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Immunomodulatory Effects Following Naked DNA Transfer in an Autoimmune Model

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Table of Contents

List of Graphs, Plates and Tables:

Abstract:

Immune modulation is one treatment modality which is being explored in the context of human diseases and disorders. Methods of such manipulation which use gene therapy have an advantage in the treatment of chronic diseases such as autoimmunity because they are less invasive and more persistent. Furthermore, naked plasmid has advantages as a vector over other methods: it is more persistent and less immunogenic and cytotoxic than viral vectors, and simpler than DNA-conjugate vectors. Thus, naked plasmid is a viable alternative treatment to study in the context of an autoimmune disease such as diabetes mellitus, despite it's disadvantages of low transfection and expression rates. Here, we demonstrate that treatment of an autoimmune model, the non-obese diabetic (NOD) mouse, with an autoantigen to which a signal sequence had been added was protective, even in the apparent absence of secretion of that gene product. In contrast, treatment with the native cDNA of the same antigen was not protective. Furthermore, we show by immunohistochemistry that gene expression is still detectable in the muscle 22 weeks after injection. Other experiments demonstrate that multiple vaccinations with the altered form of the antigen were essentially as protective as a single vaccination following by multiple injections of blank plasmid, suggesting an important role for immunostimulatory sequences in bacterial DNA in causing surveying dendritic cells to migrate out of the tissue and present antigen in draining lymph nodes. Attempts to study the results of DNA vaccination by comparing immunization via different routes were inconclusive.

We have demonstrated that DNA vaccination of an autoimmune model with a autoantigen can delay disease. The simplicity and economy of such vectors and the benefits they have for the treatment of chronic disease in contrast to more inflammatory viral vectors, support future research into their use in the treatment of autoimmune diseases.

Résumé:

La modulation immunitaire contre les maladies est un traitement exploré. Les méthodes qui utilise la thérapie génie ont certains avantages dans le cas des maladies chroniques. Elles sont généralement moins invasive. De plus, l'utilisation de plasmide ADN seul comme vecteur a l'avantage d'etre plus soutenue. Elle est aussi moins immunogénique et cytotoxique que les méthodes virales et elle est plus simples que les vecteurs de ADN conjugés. Par conséquent, la méthode du ADN seul est une alternative valable dans le traitement des maladies auto-immunes malgré que sont niveau de transfection et d'expression est bas. Notre étude montre que le traitement des souris non-obèses susceptibles de devenir diabétique (NOD) sont protégées lorsqu'un plasmide codant pour un auto-antigène modifié pour contenir une séquence signal mais apparamment pas sécrété est injecté.Contrairement, les souris NOD injectées avec le ADN du gène natif n'a pas eu d'effet. De plus, nous avons montré par immunohistochimie que l'expression de ce gène est détectable dans le muscle pendant au moins 22 semaines suivant l'injection. La vaccination multiple avec le vecteur codant l'auto-antigène modifié était aussi protecteur qu'une seule injection de ce vecteur suivit d'injections du vecteur control. Ceci suggère l'importance de séquences immuno-stimulantes qui peuvent causer les cellules dendritiques (DC) à migrer hors des tissues et à présenter les antigènes dans les noeuds lymphatiques avoisinant. Nos résultats préliminaires de la vaccination par vecteur de ADN supportent la capacité des DC dans la présentation d'antigène suite à la vaccination.

Nous avons démontré que la vaccination par l'ADN dans un modèle de maladies auto-immunes avec un auto-antigène peut retarder la maladie. Contrairement aux vecteurs viraux qui sont plus inflammatoires, la simplicité, l'économie et le bénéfice de plasmides comme vecteurs dans le traitement de maladies auto-immunes supportent plus de recherche dans leur utilisation.

1. Introduction:

1.1 Targets of Immune Modulation:

The immune system is our defense, not only against invaders that penetrate the barrier of our skin, but against the neoplastic enemies which arise within. Since Jenner's first successes with vaccination, we have attempted to take advantage of the potential of this complex system, and overcome its weaknesses. Such manipulations are to achieve one of three effects: 1) to induce a response against a previously unencountered or poorly immunogenic antigen, 2) to induce tolerance to an antigen against which a response is undesirable, 3) to deviate an existing response to a different phenotype. With regards to the second point, central tolerance, which occurs in the thymus during T cell development, appears to function mainly by deletion (negative selection) of thymocytes which possess too high an affinity for self antigen in self major histocompatibility complex (MHC) molecules. However, many experimental models suggest that peripheral tolerance also operates by inducing *anergy*, an unresponsive state, in autoreactive T cells.

The concept of immune deviation arises from the mouse, in which distinct T helper (Th) cell phenotypes can be identified. Th1 responses are characterized by the production of IFN-γ, IL-2 and TNF-β, and associated with delayed type hypersensitivity (DTH). In contrast, Th2 cells are associated with the production of IL-4, IL-5 and IL-10, and with immunity against helminths, and asthma. Th0 cells produce a variety of cytokines and are thought to be the precursors of Th1 and Th2 cells, while Th3 cells are presumed to play a regulatory role characterized by the production of the broadly immunosuppressive cytokine TGF-β. Although even in the mouse these subsets do not account for all T cells, and in humans they appear to be even less well delineated, the system is useful as a framework. Th1 cytokines tend to encourage their own

production, and suppress that of Th2 cytokines, and vice versa. In several autoimmune animal models autoantigen-specific Th2 cells and their cytokines are associated with the resolution of disease (reviewed in Fathman *et al.*, 2000)

Research of immune modulation focuses on three main pathologies. The first is infectious disease: to prevent mortality and morbidity due to pathogenic organisms, we attempt to induce protective responses without exposure to fully virulent pathogens. This has been successful with monophasic diseases to which life-long immunity is produced, but much less so with intracellular parasites such as malaria. These organisms have complex life-cycles and immunity against one stage does not protect against other stages. Further, our success in eradicating infectious disease has been restricted to the well-known example of small-pox, and in implementing vaccines across entire populations, to the developed world.

The second pathology is cancer. In this case, the immune system is hindered by the lack or sparsity of foreign antigens. Only mutated proteins or developmental antigens not normally expressed in adults are exempt from self-tolerance. Compounding this, tumours may actively suppress immune responses: many have visible infiltrations of immobilized tumour-specific lymphocytes. Thus, the problem in treating cancer is overcoming poor immunogenicity and immune suppression.

The final area in which immune modulation is sought, and the focus of this discussion, is autoimmunity. In this situation, tolerance to self is lost, and the immune system attacks self tissue.

Intolerance to self alone is not enough to cause disease. Everyone harbours auto-reactive T cells, and transient autoimmunity is common following infection or injury. Furthermore, tolerance does not extend to 'ignored' antigens, which are not present in the thymus and are

restricted in the periphery to immunologically-privileged areas such as the eye. Experimental autoimmune animal models exist, with putative human counterparts, directed against such normally 'privileged' antigens. Experimental autoimmune encephalomyelitis (EAE), which bears similarities to multiple sclerosis (MS), can be induced in mice by injecting myelin proteins in complete Freund's adjuvant (CFA). Experimental autoimmune uveitis can also be induced in susceptible strains of mice with retinal antigens or their fragments. In both cases, the antigen is sequestered where naive T cells do not traffic, but activated T cells do.

1.2 Diabetes:

However, accidental exposure to sequestered antigen can't explain all instances of autoimmune disease, because autoimmunity does not occur exclusively when T cells encounter restricted antigens. The pancreas is not immunologically privileged, yet autoimmunity against the β cells of the islets of Langerhans causes one of the most common chronic disorders of children and young adults: insulin dependent diabetes mellitus (IDDM). The inability to predict inheritance of IDDM, and the low concordance rate among monozygotic twins, suggests that there are strong predisposing environmental factors, but several genetic features are also associated with susceptibility. 60% of attributable genetic risk is HLA class II: in Caucasians there is a positive association between diabetes and the DQ8 and DQ2 haplotypes, and the most common genotype among diabetics contains both. DQ6 and DQ7 are negatively associated, and the former confers a significant negative association even in the presence of DQ8. Polymorphisms in the MHC II genes alter both peptide-binding and interactions with TCR, but how this results in the activation or suppression of an autoimmune repertoire is unknown.

Other genes that have been linked or associated with IDDM are CTLA-4 (discussed below with Costimulation) and a variable nucleotide tandem repeat upstream of the insulin gene.

The role of the latter is unclear (reviewed in Bieg *et al.*, 1998).

Environmental factors, which remain contentious, have been suggested to include viral infections that damage the pancreas, and exposure to antigens, such as Coxsackie virus and bovine serum albumin, that mimic pancreatic proteins.

Many of the targets of autoimmunity in IDDM have been identified. Interestingly, the only islet-specific antigen is insulin. Others include heat shock protein and IA-2 phosphatase. The autoantigen studied in this Masters was glutamic acid decarboxylase 65 (GAD65).

1.3 GAD65:

Two isoforms of GAD exist, GAD65 and GAD67. However, it is the former which has been the focus of diabetes research. In the central nervous system, this enzyme synthesizes the inhibitory neurotransmitter γ amino butyric acid from glutamate. GAD65 is also expressed in the testes, the ovaries and the pancreas. Its functions in these locations are unknown. Although glucose stimulates pancreatic GAD activity in both humans and rats, it seems possible that GAD's function in the pancreas may have nothing to do with its status as an autoantigen (reviewed in Lernmark, 1996, Petersen, *et al.*, 1998).

1.4 Diabetes Models:

1.4a LCMV-transgenic Model:

A highly artificial but interesting model demonstrates the absence of any species-specific property protecting antigens from autoimmune attack. In this model, mice are transgenic (Tg) for the lymphocytic choriomeningitis virus (LCMV) glycoprotein, under the control of the rat insulin promoter. Because of the tissue specificity of this promoter, the glycoprotein is expressed only in the β cells. Although this is a viral protein, it is treated as self because it is expressed from early life, and the mice do not become diabetic.

However, if these mice are exposed to LCMV, the resulting activated CD8+ cytotoxic lymphocytes (CTL) also destroy the β cells, and the mice become hyperglycemic. Perhaps even more mysterious, if these autoreactive CTL are transferred to other similarly transgenic mice which have not been infected with LCMV, they rarely cause insulitis or hyperglycemia, although they retain the ability to home to the pancreas and manifest as a peri-insular infiltrate (von Herrath *et al.*, 1997).

1.4b BioBreeding Rat:

The BioBreeding (BB) rat is an older model of diabetes, that arose spontaneously at the BioBreeding Laboratories in Ottawa. Diabetes arises equally in both sexes, and the incidence approaches 100% if the rats are kept in specific pathogen-free conditions. In contrast to the congenic diabetes-resistant strain, diabetes-prone BB rats are lymphopenic. Specifically, they are deficient of CD8+, RT6+ T cells, which apparently play a regulatory role because their depletion from diabetes-resistant rats results in rapid induction of disease (reviewed in Bieg *et al.*, 1998).

1.4c NOD Mouse:

The model used in this Masters was the Non-obese Diabetic (NOD) mouse. The NOD mouse arose in Japan from an outbred strain. Interestingly, this strain was originally intended to be the control for another sister strain, which was being selected for impaired glucose tolerance. However, the roles were reversed when spontaneous hyperglycemia developed in the NOD strain. Diabetes occurs in a high proportion of females, and a much lower proportion of males, by 35 weeks of age. The reason for this sexual discrepancy is unknown. Actual incidence in a colony depends highly on environment, and varies widely between colonies. However, the disease always progresses through a well-documented series of stages.

Initially, there is peri-insulitis, when macrophages, dendritic cells, B and T cells gather

outside the pancreas, but do not actually invade the islets. This immune assault can begin as early as 2 to 3 weeks of age, and progresses to intra-insulitis when the pancreas itself is invaded, and ultimately culminates in the destruction of the β cells. The result is a model for human autoimmune insulin dependent diabetes mellitus (IDDM), also called Type I diabetes.

Like the BB rat, and human IDDM, the NOD mouse suffers hyperglycemia, ketoacidosis, polydipsia and weight loss. The NOD mouse also displays reactivity to pancreatic antigens, and early reactivity to GAD65 (reviewed in Bieg *et al.*, 1998). Tian *et al.*, 1997 first demonstrated that at 4 weeks of age there was a Th1 response to a single epitope of GAD and that by 12 weeks of age, this had spread along the GAD molecule and to other islet antigens (insulin B chain and heat shock protein). They also demonstrated that neonatal induction of Th2 responses to an islet antigen spread to other autoantigens and protected mice from disease. The protective nature of a Th2 response to islet antigens has been demonstrated by a number of groups, such as Gallichan *et al.*, 1999 who found that mice transgenic for IL-4 in the pancreas were protected from diabetes by islet autoAg-specific T cells that secreted IL-4 and IL-10. These cells counter-regulated continued underlying destructive autoreactivity, because neutralizing IL-4 and IL-10 restored susceptibility to diabetes.

There have been conflicting observations about the relative importance of CD4+ versus CD8+ T cells in the induction of disease. The generally-accepted conclusion is that CD4+ T cells are necessary, but the process is most efficient with both CD4+ and CD8+ cells together. This is supported by recent work by Ablamunits *et al.*, 1999. They found that CD4+ islet-associated cells from non-diabetic NOD mice could transfer disease to NOD.*scid* mice, while pure populations of CD8+ cells could not. However IDDM transfer was most effective when both CD4+ and CD8+ T cells were adoptively transferred.

Although diabetes in the NOD mouse is strongly associated with T cells, Serreze *et al.*, 1998 demonstrated that NOD mice lacking B cells are resistant to disease. T cell responses to a control antigen were normal in these mice, but reactivity against GAD was eliminated. Susceptibility to diabetes could be restored by irradiating these mice and reconstituting them with syngeneic bone marrow and B cells, but not by infusing them with Ig from diabetic NOD mice. This indicates that the B cells play some role other than autoAb production, and the authors suggest that they present islet antigens to prime the autoimmune response.

The advantages of the NOD mouse as a model of autoimmunity, and the reason it was used in this thesis, are the predictable progression of the disease, and the ease with which this can be monitored: glycemia can be measured with a glucose meter that reads colourimetric strips. The standard threshold beyond which an animal is considered diabetic is 11.0 mmol/l, and disease scores can also be assigned by grading individual islets in Hematoxylin and Eosin stained sections of paraffin-embedded tissue (reviewed in Leiter *et al.*, 1999).

1.5 Methods of Immune Modulation:

There are at least five methods to suppress or deviate an immune response. The most potent and least specific method is general immunosuppression. This is used following organ transplantation, when the alternative is graft rejection and death, and in severe autoimmunities such as systemic lupus erythematosus (SLE) which have poor survival rates. Unfortunately, increased susceptibility to infection and neoplasm is a serious side-effect.

Immunizing against infectious disease is the oldest and most often clinically applied strategy to induce a response. A vaccine can be an attenuated live organism, or a particular epitope, such as viral capsid protein. While epitope vaccination excludes reversion to pathogenicity, it gives a much narrower immunity.

Several vaccination strategies have also been applied to *suppressing* an immune response in the context of autoimmunity.

1.5a Vaccination Against Autoantigens:

As mentioned above, Th2 and/or Th3 cells specific to autoantigens are frequently associated with the amelioration of, or protection from, disease. Both bystander suppression and phenomena termed 'infectious tolerance' in transplantation models are produced (Tian *et al.*, 1997). In the first, autoreactive Th2 or Th3 cells home to the target organ and release anti-inflammatory cytokines, suppressing local effector T cells independent of shared specificity. In the second, Th2 immunity (and possibly other anti-inflammatory responses) can spread to adjacent epitopes and even related proteins, a protective version of epitope-spreading (Tian *et al.*, 1997)

However, elements of a Th2 response may not be enough: *in vitro*-derived Th2 cell lines frequently fail to protect recipients when co-transferred with autoreactive Th1 cells. Furthermore, although the NOD mouse is protected from disease when made transgenic for IL-4 or TGF-β in the pancreatic islets, these transgenic islets are swiftly destroyed when transplanted into wild-type diabetic mice (reviewed in Tian *et al.*, 1999). In addition, despite the finding that wild-type NOD mice treated with an IL-12 antagonist were protected by a Th2 response, the absence of IL-12 was not enough to protect NOD mice. IL-12-deficient NOD mice, despite impaired responses to self and exogenous antigen, progressed to diabetes and showed no augmented induction of Th2 cells (Trembleau *et al.*, 1999).

Despite this, a recent study has demonstrated protection from EAE when encephalitogenic Th1 cells were co-incubated with a Th2 clone specific for an altered peptide ligand (APL) of proteolipid protein (PLP). Proteolipid protein induces EAE in susceptible

strains of mice when injected with CFA, and an APL is a T cell epitope in which the T cell receptor contact residues have been substituted. The effect of co-incubation was abrogated by combinations of neutralizing Ab to IL-4, IL-10, IL-13 and TGF-β (Young *et al.*, 2000). However, these results don't entirely refute earlier data suggesting that co-transfer of specific Th1 and Th2 cells couldn't prevent transfer of disease, while early exposure to a biased Th2 response, *in vivo* or *in vitro*, could. Interestingly, while later transfer of Th2 clones generally failed to be protective, their induction by APL administration was ameliorative in established disease (reviewed in Seroogy *et al.*, 2000).

One of the early papers observing protective immunization in autoimmune models was done by Muir *et al.*, 1995. They found subcutaneous injections of B-chain insulin induced a protective response in very young NOD mice. In agreement with the theory of immune deviation, and contrary to expectations if protection arose from anergy or deletion, treated and control mice displayed comparable insulitis. In the treatment group, this insulitis was associated with much lower levels of IFN-γ. Co-transfer to irradiated NOD mice of spleen cells from immunized animals protected the recipients from diabetes.

Later work demonstrated similar protection in NOD mice from vaccination with GAD65 and 67, in EAE, from myelin basic protein (MBP), PLP or APLs, in collagen-induced arthritis (CIA), from collagen type II protein (CII), in uveitis (EAU), from inter-photoreceptor retinoid-binding protein (IRBP), and in thyroiditis, from thyroglobulin (reviewed in Liblau *et al.*, 1997).

In more recent work with a systemic disease (versus organ-specific ones, discussed above) Kaliyaperumal *et al.*, 1999 immunized (SWR × NZB)F_1 mice, a model of SLE, with autoepitopes from core histones. One of the characteristics of lupus in both humans and mice

is the production of autoAb against DNA and nucleosomes. The most commonly fatal complication is lupus nephritis. Thus, this group chose epitopes critical for nephritis-inducing T cells, a rational approach because although T cells are not directly involved in producing autoAb, they are indirectly involved through the activation of autoreactive B cells. Intravenous (i.v.) administration of histone epitopes was significantly protective, but interestingly not associated with a change in cytokine profile, suggesting anergy and not deviation of autoreactive T cells.

It should also be mentioned that treatment with autoantigens is not always protective. In an attempt to prevent diabetes in NOD mice, Geng *et al.*, 1998 made several lines that were transgenic for mutated GAD65 that differed in only two amino acids from native GAD65 (Geng *et al.*, 1998). These mutations abrogated enzymatic activity and were introduced because previous attempts to make NOD mice transgenic for GAD65 had failed, and the authors suspected that this was due to a lethal effect of unregulated GABA production. Despite the fact that intrathymic injection of GAD65 is protective in NOD mice, and that transgenic mice expressed the mutated GAD65 in the thymus, the line that most highly expressed the transgene suffered exacerbated diabetes (reviewed in Lernmark, 1996).

1.5b Oral Tolerance:

Another method to induce tolerance is oral administration of antigen. Fed antigen is processed by the gut-associated lymphoid tissue (GALT), which has the dual roles of protecting against pathogens and preventing inappropriate responses to harmless ingested proteins. Accordingly, orally-administered T-dependant soluble protein induces tolerance, while T-independent polysaccharides and particulate antigen do not. Low doses of tolerogenic antigen result in active suppression of reactive cells, by regulatory T cells secreting TGF-β, IL-4 and IL-

10, while high doses cause apoptosis (reviewed in Krause *et al.*, 2000).

Both peripheral and gut-resident APC present fed antigen to T cells, as within 6 hours of feeding, CD69, an early marker of activation, is upregulated on peripheral T cells. In the gut, there are a number of APC with access to antigen, including intestinal epithelial cells, B cells, macrophages, and dendritic cells (DC). However, a number of studies have suggested a central importance for DC (reviewed in Whiteacre *et al.*, 2000). Among these is work done by with Flt-3L, a hematopoietic growth factor that dramatically increases *in vivo* systemic levels of DC (reviewed in Shurin *et al.*, 1997). It was demonstrated that T cells from mice treated with Flt-3L prior to ovalbumin (OVA)-feeding proliferated significantly less upon *in vitro* restimulation after OVA immunization (Viney *et al.*, 1998)

In the context of autoimmunity, oral tolerance has been studied in a variety of animal models. Interestingly, not only does feeding of CII ameliorate or prevent CIA, but also adjuvant arthritis, pristane-induced arthritis and silicone-induced arthritis (reviewed in Whiteacre *et al.*, 2000). This suggests a mechanism involving active suppression, and clinical relevance, since the inducing antigen is not known in many human autoimmune diseases.

Other evidence for active suppression consequent to antigen feeding was found in the NOD mouse, where oral or nasal administration of insulin or GAD delayed or prevented overt diabetes, while increasing IL-4, IL-10 and TGF-β production, and decreasing IFN-γ production in the islets. Interestingly, the LCMV-Tg diabetes model is also protected from hyperglycemia following viral infection, if the mice are fed insulin.

Other Th1-associated, organ specific autoimmune models are amenable to treatment with orally or nasally administered autoantigens. These include EAE, experimental autoimmune thyroiditis (EAT), experimental autoimmune uveitis (EAU) and experimental autoimmune

neuritis (EAN) (reviewed in Whiteacre *et al.*, 2000, Krause *et al.*, 2000). Zhu *et al.*, 1998 found that nasal administration of a relevant antigen delayed EAN. More recently, Xu *et al.*, 2000 demonstrated that nasal co-administration of a MBP peptide and IL-4 delayed onset and ameliorated EAE in Lewis rats, even when given after disease induction.

However, when Bellman *et al.*, 1998 treated BB rats with oral insulin, they found that insulin alone transiently delayed disease, while insulin administered with a bacterial adjuvant significantly exacerbated disease. Treatment of NOD mice with fed insulin and the same bacterial adjuvant was protective (Hartmann *et al.*, 1997).

Interestingly, despite it's generally amelioratory effects on organ-specific autoimmunity, oral tolerance is not protective in the systemic autoimmune model SLE (reviewed in Whiteacre *et al.*, 2000, Krause *et al.*, 2000).

Clinical trials attempting to induce oral tolerance in MS were disappointing, but recent observations indicate homogenous antigen (MBP versus whole myelin) is more protective, and suggest new strategies (reviewed in Whiteacre *et al.*, 2000).

1.5c Vaccination Against Autoreactive T cells:

Vaccination with autoreactive T cells themselves can also control autoimmune disease. In contrast to models where disease is *induced* by adoptive transfer, in protective protocols the T cells are first attenuated. Gearon *et al.*, 1997 found that peritoneal injection of attenuated NOD T cells from diabetic mice, but not other strains, significantly prolonged diabetes-free survival of recipients. However, there was a sudden increase in diabetes incidence following the final injection indicating that vaccination only delayed disease in some mice. This supported existing evidence that tolerance requires a persistent expression of antigen. This may not be obvious in most experimental autoimmune models because, unlike the NOD mouse, they are induced and

many spontaneously resolve, as with EAE.

Clinical trials in MS patients suggest T cell vaccination (TCV) with MBP-reactive T cells protects from disease by clonally deleting MBP-reactive T cells (Hermans *et al.*, 2000, Correale *et al.*, 2000). Specifically, CD8+ anti-clonotypic T cells were induced against the vaccine clones.

This contrasts with data obtained by another group in a TCR-Tg mouse, in which the TCR is specific for myelin and TCV protected from EAE by non-deletional cytokine-mediated suppression (Matejuk *et al.*, 2000).

