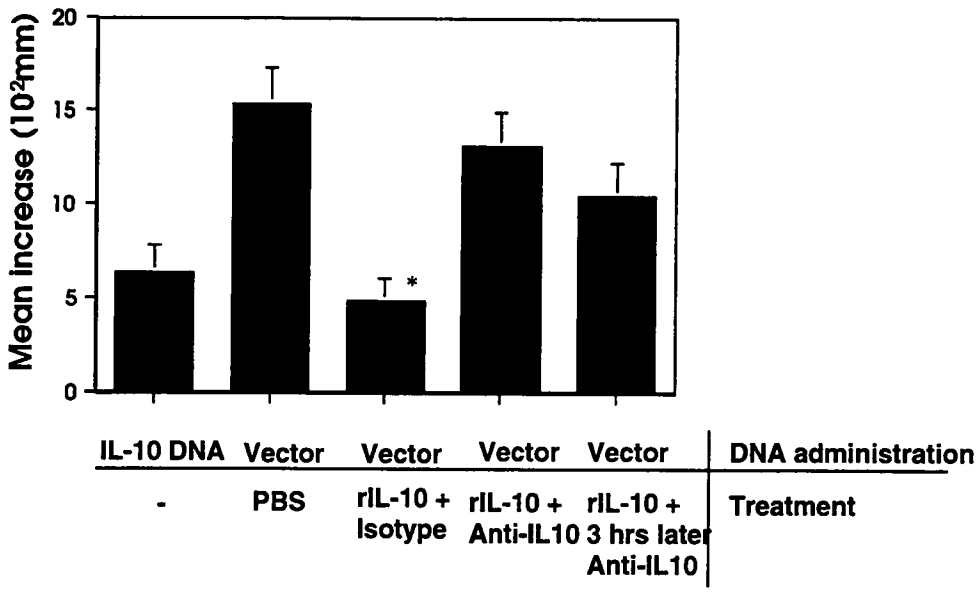

A
B
C
D
E
F

Figure 3. Local anti-IL-10 does not restore the suppressed DTH reaction by IL-10 DNA. Groups of HSV-immune mice received i.n. treatment of 200 μg of IL-10 DNA or vector. At 28 days post DNA treatment, the vector treated mice were tested for the DTH responses as described in Materials and Methods (upper panel). Some of the mice were injected into right ear pinnae with 20 μl of HSV-1 KOS and with 80 ng of IL-10 protein. At the same time or three hours later 1 μg of isotype or anti-IL-10 antibody was injected into the ear pinnae. The IL-10 DNA treated mice were injected with HSV-1 KOS or vero cell extract along with either 1 μg of isotype or anti-IL-10 antibody (lower panel). The increase in ear thickness was measured at 48 hours later. Each group consists of at least 7 mice. Similar results were obtained from the IL-10 DNA treated mice tested at day 10 post DNA treatment. * Statistically significant between IL-10 protein treated and either PBS or IL-10 protein plus anti IL-10 DNA received group ($p < 0.05$).


Mean increase (10²mm)
20
15
10
5
0
*
IL-10 DNA
Vector
Vector
Vector
Vector
DNA administration
-
PBS
rIL-10 + Isotype
rIL-10 + Anti-IL10
rIL-10 + 3 hrs later Anti-IL10
Treatment