It should be noted that when Hermans *et al.* followed vaccinated patients over time, MBP-reactive T cells re-emerged in 2 to 5 years. These cells differed in clonal origin from the original vaccine clones. Their appearance coincided with clinical relapse in two patients. The original clone did not reappear, and additional vaccinations against the new clones abolished myelin reactivity, albeit with lowered efficiency. These new clones shared functional reactivities with the old ones and had not shifted towards a Th2 or Th3 phenotype. Thus short duration exposure to an indirect method of inducing tolerance (via the TCR instead of antigen) may not be a suitable treatment for chronic human autoimmunity.

1.5d Cytokine Administration and Interference with Costimulation:

Two other methods by which the immune system might be modulated are cytokine administration and interference with costimulation. Neither of these methods is antigen specific, with the advantage of broad effect, but the possibility of undesirable systemic effects.

In particular, the administration of cytokines in boluses can lead to transiently toxic levels. Furthermore, due to short half-lives, prolonged therapeutic levels are unattainable without continuous intravenous (i.v.) administration. Some groups have tried to lessen the systemic effects by inhibiting proinflammatory cytokines, instead of administering anti-inflammatory

ones. Baker *et al.*, 1994 treated chronic relapsing EAE (CREAE) with either anti-tumour necrosis factor (TNF) antibodies (Ab) or soluble fusion proteins of human TNF receptors and immunoglobulin (Ig). Both treatments significantly delayed the onset of disease, but were ultimately unable to prevent relapses. Interestingly, intracranial (i.c.) injections were effective at 100 fold lower doses than intraperitoneal (i.p) injections. This suggests that a lack of antigen specificity can be compensated for by administration local to the inflammation. The blood brain barrier is an additional problem in EAE, hindering the entry of systemic factors, but serves to keep factors already in the brain from diffusing into the general circulation. However, as relapses occurred in this and other models soon after cessation of treatment, it's clear this method suffers from transience. This was further illustrated in clinical trials with RA patients, in which treatment with an anti-TNF Ab greatly reduced inflammation, but disease returned 2 to 4 months later (reviewed in Chernajovsky *et al.*, 1995a)

Another way to lend some specificity to cytokine treatment is administration in conjunction with antigen. Zhu *et al.*, 1998 demonstrated this, as mentioned above, as did Inobe *et al.* before them. Both groups found that combining antigen and cytokine allowed lower doses of both. Inobe *et al.*, 1998 were also working with an oral tolerance model, feeding MBP to EAE-susceptible mice. They found that either i.p. or oral treatment with IL-4 encouraged the development of TGF-β-producing regulatory T cells.

More recently, Rothe *et al.*, 1999 experimented with treating prediabetic NOD mice with the novel cytokine IL-18. This cytokine shares structural similarities with IL-1, and the ability to drive differentiation towards a Th1 phenotype with IL-12. Despite this, treatment delayed diabetes and suppressed it altogether in 30% of the test group, and appeared to arrest the shift towards IFN-γ production seen in control mice. These results might concur with other findings

of neutralizing anti-cytokine Ab, discussed below with DNA vaccination. However, this group did not look for such anti-IL-18 Ab following their treatment.

The requirement of T cells for costimulation provides another avenue through which their responses can be controlled. In order to become activated, a T cell requires two signals from the antigen presenting cell (APC). The first signal is antigen-specific: the TCR interacts with a relevant MHC-peptide complex on the APC. The second signal, or costimulation, is not antigen-specific, and the predominant pathway is through CD28 on the T cell ligating either B7.1 or B7.2 on the APC. CD28 is constitutively expressed on the majority of resting human and mouse T cells. Following costimulation and activation, CTLA-4, another receptor for B7 molecules, is upregulated. Although B7.1 and B7.2 appear to be somewhat redundant functionally, they are distinct. Both interact with CTLA-4 with 20 to 50 fold higher affinity than they do CD28, but B7.2 is upregulated on activated APC 12 to 18 hours sooner than B7.1. B7.2 is also much more sensitive to upregulation by IL-4 or IFN-γ.

In contrast, CD28 and CTLA-4 appear to be functional opposites. CD28 engagement induces IL-2 production, upregulates a range of cytokines and expression of all chains of the IL-2 receptor, prevents T cell anergy and apoptosis and lowers the threshold of TCR engagement required for activation. Th1 clones are more sensitive to anergy in the absence of CD28 signaling than naive and memory T cells, or Th2 clones. As CTLA-4 has a much higher affinity for B7 than CD28, a soluble CTLA-4/immunoglobulin fusion protein (CTLA4-Ig) can be used to block CD28-B7 ligation. Interestingly, knocking out the CD28 gene or treating mice with CTLA4-Ig biases T cells towards the Th1 phenotype.

Initially, there was some controversy over CTLA-4, since some suggested that it also costimulates T cell activation. However, evidence now suggests that CTLA-4 ligation is

inhibitory. Blocking CTLA-4/B7 binding *in vitro* increases T cell proliferation and cytokine expression. In addition, murine CTLA-4 knockouts (KO) exhibit early autoimmune disease characterized by massive lymphoproliferation, autoimmunity and death at 4 to 5 weeks of age (reviewed in Bluestone *et al.*, 1999). CTLA-4 might act by inducing CD4+ T cells to produce TGF-β (Chen *et al.*, 1998b)

Cameron *et al.*, 1997 compared the effects of treating NOD mice with IL-4 or anti-CD28 monoclonal Ab (anti-CD28 mAb). They had previously demonstrated that NOD T cells are proliferatively hyporesponsive following TCR ligation from the time the mouse is 3 weeks old, and that anti-CD28 mAb treatment provided costimulation and restored proliferation. As mentioned above, lack of CD28 signaling tends to bias towards Th1, and Th2 function appears to be more compromised in the NOD mouse (Arreaza *et al.*, 1997). Recent work by Dahlen *et al.* suggest that the defect that is compensated for by anti-CD28 treatment is a reduced expression by NOD macrophages, DC and T cells of B7.2. This deficiency appears to interfere with the full activation of T cells, and the consequent upregulation of CTLA-4. The authors suggest that this could impair downregulation of immune responses (Dahlen *et al.*, 2000). This is similar to the suggestion of Trembleau *et al.* to explain the conflicting results between NOD mice treated with IL-12 antagonist, and those deficient in IL-12 production from birth: that the congenital lack of IL-12 interferes with the development of a regulatory mechanism (Trembleau *et al.*, 1999).

Cameron *et al.* found that anti-CD28 Ab treatment completely protected NOD mice from diabetes, but only if given early, while IL-4 was protective if given at up to 12 weeks of age. The mAb activated Th2 cells and augmented their production of IL-2 and IL-4. This experiment was probably more informative about innate defects in the NOD mouse during the inductive

phase of diabetes than it was about potential treatments. Interestingly, although anti-IL-4 treatment abrogated the protection mediated by anti-CD28 mAb, there were mechanistic distinctions between the two treatments. IL-4 prevented the migration of T cells into the pancreas. CD28 signaling did not, but induced a non-destructive, peri-insular infiltrate (reviewed in Cameron *et al.*, 1997).

These results conflict directly with a variety of other disease models. CD28-deficient mice are resistant to CIA. This could not be overcome by repeated immunizations with CII in CFA (Tada *et al.*, 1999). However, Tada *et al.* also found Th2 responses to be more impaired in the absence of CD28 signaling.

In agreement with the results of Tada *et al.*, Reynolds *et al.*, 2000 found CTLA4-Ig protected rats from experimental autoimmune glomerulonephritis (EAG), an animal model of Goodpasture's disease. This group also found evidence that B7.1 and B7.2 are not entirely redundant, as treatment with a mutant CTLA4-Ig that blocked only B7.1 was similarly protective.

A final note: Girvin *et al.*, 2000 studied CD28-deficient and anti-B7 treated NOD mice for resistance to EAE induction. They found disease severity was significantly reduced, in agreement with other models of EAE. Thus, amelioration of disease with increased CD28 signaling is specific to diabetes in NOD mice, and not all autoimmune reactions in NOD mice.

1.6 Gene Therapy:

Gene therapy is an alternate method to modulate the immune system that has the potential to overcome the limitations of protein administration.

Some proteins are very difficult and expensive to purify in useful quantities. In EAE/MS, the blood-brain barrier can prevent therapeutic molecules from gaining access to their site of action. This can be overcome, as in Baker *et al.*, with repeated i.c. injections, but these are

invasive and technically-demanding.

Gene therapy has the potential for local delivery, such as by the transfection of autoreactive T cells. Even in the case of local injection, only one or two treatments might be necessary. Gene therapy could encompass any of the treatment modalities using protein, in addition to others employing anti-sense RNA or oligonucleotides, with the additional advantages of persistent expression and no necessity for protein purification. Furthermore, inducible systems such as the TetOn/TetOff system could allow expression to be turned on or off, as can engineering a 'suicide gene' into transduced cells.

1.6a Viral Gene Transfer:

Methods of gene transfer can be divided into viral, and non-viral. Viral vectors are 'gutted' of all or most of their own genes, and the gap filled with the cDNA of choice. The strong viral promoters are left intact to drive expression. This has the effect of rendering the virus replication-defective so that it can't disseminate within the host, or spread to others. The advantages of viral methods over non-viral ones are high transfection rates, and rapid expression. Disadvantages, such as immunogenicity, depend upon the vector used.

1.6a.i Retroviruses:

Retroviruses are small RNA viruses that replicate via a DNA intermediate that integrates itself into the host genome. With the exception of lentiviruses, retroviruses can only infect dividing cells, being unable to traverse an intact nuclear membrane, and are prone to low expression levels. Integration allows stable transfection and expression, but can also cause insertional mutagenesis (reviewed in Robbins *et al.*, 1998).

Because of their requirement for dividing cells, retroviruses are best suited for *ex vivo* transduction, where cells can be induced to proliferate, and monitored for uncontrolled

proliferation prior to reimplantation. Much of the work done with retroviruses has been in arthritis models.

Normal rabbit synovium is resistant to retroviral infection because of low cell division rates. In contrast, inflamed synovium is a good target, because of hyperplastic fibroblast-like synoviocytes. However, Marakov *et al.*, 1995 found transduced cells only in infected explants, and not in joints that had been directly injected with retrovirus. Later attempts by Ghizzani *et al.*, 1997 were more successful. This group injected retroviruses encoding the reporter gene β galactosidase (β gal), and found that their vector was able to infect the mitotic syniovial cells in an inflamed joint, and highly express β gal.

Therapeutic effect was achieved with *ex vivo* protocols. Chernajovsky *et al.*, 1995b incubated spleen cells from CII-injected mice with collagen to induce specific T cells to proliferate, retrovirally-transfected them with soluble TNF receptor (sTNF R), and transferred them into severe combined immunodeficient (SCID) mice. SCID mice were chosen because their lack of T or B cells prevents them rejecting allogeneic cells. In contrast to untransfected spleen cells, these transferred disease to a much lesser degree. In later work, Chernajovsky *et al.*, 1997 achieved similar success with lymphocytes engineered to express TGF-β. Costa *et al.*, 2000. recently confirmed that, in contrast to polyclonal activation, exposure to cognate antigen resulted in transduction of only antigen-specific T cells with their retroviral vector pGCIRES. One potential problem with treating chronic autoimmunity with engineered T cells is the short life span of most such activated T cells.

In contrast, synovial cells such as Marakov *et al.*, 1996 transfected *ex vivo* with the secreted interleukin 1 receptor antagonist (sIL-1ra) gene can persist indefinitely. sIL-1ra was chosen because it is a naturally occurring antagonist of IL-1. In bacterial cell wall (BCW)-induced

arthritis, treatment ameliorated induced recurrence of arthritis, joint erosion and pannus formation. Similarly, when *ex vivo* transduced RA synovial fibroblasts (RA-SF) were co-transferred into SCID mice with normal human cartilage, the sIL-1ra protected normal cartilage from chondrocyte-mediated cartilage degradation (Muller-Ladner *et al.*, 1997). Synoviocytes transfected with IFN-β protected mice from CIA, and ameliorated established arthritis (Triantaphyllopoulos *et al.*, 1998).

The other disease model studied for the benefits of retroviral gene therapy is EAE. Shaw *et al.*, 1997 demonstrated that retrovirally-transduced encephalitogenic T cells could deliver IL-4 locally to the CNS and protect mice from disease induction and the same group later demonstrated that disease could be exacerbated by local delivery of TNF, refuting earlier observations that TNF KO mice suffered protracted EAE (Dal Canto *et al.*, 1999). Chen *et al.*, 1998a similarly achieved a protective effect with MBP-reactive T cells engineered to express TGF-β. Transduced T cells specific for an irrelevant antigen were not protective.

Croxford *et al.*, 2000 achieved impressive results by injecting a fibroblast line retrovirally-engineered to produce sTNFR into the CNS of mice. The cells and their product were still detectable three months later. Furthermore, they significantly ameliorated both acute and CREAE. Because the fibroblast line was immortalized using temperature sensitive SV40 large T antigen, inactivated following implantation, the fibroblast line was not tumorigenic *in vivo*. This therapy was effective at protein levels 1000-fold lower than protein injection.

Other than cytokines and their inhibitors, retroviral vectors have also been used to deliver antigen-immunoglobulin fusion proteins to induce tolerance. Kang *et al.*, 1999 found that the recipients of bone-marrow or B cells transduced with an epitope from the λ virus became unresponsive to that epitope. This effect was enhanced if the epitope was fused with the murine

IgG heavy chain. In the context of autoimmunity, similarly transduced B cells were able to protect mice from EAU. The B cells carried the gene for IRBP, and were protective if administered 10 days before, at the time of, or, most significantly, 7 days after uveitogenic treatment (Agarwal *et al.*, 2000).

1.6a.ii Adenoviruses:

In contrast to retroviruses, adenoviruses can infect non-dividing cells, and are efficient transducers *in vivo*. They are DNA viruses, and thus pose no risk of insertional mutagenesis. Their tropism can be altered by modifying their receptors, and gene expression can be controlled by cell-specific promoters. The main difficulty with adenoviruses is their immunogenicity, which can cause infected cells to be killed by the immune system, and toxicity. Removal of viral genes to mitigate this frequently lowers gene expression or shortens its duration dramatically, and may not address the entire problem. It was recently shown that adenoviruses can trigger an inflammatory response just by binding their receptors on synoviocytes (reviewed in Pap *et al.*, 2000) Furthermore, neutralizing Ab responses can interfere with subsequent, and perhaps even initial, treatments: Chirmule *et al.*, 1999 found almost all subjects already had humoral and/or cellular immunity against adenoviruses. Furthermore, completely gutted viruses can be difficult to separate from the helper viruses required for their propagation (reviewed in Robbins *et al.*, 1998).

Despite their being less appropriate for treating autoimmunity than the more innocuous and persistent retrovirus, a considerable amount of work has been done with adenoviruses in this area. As with retroviruses, the majority was with models of arthritis.

Both activated T cells and hyperplastic synoviocytes express high levels of Fas, a molecule that induces a death pathway following ligation by Fas-ligand (FasL). Unfortunately,

FasL levels are low in the arthritic joint. Zhang *et al.*, 1997 took advantage of the high expression of Fas by injecting an adenovirus encoding FasL into arthritic murine joints three days *after* disease onset. They found increased apoptosis in treated joints, and ameliorated disease in their CIA model.

In keeping with dichotomous results mentioned above with regards to TNF blockade and CIA, Quattrocchi *et al.*, 1999 found that i.v. injection of adenovirus encoding sTNFR caused early improvement in their CIA model, followed by significant deterioration. Although it has been reported that the liver is the main site of transduction following i.v. administration, and that expression here of reporter genes such as firefly luciferase (lux) and β gal was all but undetectable by day 14 post-injection (Kurata *et al.*, 1999), Quattrocchi *et al.* were still detecting measurable quantities of TNFR in serum up to 21 days, so the sudden disappearance of the gene product does not explain a worsening of disease at day 15 to day 18. It is possible that a down regulatory mechanism was interfered with.

Woods *et al.* 1999, 2000 found that conditioned media from RA synovial tissue explants or fibroblasts infected with adenoviruses encoding IL-4 or IL-13 contained decreased levels of a number of inflammatory mediators. While applying a similar strategy to CIA, Lubberts *et al.*, 1999 found that adenovirus itself could exacerbate arthritis and this was aggravated by overexpression of IL-4. Paradoxically, chondrocyte death and cartilage erosion was reduced in adenovirus/IL-4 treated joints, though this was in agreement with other results of treating rat adjuvant arthritis with IL-4 (reviewed in Pan *et al.*, 1999).

IL-10 is known both for anti- and inflammatory properties, which may explain ambivalent results achieved with its use in arthritis models (reviewed Martino *et al.*, 2000a) Because of this, two groups chose to insert the Epstein Barr viral homologue of IL-10 (vIL-10)

into their adenoviral vector. This viral protein has only the anti-inflammatory properties of its mammalian counterpart. In the CIA model, Apparailly *et al.*, 1998 found benefit following systemic administration, while Whalen *et al.*, 1999 saw amelioration of disease only with intra-articular injection. Oddly, although Whalen *et al.* measured no vIL-10 in the serum, they found that uninjected paws were also protected in adenovirus-vIL-10 treated mice for up to 70 days. Although they demonstrated complete protection, the titre of virus necessary to achieve this was very close to their own definition of toxic. The dosee required by the first group to achieve their systemic effects were even higher. In agreement with Lubberts *et al.*, Apparailly *et al.* found that adenovirus alone caused inflammation in the joint.

Although the early inducer phase of EAE was vulnerable to interference with costimulation, as discussed above, the later effector phases seemed refractory to treatment with CTLA4-Ig. Croxford *et al.*, 1998a overcame this with direct i.c. injection of adenoviral vector encoding CTLA4-Ig, and found it more effective than a single injection of CTLA-Ig protein. Takiguchi *et al.*, 2000 chose a similar adenovirus/CTLA4-Ig vector to treat lupus nephritis in susceptible mice. They had previously demonstrated amelioration with protein injection, but wanted to overcome the need for repeated injections. A single i.v injection of their vector produced serum CTLA4-Ig levels that were still high at 20 weeks, significantly reduced nephritis and greatly prolonged survival.

1.6a.iii Herpes Simplex Virus:

Herpes simplex virus (HSV) has some of the advantages of adenovirus. It can infect both dividing and non-dividing cells, has a broad tropism and is a DNA virus, so will not integrate into the host genome. Furthermore, the HSV genome can carry very large inserts, and thus multiple genes, and is highly infectious. Unfortunately, HSV infection is quite toxic and

immunogenic. This has been addressed by deleting many viral genes, but as with adenovirus, purification of vector from helper viruses is technically challenging and titres are low. An even more serious barrier to the use of HSV in chronic autoimmune disease is the tendency for transgene expression to shut down within a week of infection (reviewed in Robbins *et al.*, 1998).

Despite this, Oligino *et al.*, 1999 treated an IL-1β overexpression-induced model of arthritis with intra-articular injection of HSV encoding sIL-1ra. Previous attempts with an adenoviral vector demonstrated benefit, but produced only transient expression, presumably due to anti-viral responses. Although they demonstrated biological relevance in protecting injected joints, transgene expression was only detectable for seven days. Given the natural trophism of HSV for neurons, EAE is perhaps a better target for HSV vectors. Martino *et al.*, 2000b administered HSV/IL-4 i.c. and found no evidence of vector toxicity, and detectable IL-4 for up to 4 weeks. Disease was ameliorated even if the vector was administered as late as at the first signs of EAE.

1.6a.iv Adeno-associated Virus:

Adeno-associated virus (AAV) is a single-stranded DNA virus that, even in its wild-type state, is replication-defective without a helper virus such as adenovirus or HSV. The wild-type virus integrates harmlessly into a specific locus on human chromosome 19. However, 'gutted' AAV vectors lose this specificity and either integrate randomly, or remain episomal. In the latter case, gene expression occurs only after second strand synthesis. Neurons and myocytes seem most efficient at providing this rate-limiting step, and long-term stable expression results. Unfortunately, AAV's genome can accommodate only small inserts (reviewed in Robbins *et al.*, 1998). Guy *et al.*, 1998 treated optic neuritis associated with EAE with AAV carrying the catalase gene. Catalase was chosen because it is a free radical scavenger and reactive oxygen species

mediate demyelination and blood-brain barrier breakdown in EAE, and because treatment with catalase protein is limited by its half-life and inability to cross the intact blood-brain barrier. The result was protection from demyelination of optic nerve head swelling, and transduction in the optic nerve persisted for at least 2 years after injection.

Recently, Pan *et al.*, 1999 demonstrated another interesting property of AAV vectors. When injected into acutely arthritic rat joints, AAV transgene expression directly correlated with disease severity. Furthermore, expression diminished to basal levels when inflammation subsided, and could be re-induced to high levels by repeated insult and renewed inflammation. This is first demonstration of disease-state regulated transgene expression.

1.6b Non-viral Gene Transfer:

Although viral vectors are more efficient than non-viral methods of gene transfer, they carry the risk of immunogenicity or oncogenesis. Also, their application may be limited by low titres or short term expression. Because of this, research continues into non-viral methods which, despite their own disadvantages, are easier and cheaper to produce and carry a much lower risk of inducing inflammation. This has obvious advantages in the context of treating chronic autoimmune disorders. The components that compose an expression plasmid are illustrated in Figure1.1: a bacterial origin of replication that allows the plasmid to be amplified in bacteria, an antibiotic resistance gene to allow transformed bacteria

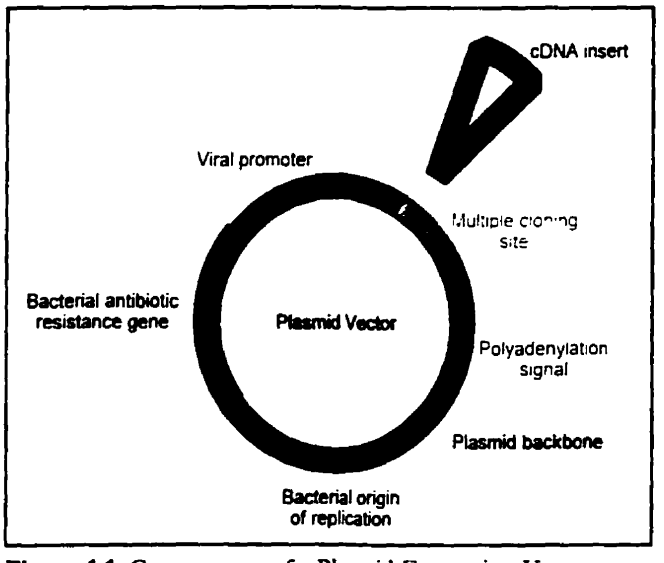


Figure 1.1: Components of a Plasmid Expression Vector

to be positively selected, a viral promoter to drive expression in mammalian cells, a multiple cloning site with a variety of enzyme restriction sites to facilitate cloning into the vector, and a polyadenylation signal to stabilize the resulting mRNA transcript. The remainder of the plasmid is called the 'backbone'.

There are a variety of methods for introducing such a construct into mammalian cells.

1.6b.i Liposomes and DNA Conjugates:

Liposomes are bilayered membranes formed of amphipathic molecules such as phospholipids surrounding an aqueous core, and can be either anionic and cationic. They are non-pathogenic, cheap and easy to produce, and offer a great deal of flexibility in size (reviewed in Gurunathan *et al.*, 2000). Unfortunately, they are inefficient. Mathisen *et al.*, 1997 circumvented this problem by priming *in vivo* with PLP and then transfecting peptide-reactive T cells *in vitro*. These T cells, carrying the IL-10 gene under the control of the IL-2 promoter, were able to inhibit EAE induction and ameliorate ongoing disease. These results must be interpreted with care because the transfected clone already had a Th2 phenotype. However, this work displayed a clear advantage over similar work done by Shaw *et al.* with retrovirally-transduced MBP-specific T cell hybridomas: the T cells used by Mathisen *et al.* did not overgrow and kill their hosts.

Croxford *et al.*, 1998b opted to inject their DNA-liposome complex local to the inflammation in their EAE model. Although they found intramuscular (i.m.) and i.c. injection of naked DNA and local injection of recombinant cytokines to be largely ineffective, DNA-liposome complexes encoding IFN-β, IL-4, TGF-β and sTNFR significantly ameliorated disease. Triantaphyllopoulos *et al.*, 1998 also injected DNA-liposome complexes i.c. in EAE. Using cationic liposomes, they delivered the genes encoding IFNβ and sTNFR, and found a significant

reduction in the clinical score of acute disease.

In contrast to Croxford *et al.*'s findings that IL-10 had no therapeutic value in EAE, Batteux *et al.*, 1999a demonstrated that it could be curative in EAT. This group also found naked DNA injection to be inefficient. However, DNA and a combination of liposomes and poly-lysine inhibited the disease process even if it was already in progress.

A rabbit model of osteoarthritis also proved amenable to treatment with liposome/DNA complexes encoding sIL-1ra, despite this tissue having proven refractory to other non-viral methods. Fernandes *et al.*, 1999 found that intra-articular injection resulted in less severe lesions and no increase in synovial inflammation, in contrast to previous results with retroviruses, despite the fact that xenogeneic sIL-1ra was used.

1.6b.ii Gene Gun:

Also called 'biolistics', in this method microscopic gold particles are coated with DNA and shot into the tissue with a helium gun. The coated particles are cheaply and easily made, and transfect both dividing and quiescent cells. This method is very efficient at transfecting skin, and requires 100 fold less DNA than naked plasmid injection. Unfortunately, epidermal cells turn over rapidly and transfecting more persistent myocytes requires that muscle be surgically exposed. This technique is better suited to treating skin cancers than chronic autoimmune disease. Nevertheless, some work has been done in this area.

Cameron *et al.*, 2000 compared expression of an IL-4 gene in the context of either a conventional DNA vector, or one with an EBV episomal maintenance replicon. The latter gave higher expression, and more significant protection from disease. However expression was still short-lived.

1.6b.iii Other DNA Conjugates:

Other DNA conjugates exist, also designed to protect DNA and increase transfection efficiency, such as cochleates and microparticle encapsulation. As the majority of work with these is in the context of vaccinating against infectious organisms, they will not be discussed.

1.7 Naked DNA Vaccines:

Direct injection of naked DNA is another method of introducing genes into somatic cells, and has the advantages of gene therapy while avoiding many disadvantages of the above methods. Although naked plasmids lack the transfection efficiency of viruses, they are much easier to synthesize. They can take advantage of the persistence of muscle cells as protein factories without requiring surgical removal of the skin, and are easily produced. The absence of viral proteins lessens the risk of immune responses either neutralizing the product or destroying the transfected cell. Chun *et al.*, 1999a found that although viral expression vectors produced superior short-term results, expression of naked plasmid DNA was much more persistent and not subject to neutralizing immunity. I.m. injection will be the main focus of this discussion, but plasmid DNA has also been given through the mucosa (Chun *et al.*, 1999b) and injected into the thyroid (Batteux *et al.*, 1999b) in the treatment of autoimmune models.

The technique is poorly suited to rapidly dividing cell populations because the plasmid remains episomal and does not replicate.. The main disadvantages of naked DNA gene transfer are low transfection rates and expression levels. In addition, although gene expression can theoretically persist indefinitely, in practice it abates over time.

The field essentially began when Wolff *et al.*, 1990 demonstrated that reporter genes could be expressed from muscle following naked DNA or RNA injection, and was still detectable four months after injection. *In vitro*, rapidly dividing cells lost expression more quickly than more slowly replicating cells of the same type. *In vivo*, the plasmid was neither replicated nor

integrated even after a year (Wolff *et al.*, 1992). Others went on to demonstrate humoral and cellular immunological responses in mice, and protection from lethal challenge with influenza in mice and chickens (reviewed in Guranathan *et al.*, 2000). Raz *et al.*, 1993 then showed how systemic responses could be manipulated using genes encoding cytokines. I.m. injection of plasmids carrying the genes for TGF-β, IL-2 or IL-4 could alter the response to a foreign protein given at another site. Furthermore, IL-2 could also increase, and TGF-β decrease, anti-chromatin Ab in lupus-prone mice.

1.7a DNA Gene Transfer and Autoimmune Models:

In a later paper, Raz *et al.*, 1995 went on to demonstrate that survival in the MRL/lpr/lpr model of SLE was significantly enhanced following monthly i.m injections of plasmid encoding TGF-β. In agreement with their earlier findings, anti-chromatin Ab were decreased. In addition, renal function deteriorated less, corresponding with lower clinical scores of glomerulae and less fibrosis and interstitial inflammation in the renal tissue. This is of note, because TGF-β is known to be pro-fibrotic. As previously observed, IL-2 worsened disease.

As mentioned above with the results of Rothe *et al.*, regulatory anti-chemokine Ab can be induced against inflammatory chemokines. Youssef *et al.*, 1998 treated mice i.m. with cDNA encoding the chemokines MIP-1α and MCP-1, which are highly expressed in the CNS at the onset of EAE. They found that EAE could not be induced in these mice even 2 months after DNA injection. This is interesting since, at the onset of disease, the blood-brain barrier is intact and should still present an impediment to Ab molecules. They found that vaccination with chemokine cDNA augmented existing anti-chemokine reactivity in EAE susceptible rats.

Also interesting, injecting the cDNA for MIP-1β, another chemokine highly expressed during disease induction, aggravated disease. Inducing Ab to the chemokine RANTES,

expression of which is associated with resolution of EAE, was not protective although repeated administrations of blank plasmid was. Also in the rat EAE model, Lobell *et al.*, 1998 injected plasmid encoding an immunodominant peptide of MBP fused with Ig and demonstrated protection from disease induction. T cells from vaccinated mice demonstrated the same proliferative response to MBP as T cells from control mice, and did not produce more IL-4 or IL-10, but secreted significantly less IFN-γ. DNA vaccination with the same peptide alone (not as an Ig fusion protein) was not protective. Ruiz *et al.*, 1999 were also successful with a plasmid encoding minigenes for either a PLP peptide or an APL. In agreement with Lobell *et al.*, they found lower IFN-γ levels and suggested that it was due to anergy of reactive T cells.

In the LCMV-Tg model of diabetes, Coon *et al.*, 1999 found that although DNA vaccination with the insulin B chain was protective, DNA vaccination with the viral protein for which the mice were Tg was not. This suggests that not all autoantigens can be used to elicit a regulatory response.

Administration of plasmids encoding cytokines or their inhibitors has also been protective in autoimmune models. Nitta *et al.*, 1998 found that although i.m. injection of a plasmid encoding IL-10 had no significant effect on insulitis in 13 week old NOD mice, the incidence of diabetes was markedly reduced. This suggests an immune deviation mechanism, with a protective infiltrate induced in place of a destructive one. Prud'homme and colleagues have also protected NOD mice from diabetes or delayed disease onset with i.m. injection of plasmids encoding TGF-β, IL-4/Ig fusion proteins and a soluble IFN-γ receptor Ig fusion protein (sIFN-γR/Ig) (Prud'homme *et al.*, 1999, Piccirillo *et al.*, 1998, Chang *et al.*, 1999).

The same vector encoding sIFN-γR/Ig was also protective in the MRL/lpr/lpr model of SLE. Lawson *et al.*, 2000 found that sIFN-γR/Ig significantly decreased serum IFN-γ levels.

Expression could be considerably improved by electroporation of the injection site. Survival was also increased by electroporation, with 90% of treated mice still alive at 11 months of age, while control mice were dead by 7 ½ months. Most impressively, with electroporation the plasmid was highly protective even when given to mice already exhibiting advanced disease. Significant in the context of lupus, which is characterized by anti-DNA Ab, naked DNA injection reduced these Ab. Induction of anti-DNA Ab had been a concern in DNA vaccination.

DNA vaccination has also been applied to TCR-based vaccination strategies, in a rat model of autoimmune myocarditis (EAC). Matsumoto *et al.*, 2000 determined the TCR β-chain spectrotype of autoreactive T cells by analyzing myocardial infiltrates in diseased rats. They then treated other rats with DNA encoding these spectrotypes a week prior to disease induction. They found two i.m vaccinations with DNA encoding a combination of two spectrotypes was as effective as 21 consecutive days of treatment with anti-TCR Ab against the same spectrotypes.

Wildbaum *et al.*, 2000 attempted to clarify the role of FasL in EAE. The role of Fas and FasL in autoimmunity is somewhat ambiguous, because the lack of Fas is associated with SLE, but resistance to EAE. Furthermore, NOD mice transgenic for FasL in the islets have accentuated disease, while Fas negative NOD mice are resistant to spontaneous and adoptively-transferred diabetes. Thus, Wildbaum *et al.* immunized rats with plasmids encoding FasL, and induced EAE two months later. Treated mice displayed markedly reduced disease, that was not due to a direct effect on T cells. The titre of anti-FasL Ab remained at a baseline in the two months following DNA vaccination, but began to rise after disease induction. *In vitro* these Ab reduced TNFα production by macrophages, but not IFN-γ production or proliferation of MBP-specific spleen cells. *In vivo*, these Ab conferred protection against disease induction when transferred into other rats and protected CNS cells from apoptotic death. Interestingly, when

these Ab were transferred into animals with late-stage disease, they delayed recovery from acute disease. The authors suggest that protecting macrophage and T cells from apoptosis at this stage may prolong disease.

1.7b Immunobiology of DNA Gene Transfer:

A number of factors affect the transfection and expression efficiency of plasmid DNA, most obviously the viral promoter: Simian virus 40 (SV40) promoter is 40 times weaker than the Cytomegalovirus immediate early (CMV I/E) promoter. Gene expression is also dose-dependent up to a point, beyond which larger doses inhibit expression (C.Piccirillo, personal communication). Although some researchers noted greater efficiency following administration of myotoxins and muscle healing, subsequent work did not support this, nor did findings by Wolff *et al.* that repeated injections and prolonged muscle contraction, both of which cause muscle damage, inhibited transfection (reviewed in Gurunathan *et al.*,2000, Dowty *et al.*, 1994). Finally, transfection rates and expression can be increased by electroporating the injected tissue, which causes transient gaps in cell membranes and allows DNA entry.

Obviously, the immunological response is directly related to protein expression, and thus indirectly related to factors which impinge upon this. However, there are other factors which affect the immunological outcome.

The age of the treated animal effects both expression and response. Danko *et al.*, 1997 found peak expression in mice treated at 2 weeks of age, and much lower expression in mice treated at 8 weeks. Ichino *et al.*, 1999 found that a plasmid vaccine that normally induced protective immunity against malaria caused tolerance when administered to neonatal mice. Larger doses were most tolerogenic, and the tolerant state persisted for more than a year.

A final factor controlling the immunological outcome of naked plasmid injection is one

intrinsic to the plasmid itself, residing predominantly in the backbone. Early observations, such as those by Raz *et al.*, 1996, suggested that DNA vaccination predisposed towards a Th1 response. They found intradermal (i.d.) immunization with β-gal protein induced T cells secreting predominantly IL-4 and IL-5, and Ab isotypes associated with a Th2 response. In contrast, plasmids encoding β-gal administered by the same route induced primarily IFN-γ secretion and IgG2a production. Later work showed that this effect was not due to bacterial contaminants, and the strength of response following DNA vaccination with foreign protein was in part due to adjuvant activity of the plasmid itself (Leclerc *et al.*, 1997).

At least some of this immunostimulation arose from unmethylated cytidine-phosphate-guanosine (CpG) motifs. These are 20 fold more common in bacterial than mammalian DNA, and in the latter they are heavily methylated and thus ignored. Unmethylated, in the context of plasmids or synthetic oligodinucleotides, they activate B cells to proliferate or secrete antibody and induce DC and macrophages to secrete cytokines such as IL-6, IL-12, TNF-α, IFN-γ and IFN-α that can activate natural killer (NK) cells (reviewed in Gurunathan *et al.*, 2000, Krieg, 2000). They can also induce DC to mature and stimulate CTL responses to plasmid-encoded antigens through a Th-independent mechanism (Vabulas *et al.*, 2000).

Although CpG motifs are safe and effective adjuvants in the context of DNA vaccination against infectious organisms, they are not conducive to the induction of tolerance. Artificial methylation would prevent immune responses, but methylated genes are transcriptionally-repressed in mammalian cells (Meehan *et al.*, 1992) Yew *et al.*,1999 reduced immune response significantly by eliminating just under half of the CpG dinucleotides in their reporter plasmid. Although they were administering a cationic lipid/DNA complex, they had previously shown the critical variable for inflammation to be the plasmid. They found their

altered plasmid induced significantly less IL-6, IL-12 and IFN-γ following i.v or intranasal (i.n.) administration, while producing comparable levels of reporter protein. As it is unlikely that all CpGs can be eliminated from a plasmid, as some will be intrinsic to obligatory elements, it is even more interesting that Yew *et al.*, 2000 found that chloroquine and quinacrine could inhibit the cytokine response when given in conjunction with wild-type plasmid.

Although we understand some of the effects of various elements of the plasmid itself on the immune reaction, there remains the issue of the mechanism by which an immune response occurs.

There are three predominant theories as to how antigen is presented consequent to DNA gene transfer to the muscle. As myocytes are known to produce the greatest proportion of gene product, they might be functioning as APCs. Muscles cells express MHC I, for which the normal source of peptide is endogenous protein. As gene transfer, like viral infection, depends upon host cells for protein production, transfected muscle cells almost certainly present plasmid-encoded genes in MHC I. However, myocytes do not express costimulatory molecules. Early work by Ulmer *et al.*, 1996 did not exclude transfected muscle cells functioning as APC. They found that implantation of myoblasts transfected *in vitro* with influenza nucleoprotein (NP) provided CTL and humoral responses comparable to those following i.m. NP cDNA injection and viral infection. However, injection of parental myoblasts into F_1 mice produced CTL responses that were restricted to both parental haplotypes. This suggested that antigen from the myocytes was being taken up and presented by host APCs, but demonstrated that muscle cell transfection was sufficient to elicit a CTL response.

However, Torres *et al.*, 1997 showed that ablation of muscle transfected by DNA injection within 10 minutes of inoculation had no effect on the duration or magnitude of the

resulting Ab response, and that this held for both secreted, membrane-bound and intracellular gene products. This suggested that while muscles cells could serve as a source of antigen, as in the experiments by Ulmer *et al.*, their presence was not necessary. Although it is unlikely that the muscle cells produced and released much protein in 10 minutes, antigen could still be introduced by 1) transfection of mobile cells which migrated from the site of injection and avoided ablation 2) plasmid leakage from the muscle and travel to other parts of the body. In fact, DNA is detectable by PCR in a number of locations following injection (C. Piccirillo, personal communication).

Klinman *et al.*, 1998 found that both migratory and non-migratory cells were involved in the immune response to gene gun-mediated DNA vaccination. In agreement with earlier work by Torres *et al.*, they found that immediate removal of the injection site abrogated the production of both Ab, cytokines and immunologic memory. They suggest that conflicting observations that removal of the injection site had no effect on the response were due to plasmid traveling through the blood or lymph and transfecting cells at other sites. Klinman *et al.* found that the longer the site of vaccination was left intact, up to two weeks, the stronger the resulting humoral response. Immunologic memory developed with faster kinetics and was produced in recipients of vaccinated skin grafts excised within 12 hours of vaccination, but not recipients of grafts excised more than a day after vaccination. This suggests that immunologic memory was elicited by DC that migrated to draining lymph nodes 5 to 12 hours after vaccination. Since the magnitude of the primary response continued to augment if the site was left intact for 2 weeks, Klinman *et al.* further suggest that this is dependent on the protein produced by non-migratory cells.

Corr *et al.*, 1996 finally demonstrated that myocytes were not acting as APC in a series

of elegant experiments with bone marrow chimeras. They irradiated F_1 mice, and reconstituted them with one strain of parental bone marrow, then injected them i.m. with a DNA vaccine encoding NP. Thus, the myocytes were H-$2^{b\times d}$, the APCs were H-2^b or H-2^d, and the T cell could recognize both parental haplotypes because they had been educated by the radio-resistant cells of the thymus. They found that CTL responses were restricted to the MHC I haplotype expressed by the APC. Similar results were obtained by Doe *et al.*, 1996, when they engrafted SCID mice with spleen cells from either H-2^b or H-2^d mice, and injected DNA encoding either HSV gB or human immunodeficiency virus (HIV) glycoprotein 120 (gp120). The first of these antigens is restricted to H-2^b, the second to H-2^d. Vaccinated SCID mice responded only to the antigen which their *engrafted* APC could present.

Also working in a parent to F_1 bone chimera, Iwasaki *et al.*, 1999 found immune responses following i.m. and gene gun vaccination to be dependent on professional APC. Furthermore, co-expression of genes encoding B7.2 or IL-12 did not convert transfected myocytes into APCs. Later work by Agadjanyan *et al.*, 1999 conflicts with this. They found that co-linear expression of B7.2 with their antigen in bone marrow chimeras, in which reconstituted APC did not express MHC I, allowed myocytes to activate CTLs.

Most recently, Haddad *et al.*, 2000 explored the mechanism behind the effectiveness of granulocyte macrophage colony stimulating factor (GM-CSF) as an adjuvant in DNA vaccination. Immunohistochemistry (IHC) of injected muscles showed an infiltration by immature DC following i.m. injection of plasmid encoding GM-CSF (pGM-CSF). The finding that injecting antigen cDNA and pGM-CSF at separate sites abrogated the adjuvant activity, and that increased immunogenicity was associated with CD11c+ cells supported the hypothesis that GM-CSF enhances immune responses to DNA vaccination by attracting DC to the site of

injection.

So, it appears to be APCs and not myocytes which present antigen to T cells following DNA vaccination. Although there are a few different kinds of cells that can act as APCs, Casares *et al.*, 1997 found that dendritic cells, but not B cells, isolated from mice primed in vivo by DNA vaccination could activate a specific T cell hybridomas *in vitro* in the absence of additional peptide. This and work by Manickan *et al.*, 1997 suggests DC, and not B cells or macrophages, are the critical cells for antigen presentation.

1.8 Dendritic cells:

Dendritic cells arise from CD34+ hematopoietic precursors in the bone marrow. Two separate lineages, myeloid and lymphoid, are primarily distinguished by the former's expression of CD11b, and the latter's expression of DEC 205 and CD8α. Although they are thought to differ functionally, it has been difficult to reliably assign duties uniquely to one or the other. However, in the thymus, lymphoid DC seem to be involved in central tolerance (reviewed in Bell *et al.*, 1999).

In their precursor state, DC migrate through the blood and lymphatics. They are primarily CD11c+, but express only low levels of MHC II and costimulatory molecules at their surface. Robert *et al.*, 1999 recently demonstrated that DC in the blood express a ligand that binds P- and E-selectin. These selectins are expressed at low levels on normal capillary endothelial cells, but upregulated during inflammation. Robert *et al.* observed that DC will roll along uninflamed endothelium *in vivo*, and were recruited to sites of inflammation. This suggests that they are primed to exit the blood at sites of inflammation, where they would be likely to acquire foreign antigen. DC also express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5 and CXCR1 (reviewed in Sallusto *et al.*, 1999). Upon entering the tissue and taking

up residence, they become immature DCs and are very efficient at taking up antigen but poor at presenting it. Interestingly, DEC 205 is an endocytic receptor that delivers antigens to endosomal compartments. In the skin, immature DC are called Langerhans cells (LC), in the heart, kidney, lung and dermis, they are interstitial DC. Once they encounter and take up antigen, they are triggered to traffic to a lymph node via the afferent lymphatics, as "veiled DC". The most immunogenic antigens are those best able to drive this maturation but a range of other stimuli, such as LPS, IL-1 and TNF, can also induce migration from the tissue. Interesting in the context of DNA immunization, CpG motifs induce the migration of LC from the skin, and cause downregulation of E-cadherin and α6 integrin and upregulation of ICAM-1 (Ban *et al.*, 2000). LC are also known to upregulate collagen IV collagenase, which would aid in their movement out of the tissue. DC at this stage also express the chemokine receptors CCR7, which binds secondary lymphoid tissue chemokine (SLC) (reviewed in Sallusto *et al.*, 1999). During this time they undergo maturation and a number of phenotypic and functional changes that include upregulation of MHC II, CD83, adhesion molecules, CD40, B7.1 and B7.2, and a loss of their proficiency at endocytosis, coinciding with a downregulation of Fc receptor expression. In the secondary lymphoid tissue, they are mature interdigitating DCs (IDC) and potent APCs, able to activate memory and naive T cells, and elicit both helper and cytotoxic effector functions. They secrete a number of new chemokines, such as thymus and activation regulated chemokine (TARC), macrophage-derived chemokine (MDC) and EBV-induced molecule 1 ligand chemokine (ELC), which allow them to attract other mature DC and naive or recently activated T cells (reviewed in Sallusto *et al.*, 1999). They can secrete a range of cytokines that includes IL-12, IL-4 and IFN-γ. There is also some evidence that lymphoid DC here are responsible for presenting self-antigen and maintaining peripheral tolerance (reviewed in Bell *et al.*, 1999).

1.8a Direct Transfection versus Cross-priming:

Thus DC are potent professional antigen presenting cells, and evidence (Corr *et al.*, 1996, Doe *et al.*, 1996, Iwasaki *et al.*, 1997, Casares *et al.*, 1997, Torres *et al.*, 1997) suggests that they are the critical APCs following naked DNA injection. The next logical question is: how do they acquire antigen following DNA injection?

The two predominant theories are 1) by direct transfection, 2) by cross-priming, or acquisition of antigen produced by transfected non-APC. The first depends heavily on the potency of DC as APC, because Casares *et al.*, 1997 estimated that only 0.4% of DC were transfected by naked gene transfer.

1.8a.i Direct Transfection:

Condon *et al.*, 1996 demonstrated that DC are transfected following biolistic transfection with green fluorescent protein (GFP), a spontaneously fluorescent molecule. They found cells in the draining lymph nodes with DC morphology that fluoresced and contained gold particles. Painting the skin with rhodamine, a red dye, confirmed that these GFP-positive DC originated in transfected skin. Boczkowski *et al.*, 1996 found that DC pulsed with RNA were more effective at stimulating primary CTL responses *in vitro* than protein-pulsed ones. Furthermore, DC pulsed with total RNA from tumour cells significantly protected mice from lung metastasis in the stringent B16/F10.9 tumour model. In a model where tumour cells are engineered to express β-gal, DC transfected *in vitro* to express β-gal conferred significant protection against lethal challenge, and suppressed established tumours (Song *et al.*, 1997). Others also found improved immune responses following administration of transfected DC. Mice treated with either naked DNA or transfected DC survived challenge with HSV, where those treated with transfected macrophages did not. At a higher titre of challenge, even mice vaccinated with naked DNA

succumbed, but many of those treated with transfected DC survived (Manickan *et al.*, 1997).

Inoue *et al.*, 1999 suggested a mechanism for this, demonstrating more IFN-γ production by splenocytes in response to specific antigen following treatment with transfected versus untransfected DC. This suggests the induction of a Th1 response, a correlate with protection against both neoplasm and viral infection. However, Inoue *et al.* transfected their DC with adenovirus, and viral infection might itself influence DC. Later work attempted to further elucidate the mechanisms of DC transfection. Sparwasser *et al.*, 1998 demonstrated that bacterial DNA and CpG motifs were sufficient to trigger DC maturation and activation. In response to co-culture with synthetic CpG-containing oligodinucleotides (ODN), both mature and immature DC were induced to up regulate MHC II, CD40 and B7.2, and secrete large amounts of IL-12, IL-6, and TNF-α. Akbari *et al.*, 1999 found that when they immunized their mice by scarification of the ear with cDNA for an intracellular antigen, C5, there was a specific T cell response, but no antibody response. Co-culturing transfected keratinocytes and DC produced no evidence of antigen transfer to DC, unless the keratinocytes were irradiated. Antigen expression was no longer detectable in DC after 2 weeks, but persisted in keratinocytes for another 10 weeks. However, naive transgenic T cells specific for C5 did not become activated if transferred to mice 20 days after vaccination, indicating that there was no source of stimulating antigen by this point. These results are in agreement with those of Porgador *et al.*, 1998 which found that, although gene gun particle bombardment alone induced a large influx of DC into the draining lymph nodes, it was the few directly transfected DC that were responsible for the resulting CTL activation. When cells were isolated from the draining lymph node of vaccinated mice, depletion of DC co-transfected to express β-gal and human CD4 reduced the response of a β-gal specific CD8+ T cell clone, as measured by IFN-γ production, by 60-70%.