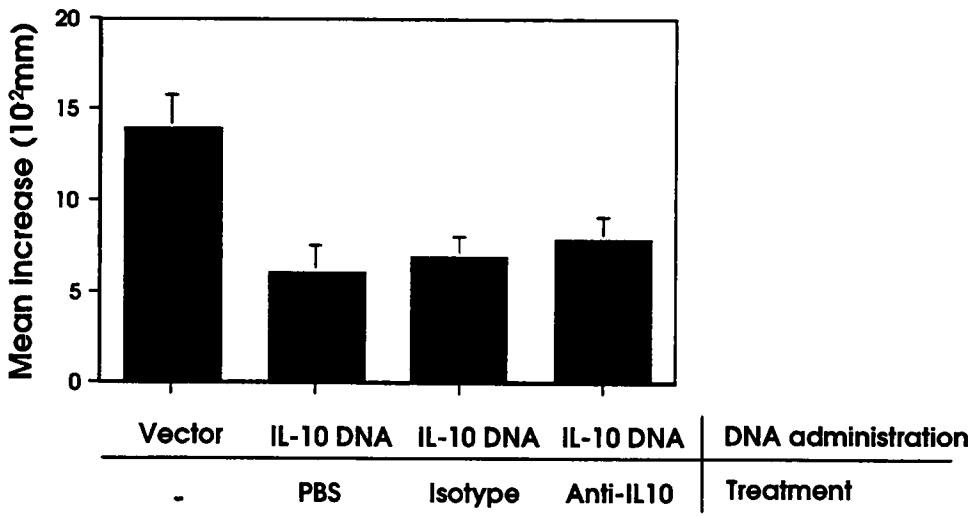
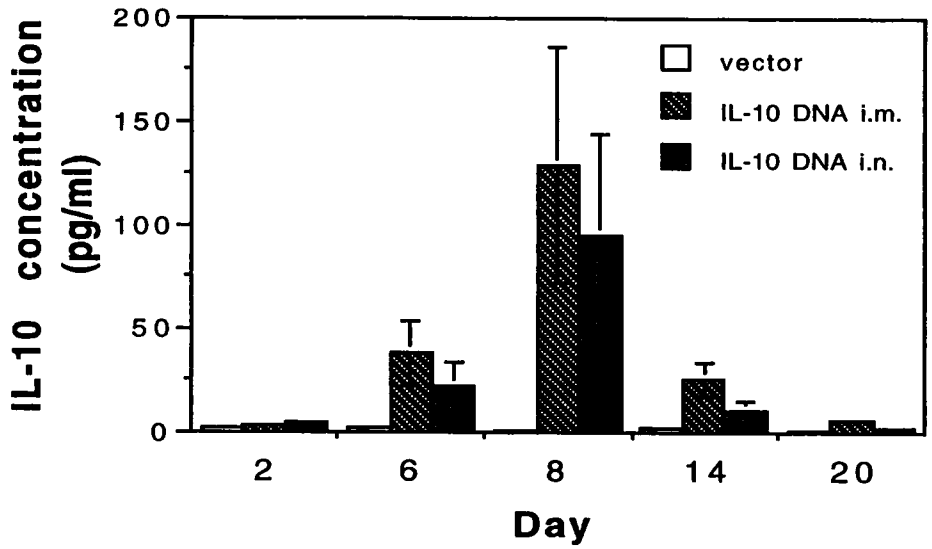

Mean increase (10²mm)
20
15
10
5
0
Vector
IL-10 DNA
IL-10 DNA
IL-10 DNA
DNA administration
-
PBS
Isotype
Anti-IL10
Treatment

Figure 4. IL-10 protein expression in serum after i.n. or i.m. treatment of IL-10 DNA. Groups of mice were given either i.m. or i.n. 200 μg of plasmid DNA encoding IL-10 or vector DNA. At different time points, serum samples were collected from the mice and individually analyzed for the presence of IL-10 protein by a standard ELISA assay. The graph represents mean concentration ± standard deviation from at least four different samples.


IL-10 concentration (pg/ml)
200
150
100
50
0
2
6
8
14
20
Day
vector
IL-10 DNA i.m.
IL-10 DNA i.n.

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PART 5. CONCLUSION

This study was commenced to evaluate the potential value of using plasmid DNA encoding cytokines to modulate virus induced inflammatory responses and to understand the mechanism by which cytokine regulates the immune system *in vivo*. In the first part of the study, the efficacy of plasmid DNA encoding cytokines administered prophylactically by different routes, systemic and mucosal surface, was tested and compared for their modulating effects on subsequent lesions caused by infection with HSV. The results demonstrated that systemic or topical treatment of IL-4 or IL-10 DNA, but not IL-2 DNA, led to a long-lasting suppression of HSV-induced cutaneous inflammatory responses. The novel finding is that effects by cytokine DNA persisted for an extended time and mucosal administration represents a useful means for immune modulation. In the case of immunoinflammatory lesions associated with corneal infection of HSV, the administration of regulatory cytokine DNAs close to inflammatory sites was necessary to achieve suppression of ocular lesions. The mechanism involved in modulation of HSV-induced inflammatory lesions was also examined. Preexposure of IL-4 DNA resulted in a significant change in Th subset balance following HSV infection, whereas IL-10 DNA inhibited Th1 type reactivity.

To further evaluate the practical application of plasmid DNA as a gene delivery system for immune regulation, the therapeutic modulatory effect of the cytokine IL-10 expressed via viral vector or plasmid DNA was assessed on DTH responses induced by prior exposure to viral infection. The data showed the superior potency of IL-10 expression via viral vectors after single administration, but the effect was only short-term and only functioned in animals lacking immunity to the viral vectors used for gene delivery. In contrast, plasmid DNA expressing IL-10 had a delayed and milder activity, but showed a far more persisting suppressive effect than that achieved by viral vectors. Furthermore, repeated administration of plasmid DNA also provided effective modulation in contrast to viral vectors. These results highlight that the naked DNA approach represents a practical therapeutic option to control chronic inflammatory lesions.