1.8a.ii Cross-priming:

However, there is also a great deal of support for the importance of cross-priming in antigen-specific responses following naked gene transfer. In experiments with F_1 into SCID bone marrow chimeras, Doe *et al.*, 1996 found anti-viral responses were elicited even if spleen cells were not transferred until weeks after DNA immunization. Given that the maximum half-life of DNA in the blood is estimated to be 30 minutes, this indicates that gene expression by APC is not obligatory for a response. Furthermore, Boyle *et al.*, 1997 examined the responses to i.m. injection of DNA encoding soluble, membrane-bound, or cytoplasmic forms of OVA (sOVA, mOVA, cOVA). The soluble form induced IgG levels, particularly IgG1, that were up to 100 fold that induced by cOVA. This suggested that the immune system had more limited access to the cell-associated forms of OVA, and that direct transfection of DC did not play a major role. This is further supported by the observation that cOVA was also weakest at priming CTL responses. Other observations suggest that directly transfected DC may play a greater role in i.d. than i.m. transfection. The former forms the majority of cases that supported direct transfection as an important mechanism. Boyle *et al.* found that mice immunized i.d. with cOVA had equivalent CTL responses to sOVA and mOVA, though continued to show poor Ig reactions. It should also be noted that gene gun administration and i.m. injection of naked DNA were already known to differ fundamentally, as the former tends to induce Th2 responses, while the latter generally biases responses towards Th1 (reviewed in Gurunathan *et al.*, 2000). Torres *et al.*, 1997 had also demonstrated that while ablation of injected muscle within 10 minutes did not affect responses, ablation of gene-gun transfected skin within this time period did eliminate responses.

As already implied, DC are well equipped to take up exogenous antigen. Norbury *et al.*,

1997 demonstrated that DC are able to phagocytose exogenous antigen, and present it in MHC I molecules. In contrast to MHC II, which is expressed only on APC, MHC I is expressed on all nucleated cells and is usually reserved for presentation of endogenously produced peptide. Professional APC, however, have a non-conventional pathway for loading MHC I with exogenous peptide because, for example, cytotoxic CD8+ T cell responses must be induced against viral infections even if the virus does not infect APC. Norbury *et al.* observed constitutive membrane ruffling and macropinocytosis in their bone-marrow derived DC *in vitro*. These DC were more efficient at presenting phagocytosed OVA than macrophages. Treatment with PMA, which enhances pinocytosis, also enhanced antigen presentation and such treated DC were able to prime a CTL response when transferred into mice.

The results of Boyle *et al.* show that DC acquire antigen if it is secreted. However, Albert *et al.*, 1998 found that human DC can also phagocytose cells that had apoptosed following viral infection. Immature DC were more efficient than mature DC, but considerably less so than macrophages. However, only immature DC were proficient at cross-presenting viral antigens. Not unexpectedly, this property was enhanced following culture with a maturation signal. Macrophages did not cross-present phagocytosed antigen from apoptotic cells, failing to induce virus-specific CTL. Rovere *et al.*, 1998 found that a murine DC line could present to both OVA-specific MHC I and MHC II-restricted T cell hybridomas, following phagocytosis of non-secreted OVA-expressing apoptotic cells Co-culture with live or necrotic OVA-expressing cells or supernatant from apoptotic cells did not result in cross-presentation. Thus DC react to apoptotic cells in a specific way, and soluble antigen released from necrotic cells does not play a role. High numbers of apoptotic cells, intended to simulate an insult such as viral infection or ischemia, induced DC to produce high levels of IL-1β and TNF-α and to upregulate maturation

markers such as MHC II, B7.2 and CD40.

Santambrogio *et al.*, 1999 demonstrated another novel way by which DC might acquire exogenous antigen for presentation. They found that immature DC expressed high levels of empty MHC II, in contrast to splenic and peritoneal B cells and macrophages, and these levels were highest in immature DC. The levels of empty MHC II decreased sharply following incubation with a peptide restricted to the MHC II haplotype, even in the absence of endocytosis. Furthermore, these peptide-loaded DC were able to activate specific T cells with significantly greater efficiency than B cells. Although the extracellular milieu *in vivo* is probably not a significant source of preprocessed peptide, MHC II has been shown to bind larger protein fragments and might be able to bind denatured or partially degraded proteins at sites of inflammation. Internalization of these MHC-protein complexes shuttling protein to endocytic compartments and back to the surface for presentation has be shown in DC.

As the ability of DC to take up exogenous antigen by a number of methods had been demonstrated, others went on to study the role of cross-priming following DNA injection. Observations by Corr *et al.*, 1999 elaborate upon earlier findings by Klinman *et al.*, demonstrating that despite the potency of directly transfected DC as APCs, the magnitude of the response is dependent on protein produced by transfected non-APCs. They found that DNA vaccination with a plasmid encoding β-gal under the control of a monocyte-specific promoter produced weak humoral and cellular responses, indicating that DC alone did not express sufficient protein to produce the strong immune response associated with DNA vaccination. In contrast, a DNA vaccine against HTLV-1 in which viral envelope protein expression was under the control of the muscle-specific desmin promoter produced a better neutralizing response then the same vaccine under the control of a viral promoter(Armand *et al.*, 2000). Most interesting, when they

injected a suppressible plasmid encoding OVA into RAG -/- mice they found that splenocytes from suppressed RAG mice could not elicit a response when transferred into anti-OVA TCR Tg recipients, while unsuppressed splenocytes could. This indicates that only in mice where DC could be loaded with protein produced by transfected non-lymphocytes did DC acquire enough antigen to elicit a strong response in recipient mice. This strongly supports the importance of cross-priming, as do further observations that mice vaccinated i.d. in the distal tail while plasmid expression was suppressed were not able to mount a primed CTL response if the tail was amputated prior to cessation of suppression. On the other hand, initially suppressed control mice whose tails were not amputated could later mount a response. This further supports the role of protein produced by non-lymphocytes in the magnitude of the resulting response.

1.9 Rationale and Objectives:

The primary objective of this thesis was to alter disease course in an autoimmune model by injection of naked plasmid encoding an autoantigen. The NOD model of diabetes was chosen because of its accessibility and the ease with which disease progress can be monitored through blood glucose levels. GAD65 was chosen as the autoantigen because of it importance early in disease: autoreactivity appears to begin with this molecule and spread to others (Tian *et al.*, 1997). Further, reactivity to GAD65 is shared by NOD mice and at least a proportion of human type I diabetics (reviewed in Bieg *et al.*, 1998), and the GAD65 protein is very difficult to purify in useful quantities. In partial fulfillment of a Honours program, the cDNA of GAD65 was subcloned into the efficient mammalian expression vector pVR1255. Another plasmid was constructed, also using pVR1255, in which the IL-4 signal sequence was added to GAD65. This was done because soluble antigens appear to be more tolerogenic than cell-associated ones. For the Masters, these constructs were injected into NOD mice, alone or in combination with

plasmids encoding cytokines, in order to assess their effects on disease progression.

The secondary objective of this thesis was to examine events following DNA vaccination, as compared to other methods of *in vivo* priming. In this case, ovalbumin (OVA) was chosen because of its availability in both protein and cDNA form. As a secreted protein, it also had a signal sequence, though not the same one as was added to GAD65. The methods to be compared were 1) base of tail injection with OVA protein in CFA, 2) i.m. DNA vaccination in the tibialis anterior and quadriceps muscles, and 3) subcutaneous injection of OVA-pulsed DC. The first was chosen as the positive control, as strong immune responses are generated by this method. The latter was chosen because of the importance of DC following DNA vaccination, and the observation that, while responses are dependent on DC presenting protein, the magnitude of the response is dependent on protein produced by other cells and acquired by the DC. Pulsing bone marrow-derived DC should overcome this limitation, and comparison with the response following DNA vaccination would highlight any aspects due to transfected muscle cells acting as something other than protein factories.

2. Materials and Methods:

2.1 Mice:

Female NOD mice were purchased from the Jackson Laboratories (Bar Harbor, ME) or Taconic Farms (Germantown, NY), at 4 -5 weeks of age. Female BALB/c mice were purchased from Charles River (Wilmington, MA) at 6 weeks of age. All animals were housed under specific pathogen-free conditions. After the age of 10 weeks, the blood sugars of NOD mice were monitored weekly with an Accu-check III glucometer (Boehringer-Mannheim, Germany). Diabetes was defined as two consecutive readings ≥11.0 mmol/l.

2.2 PCR:

Murine GAD65 in the Bluescript KS+ vector was a generous gift of H. McDevitt (Stanford University, Stanford, CA). The gene was amplified by PCR with a PTC 100 programmable thermal controller (MJ Research Inc. Watertown, MA) and cloned Pfu DNA polymerase (Stratagene, LaJolla, CA) using the following primers: sense 5'-ATATAT**GCGGC CGC**ATGGCGTCTCCTGGCTCCGG and antisense *5'*-CGAGGCG**GAATTC**TTACAAA TCTTGTCCGAGGC. Not I and EcoR I enzyme restriction sites were also added (boldface).

For the secreted form of GAD65, the desired fragment was digested from the Bluescript plasmid with Xho I and Hind III restriction enzymes. The resulting 1.8 kb fragment was isolated on a 1% agarose gel and centrifuged in an Ultra-free DA tube (Millipore, Boston, MA) at 11000 rpm. The DNA was extracted by phenol/chloroform and precipitated overnight at -20°C in 5M NaCl and isopropanol. The precipitate was pelleted by centrifuging for 20 min. at 14000 rpm, washing with 70% EtOH and centrifuging for 20 min. at 14000 rpm. This pellet was dried with a Speedvac Concentrator (model RH40-11, Savant Instruments Inc. Farmingdale, NY), and resuspended in sterile water. A 1% agarose gel confirmed the presence of a 1.8 kb fragment.

PCR then added a 10 bp overlap with the IL-4 signal sequence (IL-4 SS) to the 5' end of the fragment, and a EcoR I restriction site to the 3' end (boldface), with the following primers: sense 5'-TACCAGGAGCATGGCGTCTCCTGGCTCCGGC, antisense primer same as above. The remainder of the IL-4 SS was added by a second PCR reaction, with the following primers: sense 5'-**ATGGGTCTCAACCCCCAGCTAGTTGTCATCCTGCTCTTCTTT CTCGAATG**TACCAGGAGCATGGCGTCTC, antisense primer as above. A **Not I site** was then added 5' of the IL-4 SS by a third PCR reaction with the following primers: sense 5'-ATATAT**GCGGCCGC**ATGGGTCTCAACCCCCAGCT; antisense primer as above.

OVA in pCDNA3 was a kind gift of Dr. Ciro Piccirillo (NIH, Bethesda, MD). The insert was amplified by PCR using the following primers: sense 5'- CGAGAA*GCGGCCGC***GG TACC**ATGGGCTCCATCGGTGCAGCA, and anti-sense 5'-ACTTCA*GAATTC***ACTAG T**TTAAGGGGAAACACATCTGCC. Kpn I and Spe I sites (boldface), and Not I and EcoR I sites (italics) were also added to the OVA gene with these primers.

2.3 Plasmid Preparation and Transformation:

Inserts were subcloned into the pVR1255 expression plasmid (kind gift of VICAL Inc. San Diego, CA), previously modified to contain a multiple cloning site and from which the Lux gene had been removed. The plasmid was dephosphorylated by calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) at 37°C for 30 min. The phosphatase was then heat-inactivated. The inserts were ligated into the plasmid by T4 ligase. Negative controls contained plasmid without ligase, or with ligase and without insert.

Log phase JM109 *E. coli* (ATCC, N° 53323, Manassas, VA) were made competent by $CaCl_2$ and incubated for 24 hrs at 4°C. Cells were then transformed by addition of plasmid followed by incubation at 4°C for 30 min. and heat shocking for 3 min. at 42°C. Transformants

were grown overnight at 37°C on LB-Kanamycin plates. Negative controls were transformed with the negative controls of the ligation reactions. Later transformations were done with supercompetant DH5α *E. coli* (Life Technologies, Gaitherburg, MD), as per the manufacturer's instructions. Minipreps, from colonies grown overnight at 37°C in 1.5 ml of LB broth with 50 μg/ml of Kanamycin, confirmed successful transformations. DNA was extracted by centrifuging the bacteria at 14000 rpm for 1 min., resuspending the pellet in a 2.5% 1M Tris-HCl pH 8, 2% 0.5M EDTA, 0.9% glucose solution with 3 mg/ml of hen egg lysozyme, followed by a 5 min. incubation at room temp. Fresh 0.2% NaOH and 1% SDS solution was added and the result incubated on ice for 5 min. The DNA was extracted by phenol/chloroform and precipitated with absolute ethanol, incubated for 2 min. at room temp. and centrifuged for 5 min. at 14000 rpm, then washed a final time with 70% ethanol before being dried by Speedvac. Later minipreps were done with a QIAprep spin miniprep kit (Qiagen, Santa Clarita, CA), as per the manufacturer's instructions. After resuspension in sterile water, an aliquot was digested with Not I and EcoR I, and run on a 1% agarose gel to visualize a band of the appropriate size.

2.4 DNA Isolation and Transient Transfection:

Bacterial cultures were grown in the appropriate volume of LB-Kan overnight with shaking at 37°C. Plasmid DNA was isolated and purified by alkaline lysis using QIAfilter Endofree kits (Qiagen, Santa Clarita, CA), as per the manufacturer's instructions. The DNA concentration and purity was quantified by photospectrometry (Ultraspec II, LKB Biochrom, Cambridge, Eng.) at 260 and 280 nm, and the presence of supercoiled plasmid ascertained on a 1% agarose gel.

COS-7 cells (ATCC, Manassas, VA) were grown to 50% confluence and transfected by LIPOFECTAMINE as per the manufacturer's instructions (Life Technologies, Gaithersburg, MD).

7.5 $\times 10^4$ cells were plated per well in six well plates, in 2 ml of medium (DMEM, high glucose with L-glutamate, with 10% heat-inactivated fetal bovine serum) and grown to 75% confluence at 37°C in a CO_2 incubator. At 24, 48 and 72 hours after transfection, medium and cells were collected for Western blot.

Samples for Western blot were stored at -20°C until assayed. Samples of both cell lysates and supernatant were run on a discontinuous acrylamide gel in a SDS PAGE apparatus. Expression of the GAD65 protein was tested by Western blot as by Laemelli, using the supernatant of the GAD6 hybridomas as the primary antibody (Developmental Studies Hybridoma Bank, University of Iowa, IA). GAD6 recognizes residues 475-585/529-585 (19, 35, 36, 37). The secondary antibody was a goat anti-mouse Ig HRP conjugate (Sigma, Oakville, ON). Western blots to detect OVA expression used mouse anti-OVA, clone OVA-14 (Cederlane Laboratories, Hornby, ON) and goat anti-mouse HRP conjugate secondary antibody (Sigma, Oakville, ON). Westerns were developed using ECLPlus substrate (Amersham Pharmacia, Piscataway, NJ). All restriction enzymes, ligase, culture media and LIPOFECTAMINE were purchased from Gibco BRL (Gaithersburg, MD).

2.5 DNA and DC Injection:

Anesthetized mice received injections in both tibialis anterior muscles with 50 μg of either pVR1255 containing an insert or blank (with no gene inserted into the MCS) in 50 μl of sterile endotoxin-tested PBS, using an insulin syringe fitted with a plastic collar to limit muscle penetration. Mice receiving combinations of vectors encoding antigens and cytokines (pVR1255-IL4 or pVR1255-IL12) received two injections of 100 μg of 1:1 mixtures of plasmid in 50 μl of PBS. Mice treated with multiple injections of pVR1255-sGAD65 or pVR1255-blank were anesthetized every three weeks and injected as described.

DC were cultured as described below, washed thoroughly in sterile, endotoxin-free PBS and irradiated at 2000 rad. They were resuspended in sterile PBS and injected subcutaneously in all four paws and the sub-scapular region, with an insulin syringe. 20 μl was injected at each site, and a total of 1 million DC were injected per mouse.

For the experiments with pVR1255-OVA, plasmids were injected into the quadriceps and T.A. muscles. Muscles were then electroporated with eight pulses at 200 volts per cm^2 with a BTX Electro Square Porator (BTX Electro-square Porator (BTX, San Diego, CA)

2.6 Histology and Immunohistochemistry:

Upon diagnosis of diabetes, NOD mice were killed and their pancreas and tibialis anterior muscles removed and fixed overnight in 10% buffered formalin before being embedded in paraffin. For the first experiment, three H&E stained sections per pancreas from mice remaining non-diabetic at the termination of the experiment were scored by two independent blinded observers, using established categories of islet infiltration (0 = no evidence of infiltrate, 1 = perinsular infiltrate less than a quarter of islet circumference, 2 = peri-insular infiltrate greater than a quarter of islet circumference, 3 = intra-islet infiltration, 4 = destruction of islet architecture). For IHC, paraffin-embedded T.A. muscles were sectioned and mounted on silanized slides (Surgipath, Winnipeg, MB). These sections were deparaffinized in xylene and rehydrated through gradations of ethanol to water, then treated with 3% H_2O_2 to quench endogenous peroxidase activity, and washed twice in PBS. Sections were also incubated 30 min. with an avidin D blocking kit, and 1% goat serum to block intrinsic biotin and lower background. All incubations were done at room temperature in a humidity chamber.

Slides were then drained and incubated 45 min. with the primary antibody: either a Rabbit isotype control Ab or Rabbit anti-GAD65 (G5038, Sigma, Oakville, ON) in PBS with

1% goat serum and biotin. Slides were washed twice, and incubated with 3 drops of normal blocking serum stock and 1 drop of biotinylated goat anti-rabbit antibody stock in 10 ml of PBS for half an hour. After the slides had been washed in PBS for another 30 min., the Vectastain Elite ABC reagent was overlayed on the slides and allowing to sit for another half an hour. Finally, colour was developed with Vectastain Nova Red. This reaction was stopped by washing with water. Slides were counter-stained with Gilles Hemotoxylin, dehydrated through gradations of ethanol to xylene and mounted with glass coverslips and Vectamount. All reagents for IHC were purchased from Vector Laboratories (Burlingame, CA) unless otherwise specified.

2.7 Culture of Dendritic Cells:

Bone marrow-derived DC were cultured as in Inaba *et al.*, 1992 with some modifications. The back legs of BALB/c mice were removed and skinned, and placed immediately in complete Iscove's media (1% penicillin-streptomycin, 50 μM 2-ME, 10 heat-inactivated FBS) on ice. In a cell culture hood, femurs and tibias were striped of muscle. Epiphysis were cut off, and the bones were placed into fresh complete Iscove's media. A 10 ml syringe with a 26G ½ needle was used to flush the marrow into a conical tube. The cell suspension was then drawn up and pushed out of the syringe to disperse cell clumps. The cell suspension was then run through a Falcon 2350 70 μm nylon filter to remove pieces of bone into a fresh 50 ml conical tube (Becton Dickinson, Franklin Lakes, NJ). The cells were then washed, pelleted and resuspended in 5 ml per mouse for counting and transferred to a 14 ml conical tube. Cells were centrifuged again, and resuspended in the conditioned media of the following hybridomas: RA3 6B2 (anti-B220), GK1.5 (anti-CD4), 53.6.72 (anti-CD8) and M5/114 (anti-MHC II). This suspension was incubated on ice for half and hour, with gentle rocking. Following this, cells were pelleted again, and resuspended in 50 μl of Sheep anti-Rat IgG-coated Dynabeads per 10^7 cells and 3 to 4 ml

of media (Dynal, Oslo, Norway). This mixture was again incubated for half and hour on ice with gentle rocking. T cells, B cells and mature APCs were then depleted by putting the tubes into Dynal magnetic particle separator (Dynal, Oslo, Norway). The cells were then washed, pelleted and resuspended in 5 ml/mouse to count. Cells were plated in Falcon 3003 tissue culture plate at a density of 1 to 1.5 × 10^7 per plate with GM-CSF and 10% heat-inactivated FBS (Becton Dickinson, Franklin Lakes, NJ). On Days 2 and 3 of culture, plates were swirled gently, and 2/3 of the culture media was removed and replaced with fresh media containing the appropriate additives. On Day 6, plates were swirled more vigorously and washed to remove non- and semi-adherent cells. These were counted and replated in 25% conditioned media at a density of 5 × 10^6 per Falcon 3003 plate. DC could be matured by overnight incubation with 10μg/ml of LPS or OVA (Sigma, Oakville, ON). By Day 7, macrophages in the culture became firmly adherent, and DC were separated from these by rinsing the plate. Cells were collected, counted and pelleted for either FACs analysis or injection.

2.8 Flow Cytometry:

For FACs analysis of maturity and contaminating cell populations, DC were resuspended at 10^6/100 μl of Dulbecco's PBS with 2.5% heat-inactivated FBS. This buffer was used throughout. 100 μl of cells and 100 μl of buffer were pelleted in Nunc round bottomed 96 well plates (Life Technologies, Gaithersburg, MD). Pellets were washed twice more with 200 μl of buffer, then incubated for 15 minutes on ice with 50μl blocking buffer (Dulbecco's PBS with 10% heat-inactivated FBS). Cells were then washed once with buffer, and incubated covered on ice for 15 minutes with the primary layer. In most cases, the antibodies used were directly conjugated to fluorochromes. The following direct conjugates were used to stain for contaminating cell populations: anti-Gr-1-FITC (granulocyte marker), anti-CD3e-FITC (T cell

marker) , anti-B220-FITC, anti-IgM-FITC (B cell markers), anti-F4/80-FITC (macrophage marker). All but the anti-F4/80 were from BD PharMingen (Oakville, ON). Anti-F4/80 was from Serotec (Raleigh, NC). DC were identified by anti-CD11c-PE, anti-MHC II-FITC and anti-CD11b-biotin, all from PharMingen. Isotype controls were anti-HamsterIg-FITC, anti-RatIgG2a-FITC, anti-RatIgG2b-FITC, anti-RatIgG2b-biotin, anti-GoatIg-FITC (Cederlane, Hornby, ON) and anti-HamsterIg-PE (from Serotec, Raleigh, NC). The secondary layer used to detect CD11b was a streptavidin-Spectral Red conjugate from Southern Biotechnologies (Birmingham, AL).

Following the primary incubation, cells were washed three times. Secondary layers were performed as for primary. The pellets were then washed a further three times.

Fully stained pellets were resuspended in 200 μl of buffer and transferred to FACs tubes (Becton Dickinson, Franklin Lakes, NJ) containing 100 μl of buffer. Samples were run on FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). DC were identified by staining for both CD11c and CD11b, and maturity was assessed by brightness of MHC II staining. Cells staining double positive for F4/80 and CD11c were considered to be DC.

Dot blots, histograms and population statistics were calculated using WinMDI software.

2.9 Comparative Immunizations:

Mice were primed *in vivo* with either OVA protein, OVA cDNA or OVA-pulsed DC, and. Specifically, protein-primed mice were given 100 μg OVA in sterile PBS and Complete Freund's adjuvant (CFA) by base of tail injection, using a glass Luer-Loc syringe and a 25 G needle. Control mice received an emulsion of PBS and CFA. Mice primed with OVA cDNA received 50 μg per muscle, as described above, in both T.A. and quadriceps muscles. Following injection, the muscles were electroporated. Control mice received equal volumes of blank vector

prior to electroporation. Mice primed with pulsed DC were injected subcutaneously with 20 μl of DC suspended in sterile PBS in all four feet and subscapularly, with a total of 10^6 cells per mice. Prior to injection, DC were assessed by flow cytometry and found to be mature. Control mice received unmatured DC (See Graph 3.13). Mice were sacrificed by cervical dislocation and T cells from the draining lymph nodes were collected at Days 3, 8 and 14 following treatment. LN were placed in complete RPMI media (1% penicillin-streptomycin, 50 μM 2-ME and 10% heat-inactivated FBS) on ice, and crushed with a glass pestle until mostly fibrous tissue remained. Cell clumps were dispersed by drawing up and expelling the suspension from a 10 ml syringe. The suspension was then expelled through a steel mesh and centrifuged to pellet cells. Cells were washed once with media, then resuspended in 2 ml of media to count. Cells were centrifuged again, and finally resuspended at no greater density than 300×10^6 per ml of 1X wash buffer, provided with T cell purification columns purchased from R&D Systems (Minneapolis, MN). CD3+ T cells were then isolated by negative selection using these columns, as per the manufacturer's instructions, and cultured in RPMI 1640 or Iscove's media, with irradiated spleen cells. Single cells suspensions of spleen cells were prepared as LN cells, but resuspended at 5×10^6 cells/ml and irradiated at 2000 rad. T cell proliferation was assessed by ^{3}H-thymidine incorporation (ICN Radiochemical, Costa Mesa CA). T cell cultures were pulsed with 1 μCi/well of ^{3}H-thymidine in 10 μl of media on Day 4 of culture. 12 hours later, DNA was collected with a Brandel cell harvester (Rockville, MD), and FP-24 Whatman 934AH filters (Xymotech Biosystems, Mont Royal, QC) were placed in scintillation vials with Universol scintillation cocktail for aqueous samples (Research Products Division, Costa Mesa, CA). Counts were collected using a 1211 RackBeta Liquid Scintillation counter (Fischer Scientific, Nepeon, ON). Statistical significance between groups of NOD mice was assessed with Fischer's exact test.

3. Results:

Graph 3.1: NOD Mice Treated with Native GAD65 or Secreted GAD65

In the first experiment, NOD mice were injected in T.A. muscles with 50 μg/50 μl of plasmid DNA in sterile, endotoxin-free PBS. There were 10 mice in each group, and they received either native GAD65 (NGAD65), GAD65 to which the IL-4 signal sequence had been added (SGAD65) or blank plasmid (vector). Initially both forms of GAD65 appeared to delay diabetes in relation to the vector, however the NGAD65 treatment group soon closely resembled the control group. In contrast, the SGAD65-treated group differed significantly from the control group between weeks 16 and 18 ($p<0.05$). After this, they rapidly approached the incidence of diabetes found in the control group. Interestingly, NGAD65 appeared to increase the incidence of disease in relation to both the control and SGAD65-treated groups, though this was not statistically significant.

Plate 3.1: Immunohistochemistry of Rat and Mouse Pancreases

In order to test antibodies to be used for IHC of DNA-injected muscles, formalin-fixed paraffin-embedded sections of rat pancreas were stained with GAD1, GAD6 and a polyclonal rabbit anti-GAD65 antibody from Sigma (see Methods and Materials). The rat pancreas was chosen because it is known to strongly express GAD65. The rabbit polyclonal antibody was found to give the best signal with the lowest background, and was used for all subsequent staining. Negative controls were stained with a non-specific rabbit isotype control antibody, and demonstrated low background. A formalin-fixed, paraffin-embedded pancreas from a BALB/c mouse was also stained using the same protocol and also found to be positive for GAD65, despite published reports that GAD65 expression is very low in the mouse pancreas. In the mouse pancreas, low levels of background were detected in the lumens of ducts.

Plate 3.2: Immunohistochemistry of Injected Muscles

Mice that became diabetic were sacrificed, and their TA muscles removed, fixed in formalin and embedded in paraffin. IHC performed on the sections made from these muscles reveal low levels of background staining in control muscles. However, the diffuse signal is easily distinguishable from the positive signal evident in both NGAD65 and SGAD65 injected mice, which is clearly localized within individual muscle cells. Interestingly, NGAD65-treated muscles tended to be more intensely positive than SGAD65-treated muscles.

Plate 3.3: Immunohistochemistry of Long-term Expression of GAD65

Muscles were also taken from mice remaining euglycemic at the termination of the first experiment, fixed, embedded and stained. Interestingly, some positive muscle cells were still detectable at this time, 22 weeks after injection of plasmid DNA. Presumably as a feature of the stronger staining evident at earlier time points, muscles containing cells still positive at this time were generally those that had been treated with NGAD65.

Table 3.1: Grading of Islets

At the termination of the experiment, pancreases were also removed from euglycemic mice, fixed, embedded, sectioned and stained with H&E. There was no correlation between treatment and islet score. Surprisingly, one of the control pancreases had essentially normal islets, with an average score between the two blinded observers of 0.1. This is unusual because by 26 weeks of age, all untreated NOD mice are expected to have considerable islet infiltration.

Graph 3.2: NOD Mice Treated with Cytokines and/or Secreted GAD65

Subsequent experiments included treating NOD mice at 6 weeks of age with either SGAD65 alone, plasmid encoding IL-4 alone, or combinations of GAD65 and IL-4 or IL-12. None of the cytokine combination treatments had any significant effect on disease progression

or incidence. Interestingly, SGAD65 alone did delay disease: this group was significantly different from the control group for the ages of 20 and 21 weeks ($p<0.05$).

Graph 3.3: NOD Mice Treated with Multiple Injections of Plasmid

Due to a shortage of mice from our regular supply, Jackson Laboratories, NOD mice for this experiment were purchased from Taconic Farms. In my hands, these mice progressed to diabetes over a much longer period of time, and flip-flopped from euglycemic to hyperglycemic much more frequently. Results from these mice, while valid within the experiment, are not directly comparable with results obtained with Jackson Laboratories NOD mice. In these experiments, a single injection of SGAD65 was not protective even when administered at 4 weeks of age. However, a single injection of SGAD65 followed by multiple injections of vector was significantly protective in comparison to a single injection of vector ($p<0.05$). Statistically significant differences between the two multiply-treated groups and the PBS-injected control group, or multiple treatments with GAD65 did not arise ($p=0.07$). Interestingly, the difference between the NGAD65 and both SGAD65 groups was significant in this experiment ($p<0.05$), from week 29 to the end of the experiment.

Graphs 3.4 and 3.5: Controls for Cultured DC

Flow cytometry demonstrated that proportions of contaminating cells in DC cultures were low, as was background from isotype control antibodies. Contaminating cells types that were examined were granulocytes (Gr-1), B cells (B220) and T cells (CD3e). Initially, F4/80 was also examined as a macrophages marker, but a high proportion of F4/80 positive cells were also CD11c positive. As culture of bone marrow precursors with GM-CSF is expected to produce myeloid DC, which will express F4/80, this marker was not stained for in subsequent analyses

Graph 3.6: Unstained LPS-matured DC

Flow cytometry of unstained DC revealed a level of autofluorescence expected with cultured cells.

Graph 3.7: Isotype Controls for LPS-matured DC

Flow cytometry of DC with isotype controls for the anti-CD11c, anti-CD11b and anti-MHC II antibodies demonstrated a low level of background.

Graphs 3.8 and 3.9: CD11b and CD11c Expression on LPS-matured and Unmatured DC

Flow cytometry demonstrated that DC cultures contained the expected high numbers of cells positive for both CD11b and CD11c and that the level of expression of these markers was not altered by overnight incubation with LPS, as a maturation stimulus.

Graphs 3.10, 3.11 and 3.12: MHC II on LPS-matured and Unmatured DC

Flow cytometry demonstrated that DC responded to overnight culture with LPS by up regulating their expression of MHC II. This is demonstrated by the change in percentage of cells in the MHC II^{lo} versus the MHC II^{hi} population. The MHC II^{mod} population remained the same.

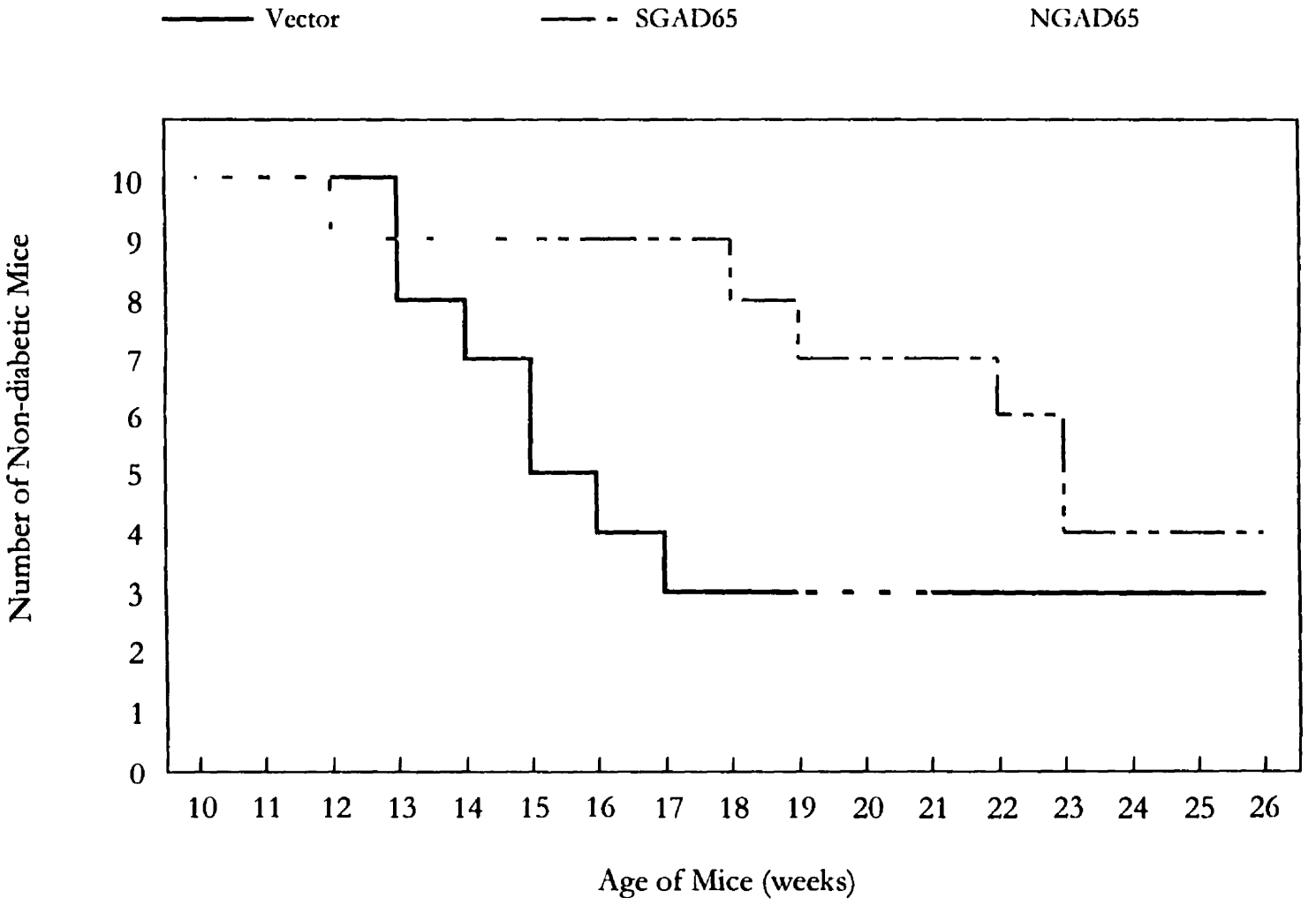
Graph 3.13: Characteristics of OVA-pulsed DC

Flow cytometry demonstrated that cells injected into BALB/c mice for comparison with other methods of in vivo priming were predominantly double positive for CD11b and CD11c, and contained an expected population of cells that highly expressed MHC II.

Table 3.2: ^{3}H-Tritium Incorporation

Although the Con A responses demonstrate the presence of both APC and T cells capable of proliferation, neither CFA/OVA nor pVR-OVA produced a measurable response upon *in vitro* restimulation. This was very unexpected: CFA/OVA at least should have been an excellant priming protocol. Of interest, the only successful priming stimuli were the DC, though they unfortunately primed as effectively against fetal bovine serum proteins as against OVA.

Graph 3.1: NOD Mice Treated with Native GAD65 or Secreted GAD65



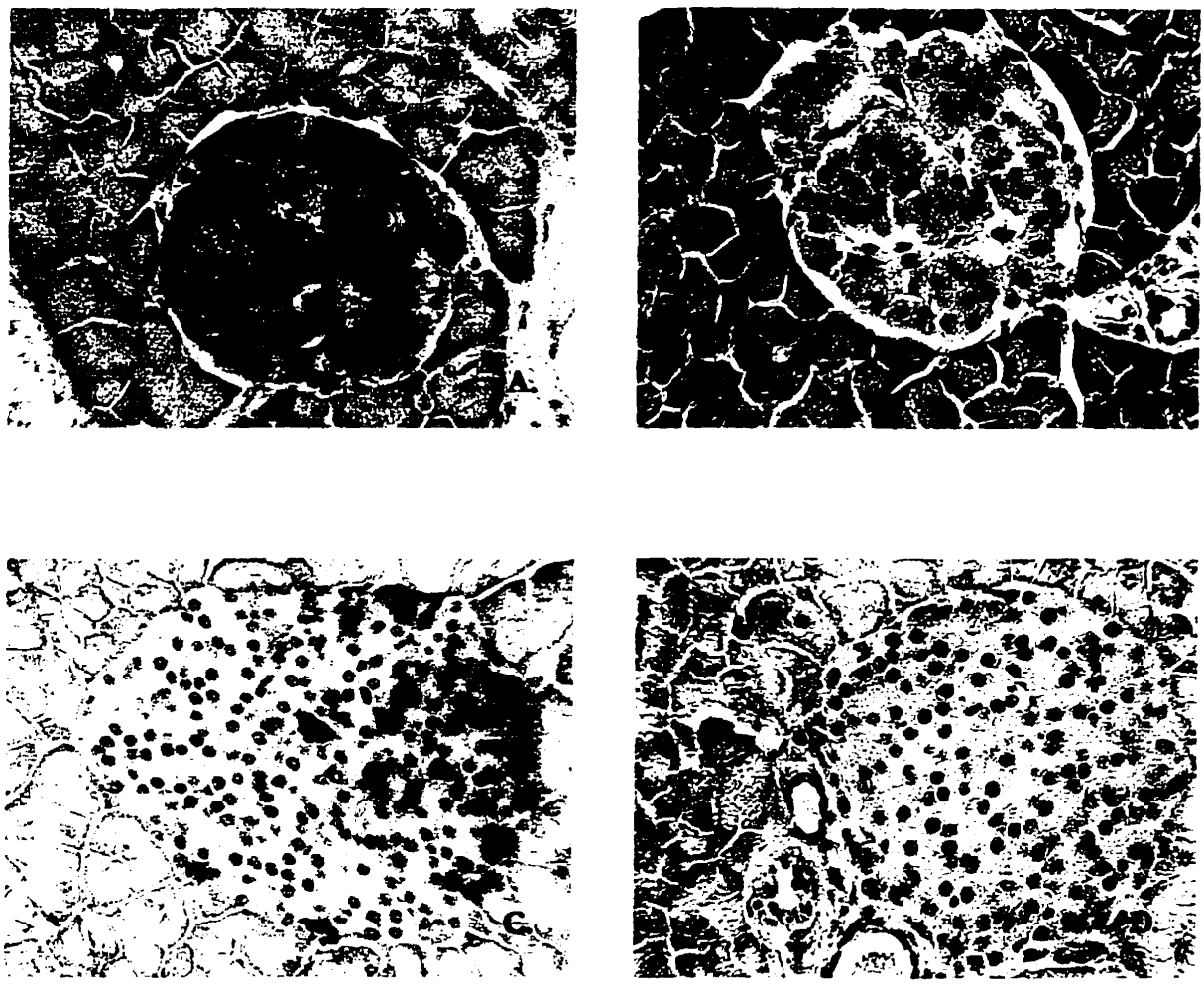


Plate 3.1 A)Rat islet, stained for GAD65 B)Rat islet, negative control C)BALB/c islet, stained for GAD65 D)BALB/c islet, negative control

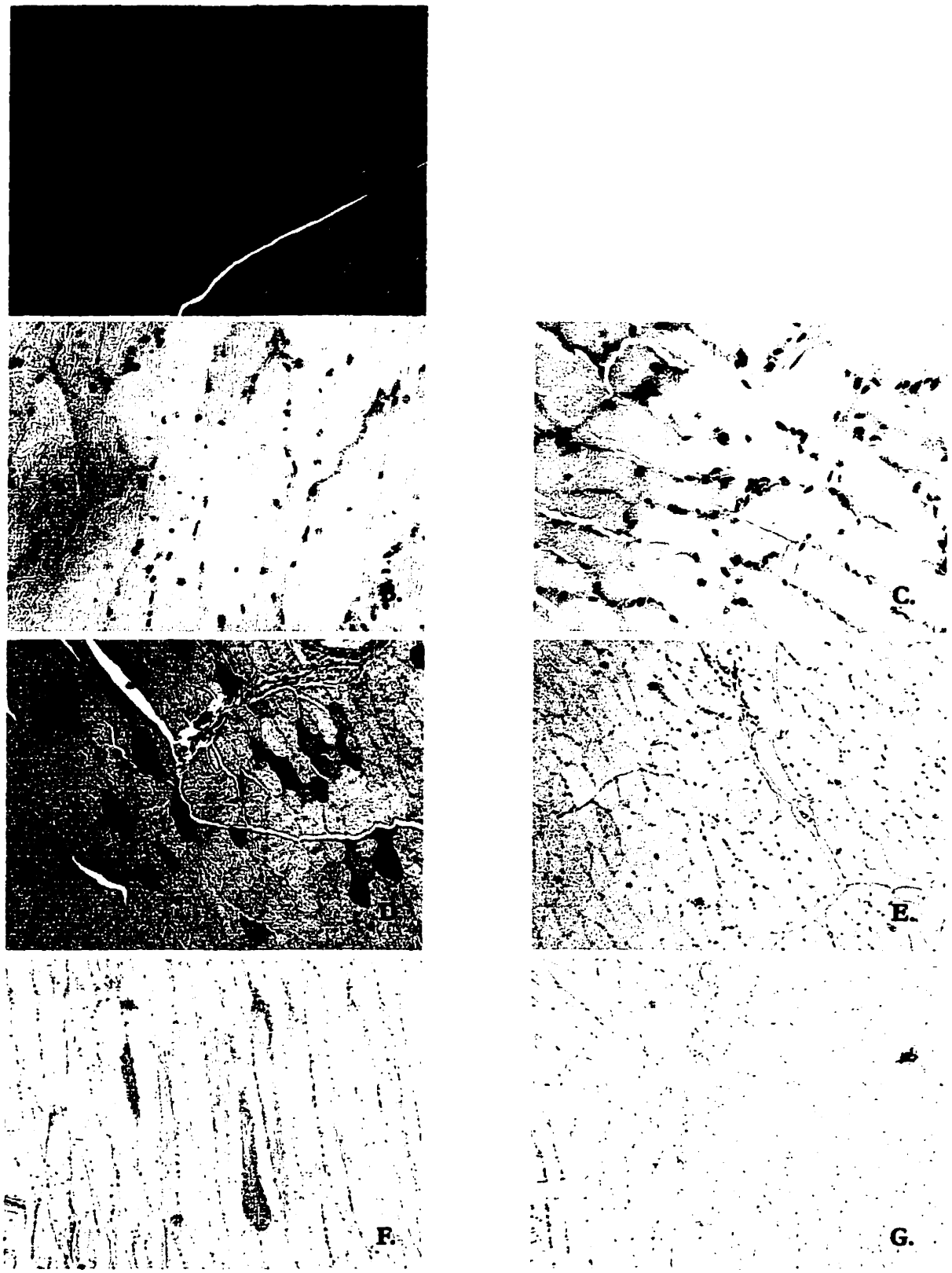


Plate 3.2: Immunohistochemistry of Injected Muscles A)H&E stained tibialis anterior B)Blank plasmid-injected muscle, stained for GAD65 C)Blank plasmid-injected muscle, negative control D)NGAD65-injected muscle, stained for GAD65 E)NGAD65-injected muscle, stained for GAD65 E)NGAD65-injected muscle, negative control F) SGAD65-injected muscle, stained for GAD65 G)SGAD65-injected muscle, negative control

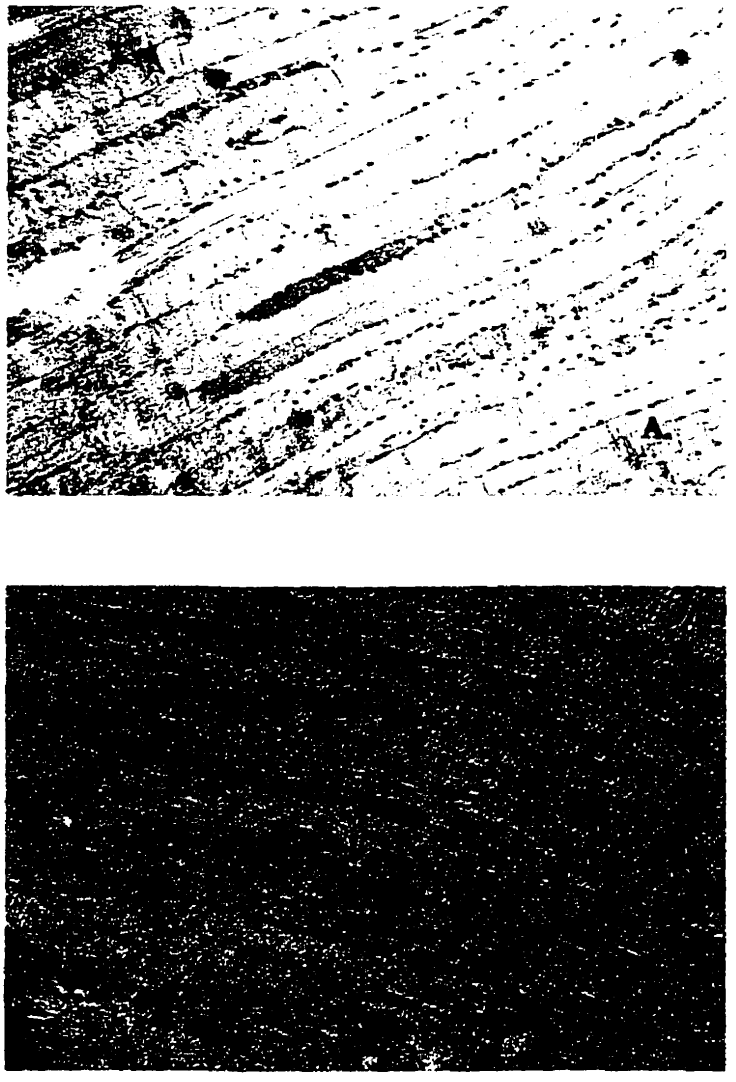
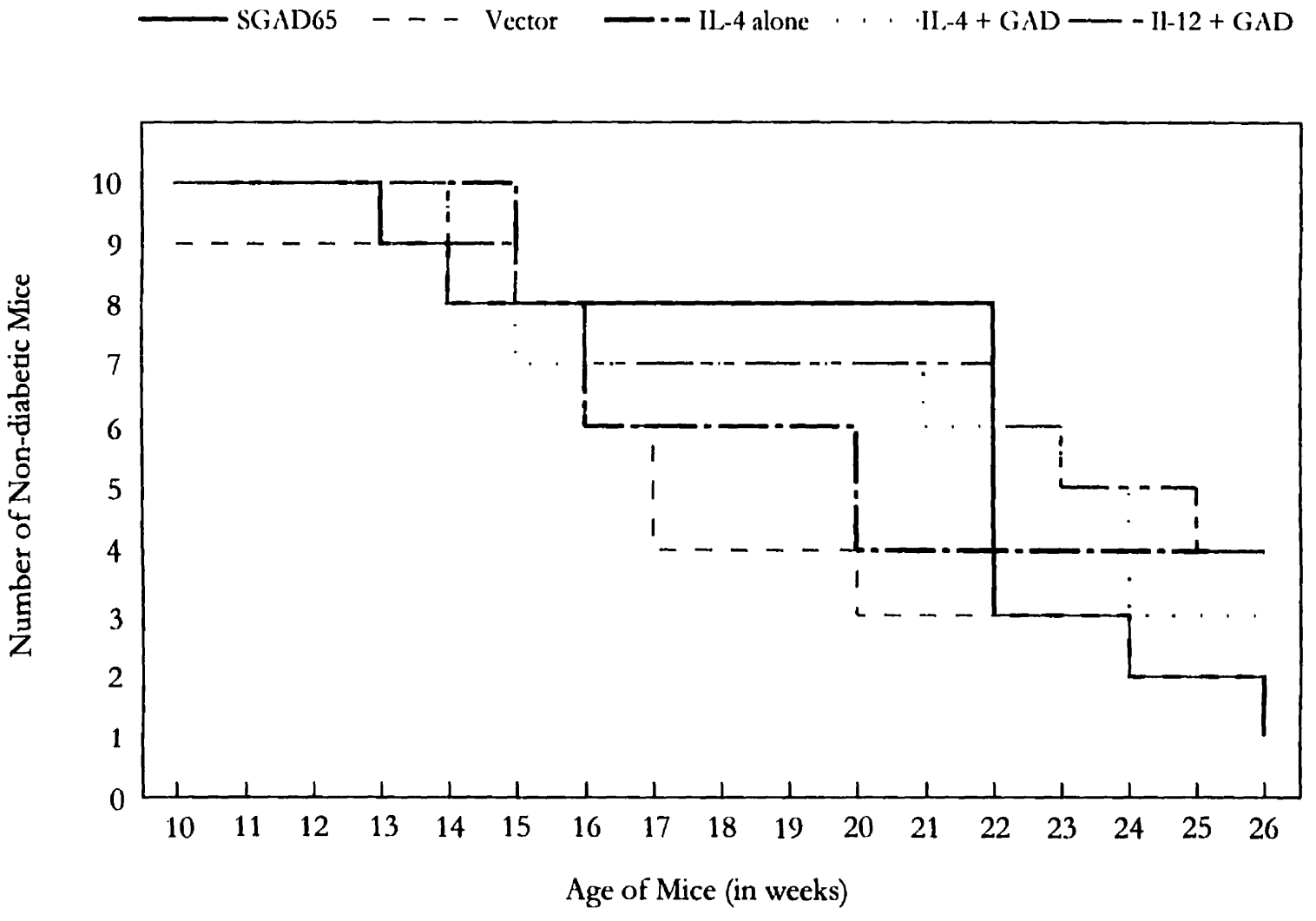