Finally, the mechanism of immunomodulation following IL-10 DNA delivery was investigated. In this part the data demonstrated that mucosal administration of plasmid DNA led

to a rapid and widespread distribution around the body via the blood stream. This distribution indicated that the gene expression could occur at multiple sites. Indeed, the expression of marker β-galactosidase was observed in the lung, lymph nodes, spleen, and even ear tissues, indicating that IL-10 protein produced at the sites following IL-10 DNA administration might actively inhibit the recruitment and activity of inflammatory cells. However, the modulatory effects and inhibited T cell functions by IL-10 DNA persisted beyond the duration of detectable plasmid encoded protein and was maintained upon adoptive transfer into a plasmid free environment. Furthermore, the unresponsiveness of T cells to antigen was readily reversible by exposure to antigen in the presence of exogenous IL-2. These data provide evidence that following exposure to IL-10, T cells are anergized and remain in an antigen-unresponsiveness state. The finding that IL-10 induces an anergic state in T cells *in vivo* was novel, and may account for the immune modulation in addition to active suppression. However, the relationship between active suppression and induction of anergy requires further clarification. For instance, it would be interesting to study when the anergic cells are induced and how long the anergized cells are maintained following IL-10 DNA administration. The adoptive transfer of the suppressed T cells taken at different time would be a good model for the approach of the issue.

Although the presence of regulatory T cells such as Tr-1 cells could not be demonstrated in the study, the possibility of induction of the regulatory Tr-1 cells following administration of plasmid DNA encoding IL-10 can not be completely ruled out. Since the frequency of HSV-Ag specific CD4 T cells is relatively low, this issue could be reanalyzed using an animal model such as DO11.10 mice or DO11.10 mice backcrossed with IL-10-/- mice since these animals have a limited T cell repertoire. Moreover, the DO11.10 x IL-10-/- animals do not make endogenous IL-10 protein. It would be a very exciting to analyze whether the DO11.10 and the DO11.10 x IL-10-/- mice show similar modulatory effect following the administration of plasmid DNA encoding IL-10. This system may provide more direct evidence whether the regulatory Tr-1 cells are involved in the modulation of Ag-specific T cell response by IL-10 DNA.

As future aspects of the induction of Ag-unresponsiveness by IL-10, the cellular and molecular mechanisms by which IL-10 induces anergic state and by which this state is maintained need to be resolved. The induction of the anergic state by IL-10 might involve multiple mechanisms. The signals following the binding of IL-10 to its receptor may inhibit the DNA binding activity of NF-κB and several transcription factors that are essential for the production of IL-2. IL-10 signaling might directly inhibit the G0/G1 cell cycle progression of antigen specific T cells by downregulating cyclins and upregulating inhibitor kinase, $p27^{kip1}$. In addition, IL-10 may induce suppressor molecules that inhibit cytokine and costimulatory signaling. These intracellular events could be addressed *in vitro* by measuring target protein expression or by using molecular biological techniques. It is highly likely that all of the possible mechanisms may cooperate to induce and maintain the anergic state of the antigen specific T cells.

An important aspect of our findings is that although the modulation of immune responsiveness by regulatory cytokine DNA harbors promising hopes in the clinical situations, inhibition of an unwanted inflammatory immune reaction may concomitantly render recipients more susceptible to infectious agents. In fact, our adoptive transfer studies showed that T cells from HSV primed IL-10 DNA treated mice were less protective than were T cells from HSV-primed untreated animals. Moreover, we observed that intramuscular pretreatment of IL-10 DNA caused the recipients to be more susceptible to infection by other infectious agents. This might be an important concern in the clinical situation. The modulatory effect following mucosal administration of IL-10 DNA was not indefinite and was shorter than that after intramuscular injection. This shorter duration might be a potential advantage of mucosal delivery in some clinical situations. Another perspective approach regarding this matter may be the regulation of the therapeutic protein levels according to the need of each occasion. This could be assessed by using inducible gene expression systems that could regulate protein expression when the therapeutic protein is needed.

VITAE

Sangjun Chun was born in Youngdoing-Goon, Chung-Book, Korea on October 1st, 1967. He was raised in Seoul where he attended private schools. He entered Yonsei University in 1986 and obtained his bachelor degree (major in Biology and minor in Biochemistry) in February 1990. He continued his study at the Graduate School of Yonsei University and finished with a Master of Science degree in Microbiology in February of 1992. He worked as a teaching assistant fellow and studied the nucleotide metabolism and purinergic receptor system in the Department of Physiology, Wonju College of Medicine, Yonsei University for the next three and half years.

Sangjun came to the Department of Microbiology at University of Tennessee, Knoxville, in September of 1995 and joined Dr. Rouse' laboratory in January of 1996. He won a Science Alliance Graduate Student Award of Excellence from Division of Biology in 1999 and received the Doctor of Philosophy degree from the Department of Microbiology in August of 1999.

Sangjun plans to pursue postdoctoral training in Dr. Peter Doherty's laboratory, St. Jude Children's Research Hospital.