Plate 3.3: Immunohistochemistry of Long-term Expression of GAD65 A)NGAD65-injected muscle stained for GAD65 B)NGAD65-injected muscle, negative control

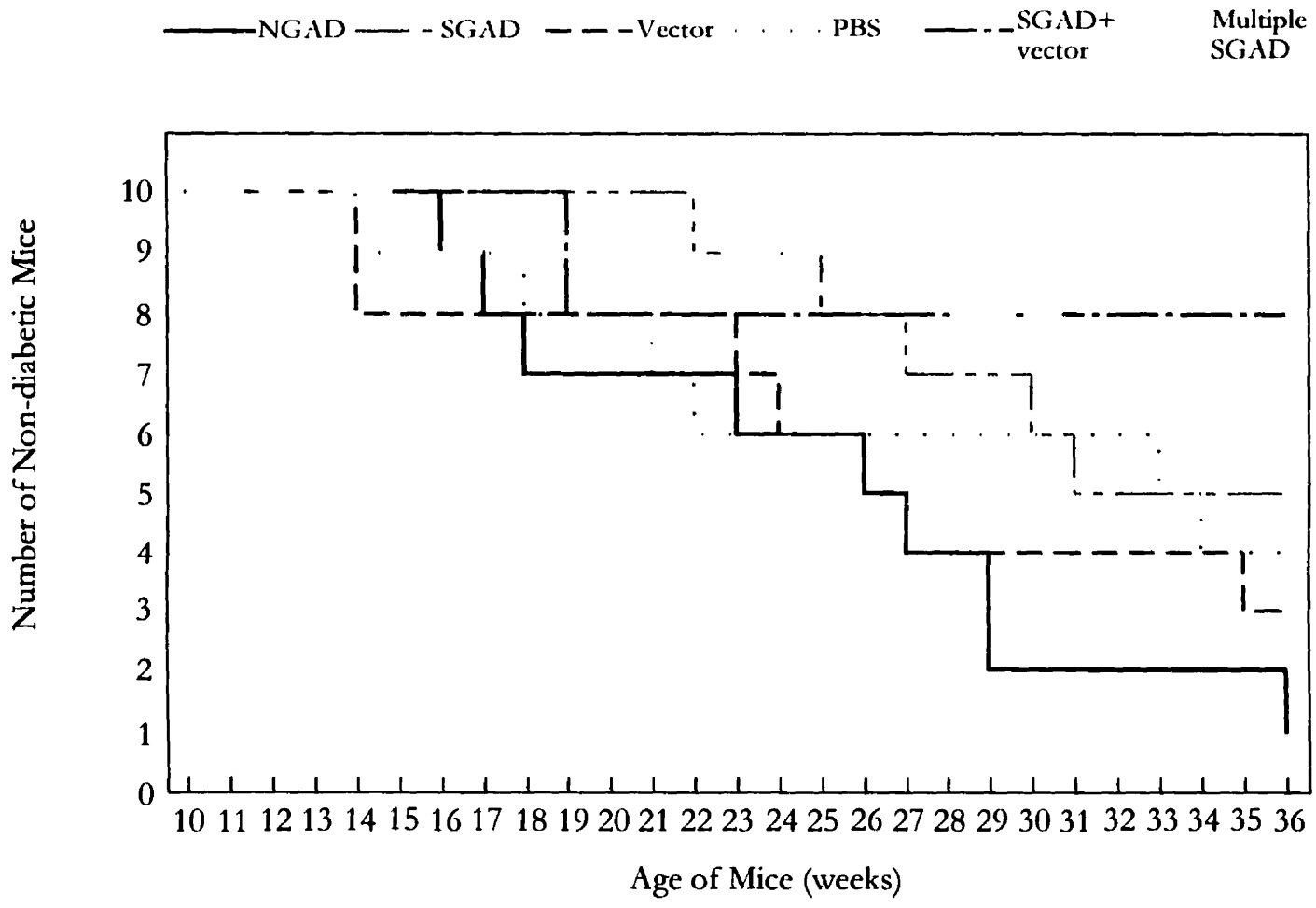
Table 3.1: Grading of Islets

Mouse	N° of Islets Graded	Average Score
SGAD1	9	0.65
SGAD2	9	1.23
SGAD3	15	2.18
SGAD4	31	0.97
CONTROL1	21	2.5
CONTROL2	78	0.1
CONTROL3	17	2.02
NGAD1	30	2.37

Graph 3.2: NOD Mice Treated with Cytokines and/or Secreted GAD65

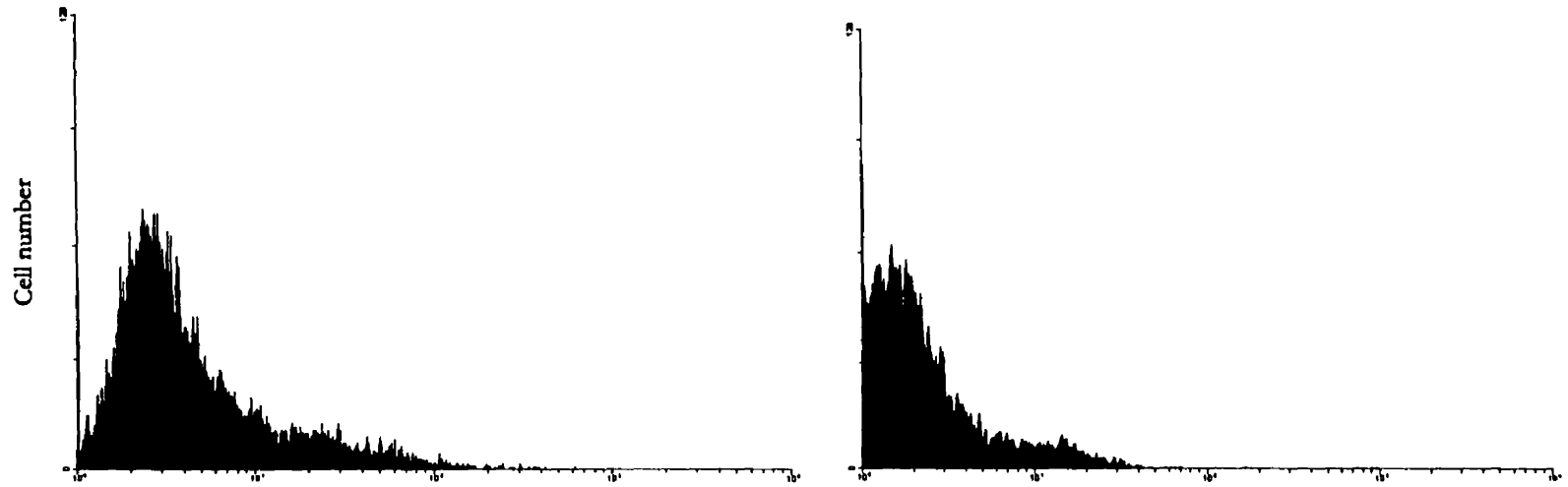


Graph 3.3: NOD Mice Treated with Multiple Injections of Plasmid

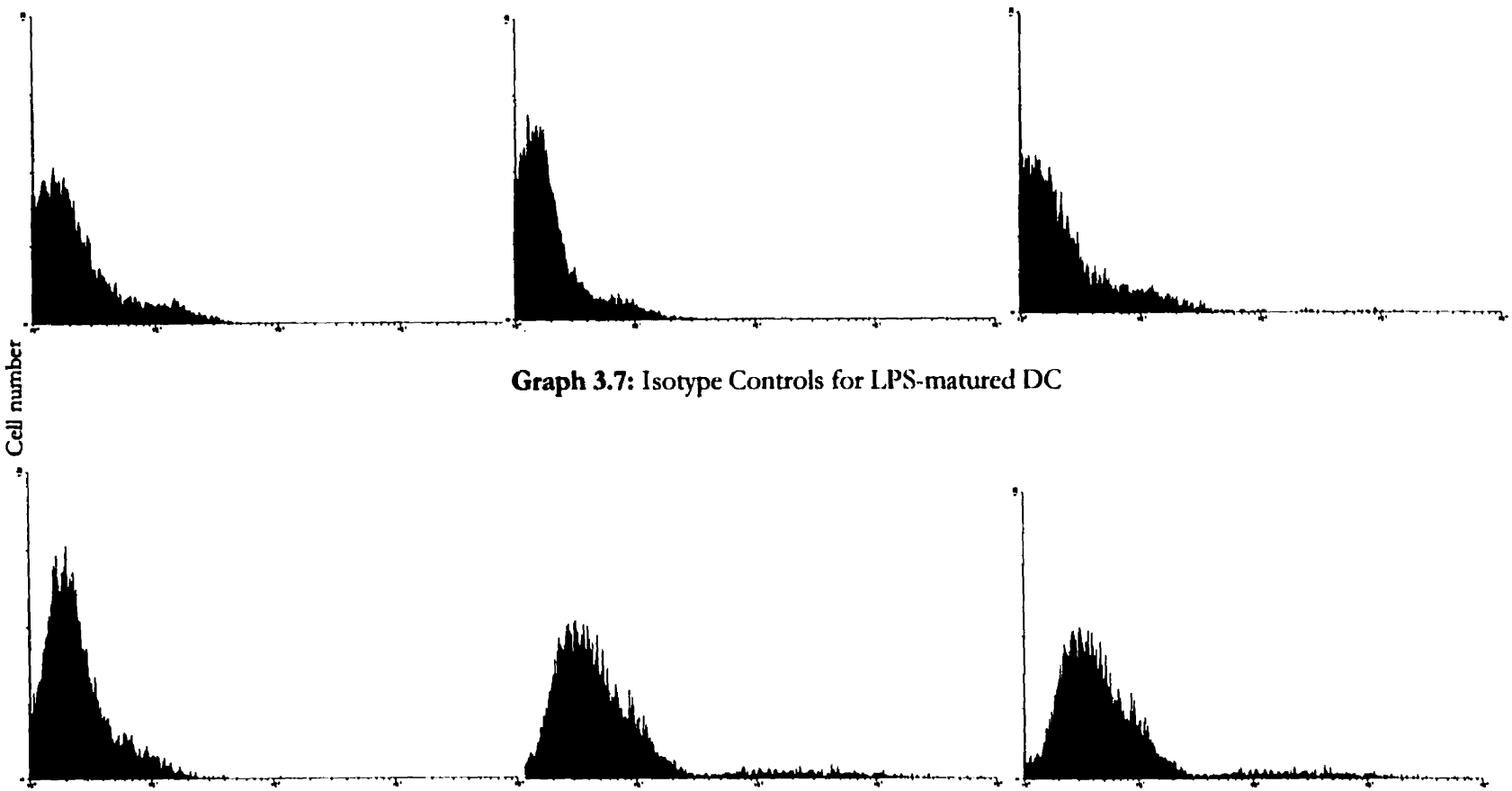


Graph 3.4: Gr-1, B220, CD3e Expression in LPS-matured DC

Graph 3.5: Isotype Controls for Contaminating Cell Markers

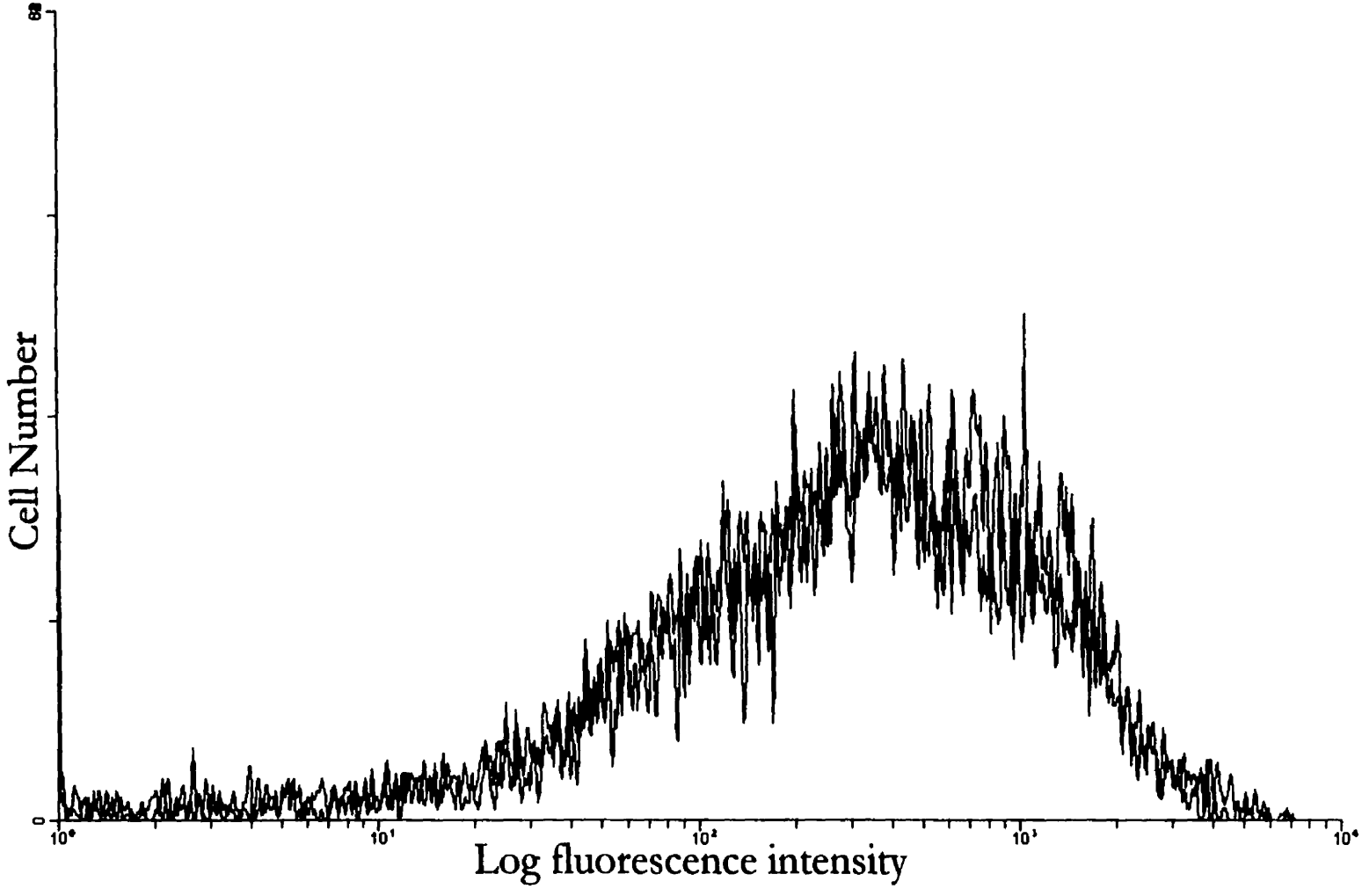


Graph 3.6: Unstained LPS-matured DC

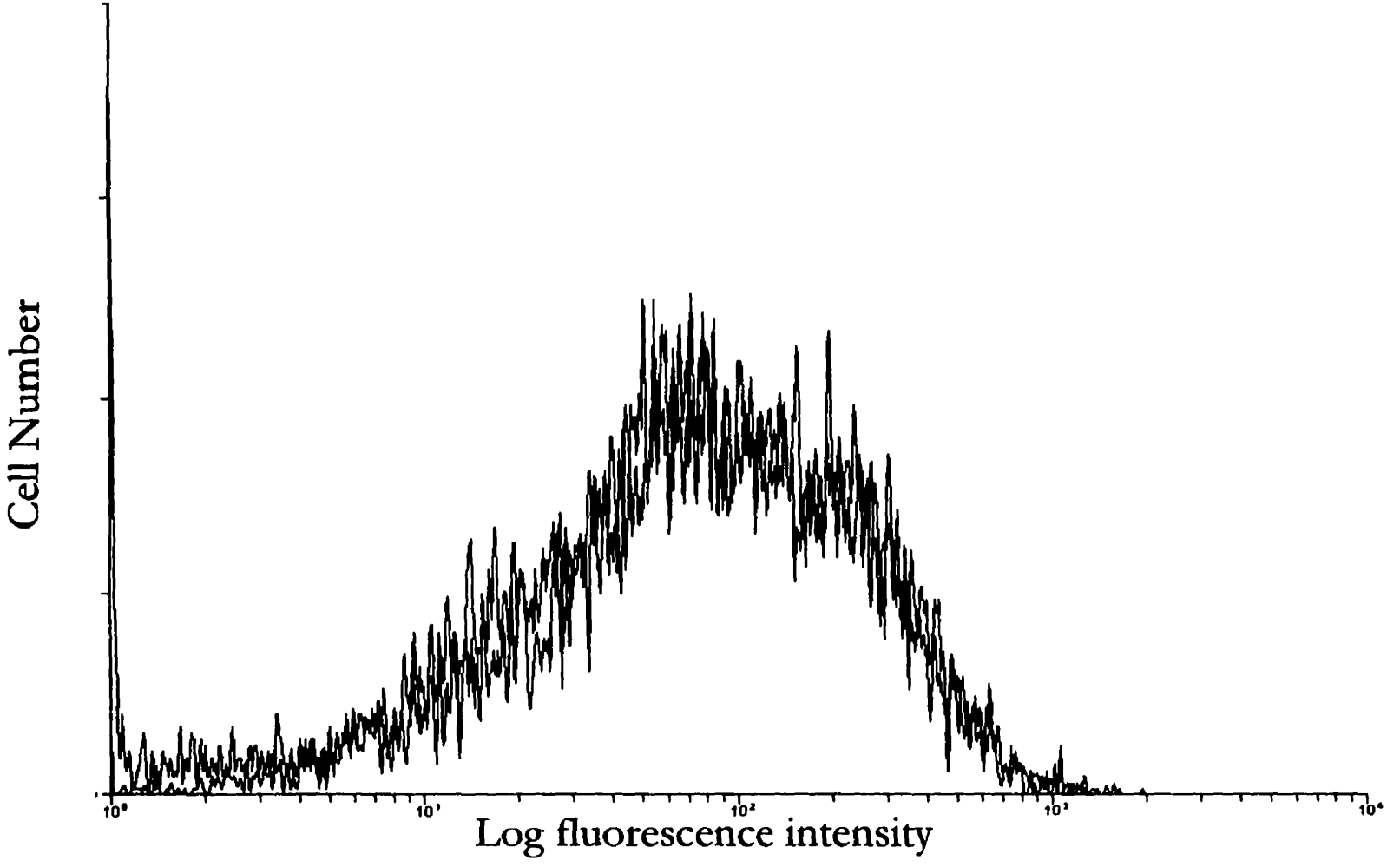


Graph 3.7: Isotype Controls for LPS-matured DC

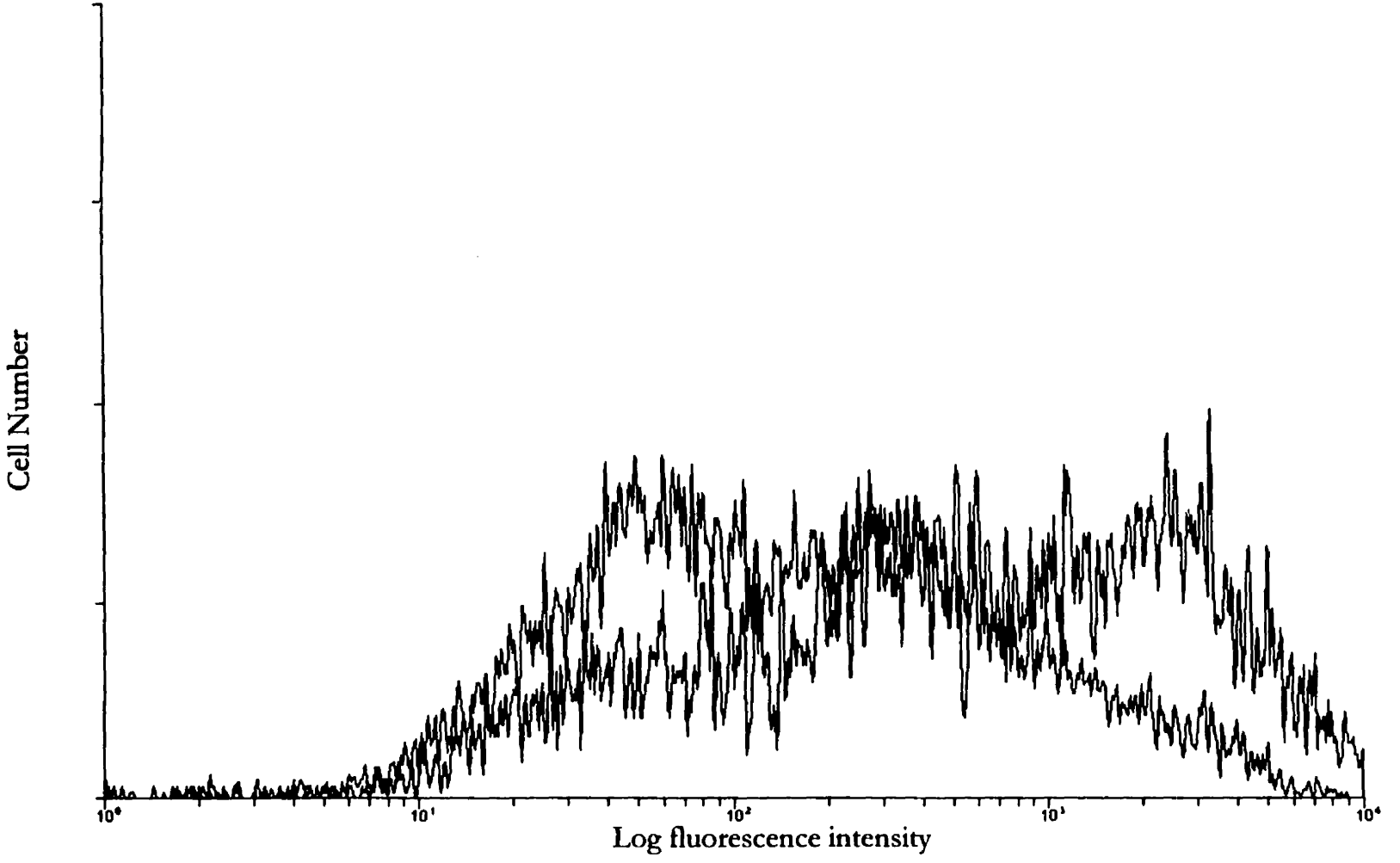
Graph 3.8: CD11b Expression on LPS-matured and Unmatured DC



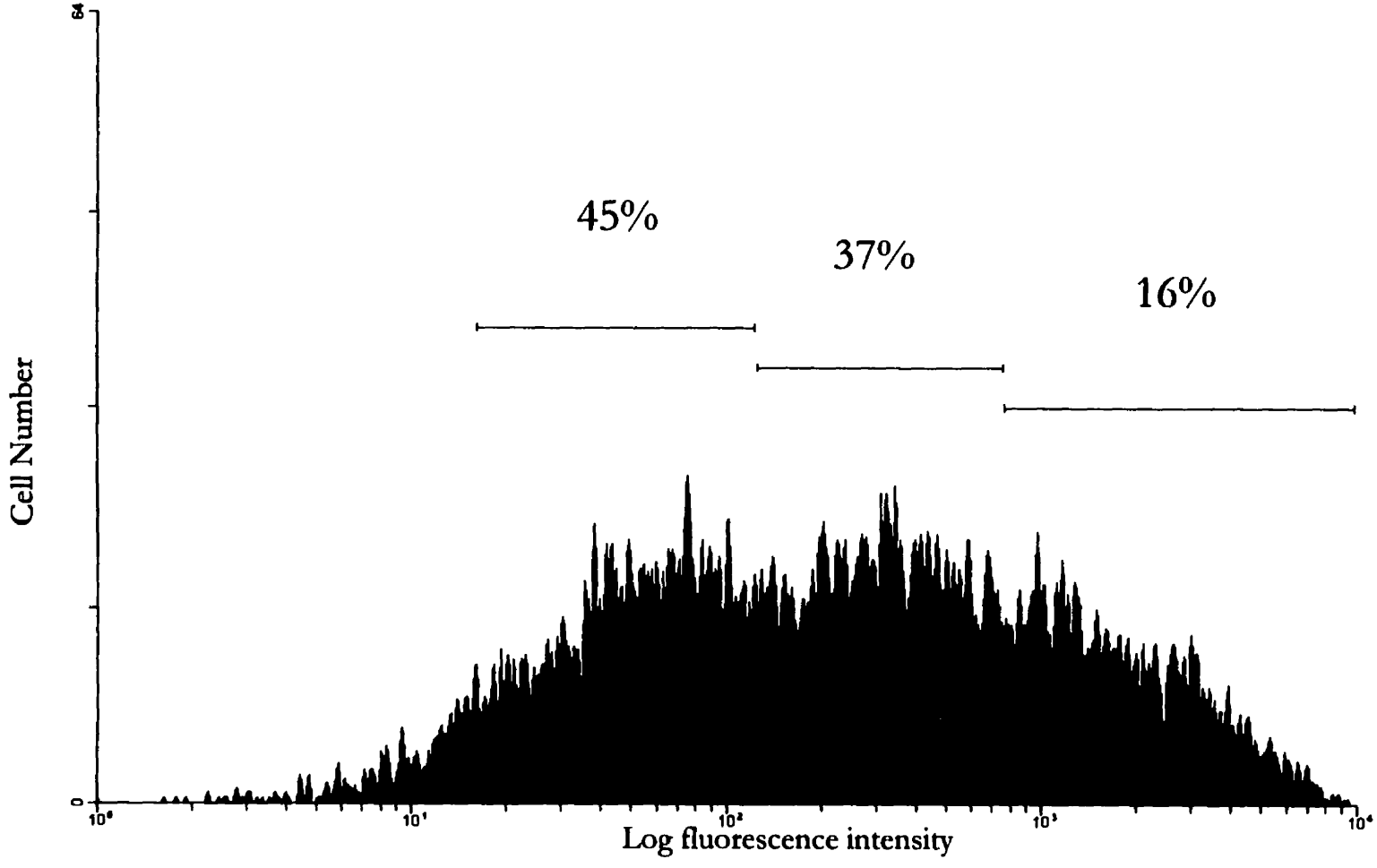
Graph 3.9: CD11c on LPS-matured and Unmatured DC



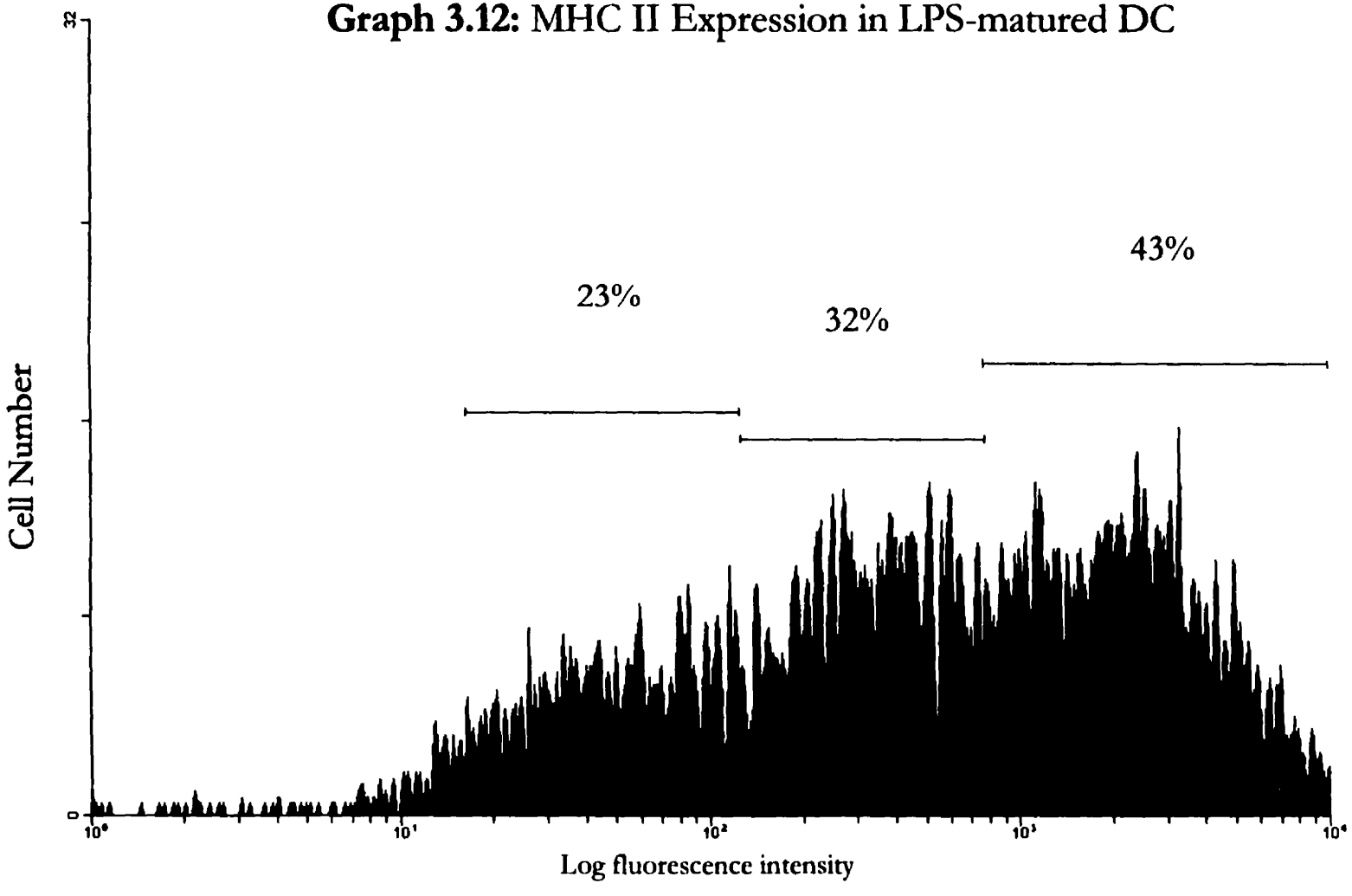
Graph 3.10: MHC II on LPS-matured and Unmatured DC



Graph 3.11: MHC II Expression in Unmatured DC



Graph 3.12: MHC II Expression in LPS-matured DC



Graph 3.13: Characteristics of OVA-pulsed DC

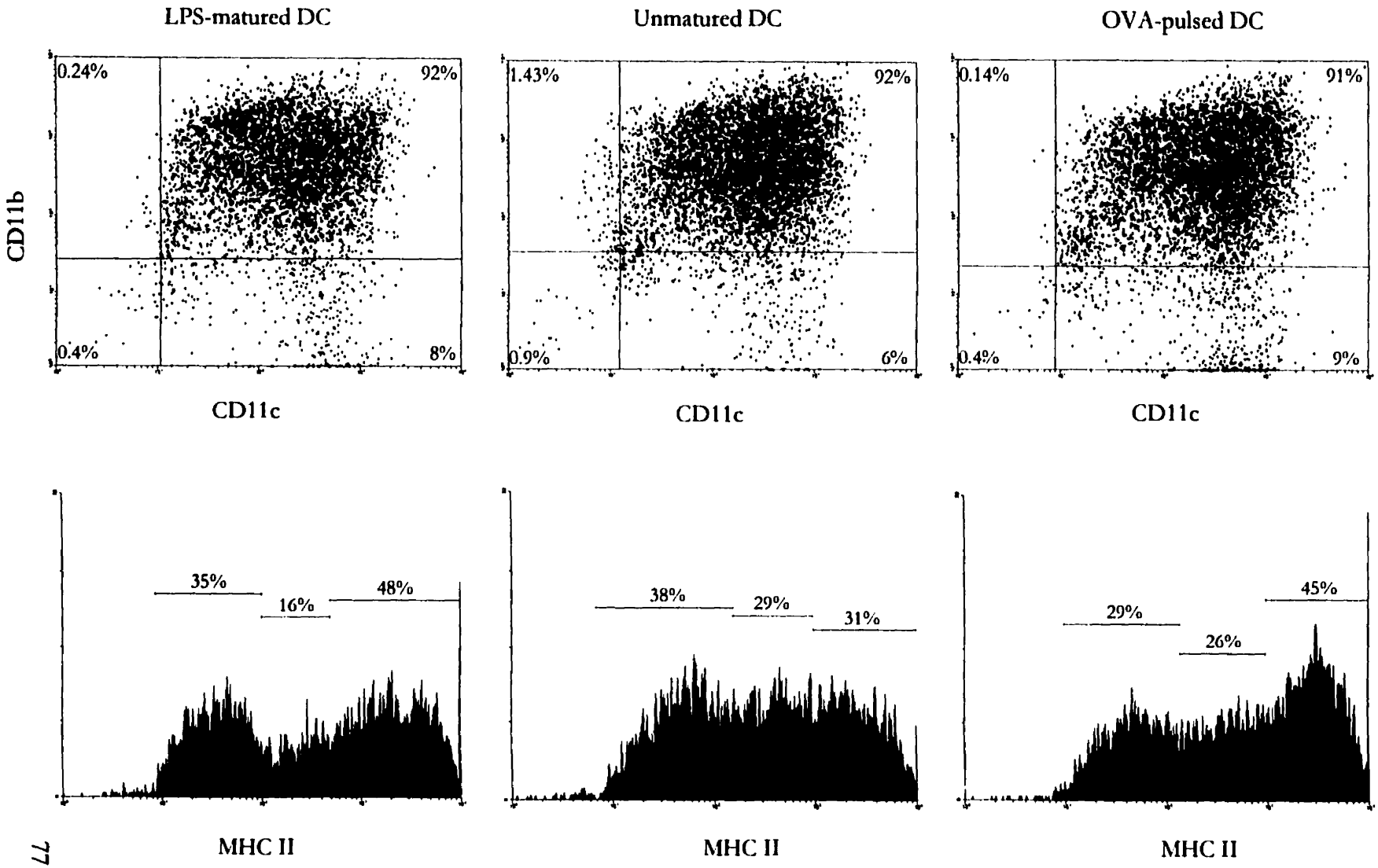


Table 3.2: ^{3}H-Tritium Incorporation

	Day 3			Day 8			Day 14		
Group	OVA	No OVA	Con A	OVA	No OVA	Con A	OVA	No OVA	Con A
pVR-blank	182	181	184808	56	66	47821	74	152	96406
pVR-OVA	122	146	89900	38	87	24977	123	122	31522
CFA/PBS	285	93	117938	172	145	86047	74	64	124498
CFA/OVA	67	100	128279	303	193	105482	82	93	108367
DC-PBS	2794	2176	79379	2105	1281	71575	1911	3463	141425
DC-OVA	5339	9066	130765	17183	12047	168809	982	776	101855

4. Discussion:

4.1 GAD65 and the NOD Mouse:

During an Honours project, two constructs were created using the pVR1255 plasmid containing the gene for GAD65, a putative primary autoantigen in both human type I diabetes and the murine diabetes model, the NOD mouse. The first construct contained the full-length native cDNA of GAD65 (NGAD65), the second contained GAD65 to which a series of overlap PCR reactions had added the IL-4 signal sequence (SGAD65). For the Masters, the incidence of diabetes was assessed following i.m. injection of these constructs, in comparison to control groups which received blank plasmid. In the first experiment, NOD mice were injected at 4 weeks of age, as therapies are generally more effective in this model when administered to young animals.

Transient transfection of COS-7 cells had demonstrated strong intracellular expression of both native and altered GAD65, but no secreted product was detectable in culture medium. Despite this apparent lack of secretion, only the plasmid containing SGAD65 was protective *in vivo*. Differences in disease incidence between the SGAD65 and blank plasmid groups were significant between the ages of 16 and 18 weeks. This is an interesting result because i.m. injection of naked plasmid is associated with a Th1 response, as is autoimmune diabetes. Thus a protective effect following administration of a diabetic autoantigen via this route is somewhat counterintuitive. Interestingly, the native form of GAD65 did appear to exacerbate disease, though this did not reach statistical significance (Graph 3.1).

Coon *et al.*, 1999 also demonstrated a protective effect following i.m. injection of plasmid encoding the insulin B chain in their LCMV-Ig diabetic model. As described in the introduction, these mice are transgenic for the LCMV glycoprotein under the control of the rat insulin

promoter. Endogenous expression of this viral antigen is thus restricted to the pancreatic β islet cells. The mice remain normoglycemic, unless infected with LCMV. The resulting T cell response eliminates the viral infection, but also destroys the β cells.

This can be prevented by i.m. DNA vaccination with insulin B chain, but not LCMV glycoprotein, although the latter did temporarily reduce LCMV-specific CTLs 3 to 4-fold. Coon *et al.* found that insulin B chain-encoding plasmid induced IL-4-producing cells, and suggest that mice were protected by the induction of bystander suppression by IL-4-secreting Th2 cells. They suggest that LCMV glycoprotein-encoding plasmids were not protective because that antigen is directly associated with a viral infection, and thus a strong Th1-inducing stimulus.

We next examined the expression of GAD65 in the mouse using IHC. Protocols were optimized in formalin-fixed paraffin-embedded sections of rat pancreas, because GAD65 expression was known to be high there. Having demonstrated a strong signal, and low background, we next examined GAD65 expression in the mouse pancreas. The mouse pancreas expresses higher levels of GAD67, and previous reports had indicated GAD65 was undetectable by IHC. In agreement with their results, we also found that the monoclonal antibody GAD6 could not detect GAD65 in the mouse pancreas (Velloso *et al.*, 1994), but the antibody from Sigma did(see Methods and Materials). However, we detected a clear signal in the pancreas of BALB/c mice. As our antibody was specific for an epitope not shared with GAD67, the likelihood of cross-reactivity is low (Plate 3.1).

Using the same protocol, GAD65 expression was detected in muscles injected with either NGAD65 or SGAD65, but not in muscles treated with blank plasmid. Interestingly, in most cases NGAD65-injected muscles stained more brightly than SGAD65-injected ones (Plate 3.2). Given the differing effects of the two treatments, this suggests that the SGAD65 product

may have been secreted by transfected muscles, as it was not by monkey kidney cells. Unfortunately, levels of protein produced following naked plasmid gene transfer are difficult to detect in the serum. Commercial ELISA kits exist to detect bioactive molecules such as cytokines, but no such kit exists to detect GAD65. We attempted to develop such ELISA, but the difficulty in purifying GAD65 protein meant that sufficient quantities were unavailable to optimize such an assay. In order to overcome this, we attempted to create stable transfectants of GAD65 with COS-7 cells. To this end, we subcloned GAD65 into the pIRES vector which encodes resistance to geneticin in a second open reading frame. 14 clones with long-term resistance to this antibiotic were tested by western blot for GAD65, and all were found to be negative for GAD65 expression. This seems to indicate that GAD65 is somehow detrimental to COS-7 cells, and was strongly selected against in the long-term.

This was apparently not the case with murine myocytes *in vivo*. Some muscles from mice sacrificed at the end of the experiments, 22 weeks after a single injection of plasmid, still demonstrated detectable expression of GAD65 (Plate 3.3).

Pancreases were also taken from those mice remaining non-diabetic at 26 weeks of age, and the pancreatic islets were graded. In agreement with the observation that mice in the SGAD group had begun to develop diabetes by this time point, there was no correlation between treatment and islet score. This suggested that by 26 weeks, although GAD65 was still being expressed detectably in some muscles, it was no longer having an effect on diabetes or islet pathology (Table 3.1).

In attempt to prolong the protective effective observed in the first experiment, two strategies were attempted. In the first, 6 week old NOD mice were injected with either SGAD65 alone, IL-4 alone, or SGAD65 in combination with IL-4 or IL-12. As IL-4 is produced by Th2

cells, it would be expected to protect from disease, while IL-12 would be expected to worsen disease. Control groups were treated with blank plasmid. Oddly, although the plasmid encoding the IL-4/IgG_1 fusion protein had been protective in previous experiments, even when given to 6 week old mice, it was not protective in this experiment (Chang *et al.*, 1999).

Neither the combinations of IL-4 and SGAD65, nor of IL-12 and GAD65 had any significant effect on disease progression or incidence. However, recent work by Serreze *et al.*, 2001 has called into question the importance of IL-4 in diabetes in the NOD mouse. This group found that IL-4 deficient NOD mice developed diabetes at the same rate and frequency as normal NOD mice, and were equally protected by the non-specific immunostimulatory chemical CFA or bacillus Calmette-Guérin (BCG) vaccine. They suggest that the Th2 cytokine profile associated with protection from diabetes by various antigen-specific and nonspecific therapies is a secondary effect, and not causally linked to protection at all. Given that the APCs of NOD mice are known to be defective in several aspects of T cell activation, Serreze *et al.* suggest that they are able to activate T cells sufficiently to cause them to attack self tissue, but not enough to cause activation-induced cell death (AICD). As Th1 cells are considered more sensitive to AICD, protocols which protect NOD mice overcome their deficiency in activating T cells enough to cause the death of Th1 but not Th2 cells. Thus the apparent shift to a Th2 profile is actually due to the 'unmasking' of a pre-existing Th2 response by the death of the Th1 cells.

Interestingly, we found that SGAD65 alone was protective when administered to 6 week old NOD mice, significantly delaying the onset of diabetes between the ages of 20 and 21 weeks. This result conflicts with an earlier study by Wiest-Ladenburger *et al.*, 1998 in which they treated 6 week old NOD, BALB/c and C57BL/6 mice with plasmids encoding either rat GAD65 or GAD67. Although they induced antibodies against GAD65 in all three strains, and demonstrated

strain-specific epitope recognition, they had no effect on diabetes incidence in the NOD mouse.

However, in more recent work, Tisch *et al.*, 2001 demonstrated that vaccination with their plasmid encoding a GAD65 peptide fusion protein was highly protective when administered in combination with IL-4. They fused fragments of GAD65 cDNA encoding three epitopes recognized by CD4+ NOD T cells to a human IgG_4F_c region. Thus, they created a secreted product that they suggest was preferentially processed and presented via the MHC II pathway. In agreement with our results, they also found that immunization with plasmid encoding IL-4 alone was not protective. However, in contrast to our findings, they observed that co-administration of GAD65 and IL-4 was protective, while GAD65 alone was not. Even more interesting, they demonstrated similar protection when 12 week old NOD mice were injected with either a combination of plasmids encoding IL-4 or GAD65, or a plasmid encoding both IL-4 and GAD65. In all cases, they monitored their mice for at least 52 weeks, demonstrating a much longer term protection than we achieved. In their study, mice treated with both IL-4 and GAD65 exhibited very low islet infiltration scores, while any such correlation in our mice was no longer evident at 26 weeks of age. These differences could arise from any of a number of factors including the presence of a signal sequence on our GAD65, and their use of only fragments of the entire sequence, to non-specific effects arising from different plasmid backbones.

In vitro restimulation of co-immunized mice showed that their T cells responded to GAD65 with augmented production of IL-4 and IL-5, indicating that a Th2-type response had been induced. Furthermore, these cells responded similarly to two other diabetic antigens, indicating that protective intermolecular epitope spreading had occurred. Control mice, and co-immunized mice who had developed diabetes, displayed Th1-type responses to these antigens.

In addition to a lack of protection, Tisch *et al.* also found that T cells from mice vaccinated with GAD65 alone produced elevated levels of IFN-γ upon *in vitro* restimulation with specific antigen. They further supported their hypothesis that protection was mediated by GAD65-specific Th2 by demonstrating that co-transfer of CD4+ T cells from IL-4/GAD65-injected mice protected recipients from adoptive transfer of diabetes.

We next examined the protective effect of multiple injections of plasmid. Unfortunately, due to a shortage of animals at the Jackson Laboratories, this study was performed with NOD mice from Taconic Farms. These mice displayed a much slower progression to diabetes. Any comparison made between experiments performed with these mice and those from Jackson Laboratories must be made with great care.

The original finding, in which a single injection of SGAD65 at 4 weeks of age delayed disease, was not duplicated in this study. Although SGAD65 did appear to delay diabetes, this did not reach statistical significance. Interestingly, the exacerbation of disease caused by NGAD65 in comparison to SGAD65, suggested in the first experiment, did reach statistical significance here.

Of note, there was essentially no difference in protection from diabetes between mice vaccinated at 4 weeks of age with SGAD65 followed by injections every three weeks with blank plasmid and those injected every three weeks withSGAD65 (Graph 3.3), despite the fact that one reached statistical significance and one didn't (see Graph 3.3). Given the IHC findings of the first experiment, in which GAD65 expression was still detectable 22 weeks after injection, it is possible that the critical correlate with protection is a continuing stimulus, in the form of bacterial DNA, causing DC to migrate from the muscle and present antigen in draining lymph nodes. In this, blank plasmid would serve as well as GAD65-encoding plasmid, assuming that

GAD65 were still expressed in the muscle.

4.2 Bone-marrow Derived DC:

Bone marrow precursors were isolated from mice between 6 and 12 weeks old, and cultured for seven days with GM-CSF in order to produce a final yield of between 2.5 and 5 million DC per mouse.

Flow cytometry staining for contaminating cell markers, in comparison with isotype controls for these antibodies, reveal low levels of granulocytes (Gr-1), B cells (B220/IgM) and T cells (CD3e) (Graphs 3.4 and 3.5). Unstained controls of LPS-matured DC demonstrate the presence of some autofluorescence, not unexpected in cultured cells, and low background from isotype controls (Graphs 3.6 and 3.7). In contrast, both unmatured and LPS-stimulated DC are strongly positive for the pan-DC marker CD11c and CD11b. The latter is a marker of the myeloid DC that are the expected predominant product of culture with GM-CSF (Graphs 3.8 and 3.9). Although the maturation stimulus LPS has no effect on CD11b or CD11c expression, MHC II expression is strongly upregulated (Graph 3.10). MHC II expression is a marker of DC maturation towards an efficient antigen-presenting cell. Bone-marrow derived DC cultures are a heterogeneous population of cells at various stages of maturation. Histograms of MHC II expression reveal three roughly delineated populations (MHC II^{lo}, MHC II^{mod} and MHC II^{hi}). In unmatured DC cultures, approximately 45% of cells are MHC II^{lo}, while only about 16% are MHC II^{hi}. In contrast, in cultures which have been incubated overnight with LPS, these percentages are reversed: 23% of cells are MHC II^{lo} and 43% are MHC II^{hi} (Graphs 3.11 and 3.12). This demonstrates that culturing precursors has produced a high proportion of DC, that are at least functional in their ability to respond appropriately to a maturation signal. Graph 3.13 demonstrates that incubation overnight with OVA also produces mature populations of DC.

Following this, an attempt was made to study the effects of DNA vaccination as compared to protein vaccination in CFA, or pulsed DC. Unfortunately, several technical difficulties were experienced. The first was the unexpectedly poor response following subcutaneous vaccination with OVA in CFA. Previous optimization experiments (data not shown) yielded *in vitro* responses lower than would be expected from the literature, but still detectable. For these initial trials, lymph nodes were collected on Day 5, so it is possible that the Day 3 and Day 8 time points used in the experiment preceded and exceded the optimal response. The second technical fault was forseen to some extent, but of an unexpected severity. As the DC were cultured in media containing FBS, both OVA-pulsed and untreated DC would be expected to prime recipient mice against bovine proteins. As *in vitro* restimulation cultures also contained these proteins, a degree of background was expected. As restimulation was done with a concentration of OVA of 100 μg/ml, it was hoped that signal versus background would still be distinguishable. This proved to be a dangerously optimistic assumption, as shown in Table 3.2. In an attempt to remedy this, the Day 14 cultures were grown in media identical to previous trials but with heat-inactivated mouse serum replacing the FBS. Unfortunately, these cultures did not proliferate, even in response to Con A, presumably indicating some human error. Naturally, duplicate cultures of the Day 14 timepoint were cultured in media containg FBS, and these proliferated as expected despite the fact that they were grown in the same batch of media in which only the serum had been substituted.

Finally, and most unexpectedly, DNA vaccination of four muscles per mouse followed by electroporation did not produce a measurable response *in vitro*.

As the time limitations placed upon a Masters preclude the optimization and repetition of this experiment for inclusion in this thesis, my discussion of these final results will be of ways

in which this optimization would be performed. The simplest change would be to replace the Day 3 time point with a Day 5 time point, thus eliminating the chance of 'missing' the optimal response to OVA/CFA. Alternatively, these timepoints could be left unchanged, and the response boosted with sub-scapular injections of OVA/CFA at a later time point. Boosting vaccinations with plasmids will apparently also be required to elicit a detectable response.

With regards to the DC, the most likely solution is to optimize their derivation from bone marrow precursors in mouse serum-containing culture. This will preclude them being loaded with foreign proteins other than OVA, and allow *in vitro* restimulations to be performed in FBS-containing culture. These conditions would be easier to optimize than those of the restimulation, because the maturation, health and phenotype of the resulting DC can be reliably assessed by flow cytometry prior to injection.

4.3 Conclusion:

In conclusion, we have demonstrated that the cDNA of GAD65 to which a secretory signal has been added delays the onset of diabetes in the NOD mouse, even when administered as late as 6 weeks of age. In contrast, plasmid encoding native GAD65 is not protective. The delay seen with SGAD65 was statisitically significant despite observations in transiently-transfected cells that strongly suggest the protein is not secreted.

The origins of the NOD mouse model preclude the study of strain differences in response to treatment, however our observation that the same treatment (SGAD65, in both T.A. at 4 weeks of age) results in delayed disease in NOD mice from Jackson Laboratories, while producing prolonged protection in NOD mice from Taconic Farms suggests not only a critical importance of environment in the NOD mouse, but also a cautionary note with regards to comparing results obtained with different NOD colonies. This is accentuated by statistically

significant worsening of disease evident in Taconic NOD mice as a result of native GAD65 treatment, that was not observed in NOD mice from Jackson Laboratories.

Our immunohistochemical results clearly demonstrate that the GAD65 cDNA was expressed in the muscle over a prolonged period of time. In fact, it was expressed long after it had ceased to effect disease progression. The equivalent protection seen from either multiple injections of SGAD65 or a single injection of SGAD65 suggest that this loss of effect maybe due to the clearing of bacterial DNA and healing of any damage caused by injection. Once these stimuli are gone, nothing induces surveying DC to exit the muscle and carry protein collected there to draining lymph nodes for presentation, and protein produced in the muscle then ceases to have an effect on immune responses in the rest of the body.

IHC also yielded the interesting results of detection of GAD65 in the murine pancreas., as previous reports suggested that levels here were undetectable even by RT-PCR. It would be interesting to test if the results of Velloso *et al.* are specific to NOD mice.

Finally, we attempted to study the mechanisms of DNA vaccination be comparing several modes of immunization. Although we successfully cultured a high purity of dendritic cells from bone precursors, the final experiment was flawed, and time limits precluded optimization of these conditions. Interestingly, DC vaccination did appear to result in a strong response to both OVA and serum proteins (Table 3.2).

However, we have demonstrated that DNA vaccination of an autoimmune model with a autoantigen can delay disease. Given the ease and economy with which such vectors can be produced, and the benefits they have for the treatment of chronic disease in contrast to more inflammatory or cytotoxic viral vectors, we believe our results support future research into the treatment of autoimmune disease with naked plasmid gene therapy.

5. References:

Ablamunits, V., Elias, D. and Cohen, I.R. (1999) The pathogenicity of islet-infiltrating lymphocytes in the non-obese diabetic (NOD) mouse.. *Clin. Exp. Immunol.* 115: 260-267.

Agadjanyan, M.G., Kim, J.J., Trivedi, N., Wilson, D.M., Monzavi-Karbassi, B., Morrison, L.D., Nottingham, L.K., Dentchev, T., Tsai, A., Dang, K., Chalian, A.A., Maldonado, M.A., Williams, W.V. and Weiner, D.B. (1999) CD86 (B7.2) can function to drive MHC-restricted antigen specific CTL responses *in vivo*. *J. Immunol.* 162: 3417-3427.

Agarwal, R.K., Kang, Y., Zambidis, E., Scott, D.W., Chan, C.-C. and Caspi, R.R. (2000) Retroviral gene therapy with an immunoglobulin-antigen fusion construct protects from experimental autoimmune uveitis. *J. Clin. Invest.* 106: 245-252.

Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D. and Stockinger, B. (1999) DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J. Exp. Med.* 189: 169-177.

Albert, M.L., Pearce, S.F.A., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L. and Bhardwaj, N. (1998) Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta_5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188: 1359-1368.

Apparailly, F.C., Verwaerde, C., Jacquet, C., Auriault, C., Sany, J. and Jorgensen, C. (1998) Adenovirus mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. *J.Immunol.* 160: 5213-5220.

Armand, M.-A., Grange, M.-P., Paulin, D. and Desgranges, C. (2000) Targeted expression of HTLV-1 envelope proteins in muscle by DNA immunization of mice. *Vaccine* 18: 2212-2222.

Arreaza, G.A., Cameron, M.J., Jaramillo, A., Gill, B.M., Hardy, D., Laupland, K.B., Rapoport, M.J., Zucker, P., Chakrabarti, S., Chensue, S.W., Qin, H.-Y., Singh, B. and Delovitch, T.L. (1997) Neonatal activation of CD28 signaling overcomes T cell anergy and prevents autoimmune diabetes by an IL-4-dependent mechanism. *J. Clin. Invest.* 100: 2243-2253.

Baker, D., Butler, D., Scallon, B.J., O'Neill, J.K., Turk, J.L. and Feldman, M. (1994) Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur. J. Immunol.* 24: 2040-2048.

Ban, E., Dupre, L., Hermann, E., Rohn, W., Vendeville, C., Quatannens, B., Ricciardi-Castagnoli, P., Capron, A. and Riveau, G. (2000) CpG motifs induce Langerhans cell migration *in vivo*. *Internat. Immunol.* 12: 737-745.

Batteux, F., Trebeden, H., Charreire, J. and Chiocchia, G. (1999a) Curative treatment of experimental autoimmune thyroiditis by *in vivo* administration of plasmid DNA coding for interleukin-10. *Eur. J. Immunol.* 29: 958-963.

Batteux, F., Tourneur, L., Trebeden, H., Charreire, J and Chiocchia, G. (1999b) Gene therapy of experimental autoimmune thyroiditis by *in vivo* administration of plasmid DNA coding for Fas ligand. *J. Immunol.* 162: 603-608.

Bell, D., Young, J.W. and Banchereau, J. (1999) Dendritic cells. *Adv. Immunol.* 72: 255-324.

Bieg, S. and Lernmark, A. (1998) Diabetes. In Rose, N.R. and Mackay, I.R. (Eds.), *The Autoimmune Diseases*, Third edition.

Bluestone, J.A., Khattri, R. and van Seventer, G.A. (1999) Accessory molecules. In Paul, W.E. (Ed.), *Fundamental Immunology*, Fourth edition.

Boczkowski, S., Nair, S.K., Snyder, D. and Gilboa, E. (1996) Dendritic cells pulsed with RNA are potent antigen-presenting cells *in vitro* and *in vivo*. *J. Exp. Med.* 184: 465-472.

Boyle, J.S., Koniaras, C., and Lew, A.M. (1997) Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int. Immunol.* 9: 1897-1906.

Cameron, M.J., Guillermo, A.A., and Delovitch, T.L. (1997) Cytokine- and costimulation-mediated therapy of IDDM. *Crit. Rev. Immunol.* 17: 537-544.

Cameron, M.J., Strathdee, C.A., Holmes, K.D., Arreaza, G.A., Dekaban, G.A. and Delovitch, T.L. (2000) Biolistic-mediated interleukin 4 gene transfer prevents the onset of type 1 diabetes. *Human Gene Ther.* 11: 1647-1656

Casares, S., Inaba, K., Brumeanu, T.-D., Steinman, R.M. and Bona, C.A. (1997) Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med.* 186: 1481-1486.

Chang, Y., and Prud'homme, G.J. (1999) Intramuscular administration of expression plasmids encoding interferon-γ receptor/IgG1 or IL-4/IgG1 chimeric proteins protects from autoimmunity. *J. Gene Med.* 1: 415-423.

Chen, L.Z., Hochwald, G.M., Huang, C., Dakin, G., Tao, H., Cheng, C., Simmons, W.J., Dranoff, G. and Thorbecke, G.J. (1998a) Gene therapy in allergic encephalomyelitis using myelin basic protein-specific T cells engineered to express latent transforming growth factor-β1. *PNAS* 95:12516-12521.

Chen W. Jin W. Wahl SM. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4+ T cells. (1998b) *J. Exp. Med.* 188: 1849-57.

Chernajovsky, Y., Feldmann, M. and Maini, R.N. (1995a) Gene therapy of rheumatoid arthritis via cytokine regulation: future perspectives. *Brit. Med. Bull.* 51: 503-516.

Chernajovsky, Y., Adams, G., Podhajcer, O.L., Mueller, G.M., Robbins, P.D. and Feldman, M. (1995b) Inhibition of transfer of collagen-induced arthritis into SCID mice by ex vivo infection of spleen cells with retroviruses expressing soluble tumor necrosis factor receptor. *Gene Ther.* 2: 731-735.

Chernajovsky, Y., Adams, G., Trianphyllopoulos, K., Ledda, M.F. and Podhajcr, O.L. (1997) Pathogenic lymphoid cells engineered to express TGF-β1 ameliorate disease in a collagen-induced arthritis. *Gene Ther.* 4: 553-559.

Chirmule, N., Propert, K.J., Magosin, S.A., Qian, Y. and Wilson, J.M. (1999) Immune responses to adenoviral and adeno-associated virus in humans. *Gene Ther.* 6: 1574-1584.

Chun, S., Daheshia, M., Lee, S. and Rouse, B.T. (1999a) Immunmodulation by IL-10 gene transfer via viral vector and plasmid DNA: implications for gene therapy. *Cell. Immunol.* 194: 194-204.

Chun, S., Daheshia, M., Lee, S., Eo, S.K. and Rouse, B.T. (1999b) Distribution fate and mechanism of immune modulation following mucosal delivery of plasmid DNA encoding IL-10. *J. Immunol.* 163: 2393-2402

Condon, C., Watkins, S.C., Celluzzi, C.M., Thompson, K. and Falo Jr., L.D. (1996) DNA-based immunization by *in vivo* transfection of dendritic cells. *Nat. Med.* 2: 1122-1128.

Coon, B., A, L.-L., Whitton, L. and von Herrarth, M. (1999) DNA immunization to prevent autoimmune diabetes. *J. Clin. Invest.* 104: 189-194.

Corr, M., Lee, D.J., Carson, D.A. and Tighe, H. (1996) Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* 184: 1555-1560.

Corr, M., von Damm, A., Lee, D.L. and Tighe, H. (1999) *In vivo* priming by DNA injection occurs predominantly by antigen transfer. *J. Immunol.* 163: 4721-4727.

Correale, J., Lund, B., McMillan, M., Ko, D.Y., McCarth, K. and Weiner, L.P. (2000) T cell vaccination in secondary progressive multiple sclerosis. *J. Neuroimmunol.* 107: 130-139.

Costa, G.L., Benson, J.M., Seroogy, C.M., Achacoso, P., Fathman, C.G. and Nolan, G.P. (2000) Targeting rare populations of murine antigen-specific T lymphocytes by retroviral transduction for potential application in gene therapy for autoimmune diseases. *J. Immunol.* 164: 3581-3590.

Croxford, J.L., O'Neil, J.K., Ali, R.R., Browne, K., Byrnes, A.P., Dallman, M.J., Wood, M.J.A., Feldmann, M. and Baker, D. (1998a) Local gene therapy with CTLA4-immunoglobulin fusion protein in experimental allergic encephalomyelitis. *Eur. J. Immunol.* 28: 3904-3916.

Croxford, J.L., Triantaphyllopoulos, K., Podhajcer, O.L., Feldmann, M., Baker, D. and Chernojovsky, Y. (1998b) Cytokine gene therapy in experimental allergic encephalomyelitis by

injection of plasmid DNA-cationic liposome complex into the central nervous system. *J. Immunol.* 160: 5181-5187.

Croxford, J.L., Triantaphyllopoulos, K., Neve, R.M., Feldmann, M., Chernajovsky, Y. and Baker, D. (2000) Gene therapy for chronic relapsing experimental allergic encephalomyelitis using cells expressing a novel soluble p75 dimeric TNF receptor. *J. Immunol.* 164: 2776-2781.

Dahlen, E., Hedlund, G. and Dawe, K. (2000) Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation. *J. Immunol.* 164: 2444-2456.

Dal Canto, R.A., Shaw, M.K., Nolan, G.P., Steinman, L. and Fathman, C.G. (1999) Local delivery of TNF by retrovirus-transduced T lymphocytes exacerbates experimental autoimmune encephalomyelitis. *Clin. Immunol.* 90: 10-14.

Danka, I., Williams, P., Herweijer, H., Zhang, G., Latendresse, J.S., Bock, I. and Wolff, J.A. (1997) High expression of naked plasmid DNA in muscles of young rodents. *Hum. Mole. Gene.* 6: 1435-1443.

Doe, B., Selby, M., Barnett, S., Baenziger, J. and Walker, C. (1996) Induction of cytotoxic T lymphoctyes by intramuscular immunization with plasmid DNA is facilitated by bone-marrow derived cells. *PNAS* 93: 8578-8583.

Dowty, M.E. and Wolff, J.A. (1994) Possible mechanisms of DNA uptake in skeletal muscle. In Wolff, J.A. (Ed.), *Gene Therapeutics: Methods and Applications of Direct Gene Transfer.*

Fathman, C.G., Costa, G.L. and Seroogy, C.M. (2000) Gene therapy for autoimmune disease. *Clin. Immunol.* 95: S39-S43.

Fernandes, J., Tardif, G., Martel-Pelletier, J., Lascau-Coman, V., Dupuis, M., Moldovan, F., Sheppard, M., Krishnan, B.R. and Pelletier, J.-P. (1999) *In vivo* transfer of interleukin-1 receptor antagonist gene in osteoarthritic rabbit knee joints. *Amer. J. Path.* 154: 1159-1169.

Gallichan, W.S., Balasa, B., Davies, J.D., and Sarvetnick, N. (1999) Pancreatic IL-4 expression results in islet-reactive Th2 cells that inhibit diabetogenic lymphocytes in the nonobese diabetic mouse. *J. Immunol.* 163: 1696-1703.

Gearson, C.L., Hussain, M.J., Vergani, D., and Peakman, M. (1997) Lymphocyte vaccination protects prediabetic non-obese diabetic mice from developing diabetes mellitus. *Diabetologia* 40: 1388-1395.

Geng, L., Solimena, M., Flavell, R.A., Sherwin, R.S. and Hayday, A.C. (1998) Widespread expression of an autoantigen-GAD65 transgene does not tolerize non-obese diabetic mice and can exacerbate disease. *PNAS* 95: 10055-10060.

Ghivizzani, S.C., Lechman, E.R., Tio, C., Mule, K.M., Chada, S., McCormick, J.E., Evans, C.H.

and Robbins, P.D. (1997) Direct retrovirus-mediated gene transfer to the synovium of the rabbit knee: implications for arthritis gene therapy. *Gene Ther.* 4: 977-982.

Girvin, A.M., Dal Canto, M.C., Rhee, L., Saloman, B., Sharpe, A., Bluestone, J.A. and Miller, S.D. (2000) A critical role for B7/CD28 costimulation in experimental autoimmune encephalomyelitis: a comparative study using costimulatory molecule-deficient mice and monoclonal antibody blockade. *J. Immunol.* 164: 136-143.

Gurunathan, S., Klinman, D.M. and Seder, R.A. (2000) DNA vaccine: immunology, application, and optimization. *Annu. Rev. Immunol.* 18: 927-974.

Guy, J., Qi, X. and Hauswirth, W.W. (1998) Adeno-associated viral-mediated catalase expression suppressed optic neuritis in experimental allergic encephalomyelitis. *PNAS* 95: 13847-13852.

Haddad, D., Ramprakash, J., Sedegah, M., Charoenvit, Y., Baumgartner, R., Kumar, S., Hoffman, S.L. and Weiss, W.R. (2000) Plasmid vaccine expressing granulocyte-macrophage colony-stimulating factor attracts infiltrates including immature dendritic cells into injected muscles. *J. Immunol.* 165: 3772-3781.

Hartmann, B., Bellman, K., Ghiea, I., Kleeman, R. and Kolb, H. (1997) Oral insulin for diabetes prevention in NOD mice: potentiation by enhancing Th2 cytokine expression in the gut through bacterial adjuvant. Diabetologia

Hermans, G., Medaer, R., Raus, J. and Stinissen, P. (2000) Myelin reactive T cells after T cell vaccination in multiple sclerosis: cytokine profile and depletion by additional immunizations. *J. Neuroimmunol.* 102: 79-84.

Ichino, M., Mor, G., Conover, J., Weiss, W.R., Takeno, M., Ishii, K.J. and Klinman, D.M. (1999) Factors associated with the development of neonatal tolerance after the administration of a plasmid DNA vaccine. *J. Immunol.* 162: 3814-3818.

Inaba, K., Inaba, M., Romani, N., Aya, H., Degushi, M., Ikehara, S., Muramatsu, S. and Steinman, R.M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693-1702.

Inobe, J.-I., Slavin, A.J., Komagata, Y., Chen, Y., Liu., L. and Weiner, H.L. (1998) IL-4 is a differentiation factor for transforming growth factor-β secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. *Eur. J. Immunol.* 28: 2780-2790.

Inoue, N., Kanaoka, S., Yamasaki, S., Arii, S. and Imamura, M. (1999) Production of a specific antibody and T helper 1-dominant cytokine by dendritic cells genetically modified with an adenovirus vector. *Immunol. Letters* 70: 77-81.

Iwasaki, A., Torres, C.A.T., Ohashi, P.S., Robinson, H.L. and Barber, B.H. (1997) The dominant

role of bone-marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* 159: 11-14.

Kaliyaperumal, A., Michaels, M.A. and Datta, S.K. (1999) Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: tolerance spreading impairs pathogenic function of autoimmune T and B cells. *J. Immunol.* 162: 5775-5783.

Kang, Y., Melo, M., Deng, E., Tisch, R., El-Amine, M., and Scott, D.W. (1999) Induction of hyporesponsiveness to intact foreign protein via retroviral-mediated gene expression: The Ig scaffold is important for the induction and maintenance of immune hyporesponsiveness. *Proc. Natl. Acad. Sci. USA.* 96: 8609-8614.

Klinman, D.M., Sechler, J.M.G., Conover, J., Gu, M. and Rosenberg, A.S. (1998) Contribution of cells at the site of DNA vaccination to the generation of antigen-specific immunity and memory. *J. Immunol.* 160: 2388-2392.

Krause, I., Blank, M. and Shoenfeld, Y. (2000) Immunomodulation of experimental autoimmune diseases via oral tolerance. *Critical Rev. Immunol.* 20: 1-16.

Krieg, A.M (2000) Minding the Cs and Gs. *Mole. Ther.* 1: 209-210.

Kurata, H., Liu, C.-B., Valkova, J., Koch, A.E., Yssel, H., Hirabayashi, Y., Inoue, T., Yokota, Arai, K.-I. (1999) Recombinant adenovirus vectors for cytokine gene therapy in mice. *J. Allergy Clin. Immunol.* 103: S471-S484.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* 227:680-685.

Lawson, B.R., Prud'homme, G.J., Chang, Y., Gardner, H.A., Kuan, J., Kono, D.H. and Theofilopoulos, A.N. (2000) Treatment of murine lupus with cDNA encoding IFN-γR/Fc. *J. Clin. Invest.* 106: 207-215.

Leclerc, C., Deriaud, E., Rojas, M. and Whalen, R.G. (1997) The preferential induction of a Th1 immune response by DNA-based immunization is mediated by the immunostimulatory effect of plasmid DNA. *Cell. Immunol.* 179: 97-106.

Leiter, E.H., Gerling, I.C. and Flynn, J.C. (1999) Spontaneous insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice: Comparisons with experimentally induced IDDM. In McNeil, J.H. (Ed.), *Experimental Models of Diabetes.*

Lernmark, A. (1996) Glutamic acid decarboxylase – gene to antigen to disease. J. Intern. Med. 240: 259-277.

Liblau, R., Tisch, R., Bercovici, N. and McDevitt, H.O. (1997) Systemic antigen in the treatment of T-cell-mediated autoimmune diseases. *Immunol. Today* 18: 599-604.

Lobell, A., Weissert, R., Storch, M.K., Svanholm, C., de Graaf, K.L., Lassmann, H., Andersson, R., Olsson, T. and Wigzell, H. (1998) Vaccination with DNA encoding an immunodominant myelin basic protein peptide targeted to Fc of immunoglobulin G suppresses experimental autoimmune encephalomyelitis. *J. Exp. Med.* 187: 1543-1548.

Lubberts, E., Joosten, L.A.B., van der Bersselaar, L., Helsen, M.M.A., Bakker, A.C., van Meurs, J.B.J., Graham, F.L., Richards, C.D. and van den Berg, W.B. (1999) Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevent cartilage destruction. *J. Immunol.* 163: 4546-4556.

Makarov, S.S., Olsen, J.C., Johnston, W.N., Schwab, J.H., Anderle, S.K., Brown, R.R. and Haskill, J.S. (1995) Retrovirus mediated in vivo gene transfer to synovium in bacterial cell wall-induced arthritis in rats. *Gene Ther.* 2: 424-428.

Makarov, S.S., Olsen, J.C., Johnston, W.N., Anderle, S.K., Brown, R.R., Baldwin Jr., A.S., Haskill, J.S. and Schwab, J.H. (1996) Suppression of experimental arthritis by gene transfer of interleukin 1 receptor antagonist cDNA. *PNAS* 93: 402-406.

Manickan, E., Kanangat, S., Rouse, R.J.D., Yu, Z. and Rouse, B.T. (1997) Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells. *J. Leuk. Biol.* 61: 125-132.

Matsumoto, Y., Jee, J. and Sugisaki, M. (2000) Successful TCR-based immunotherapy for autoimmune myocarditis with DNA vaccines after rapid identification of pathogenic TCR. *J. Immunol.* 164: 2248-2254.

Martino, G., Poliani, P.L., Marconi, P.C., Comi, G. and Furlan, R. (2000a) Cytokine gene therapy of autoimmune demyelination revisited using herpes simplex virus type-1-derived vectors. *Gene Ther.* 7: 1087-1093.

Martino, G., Poliani, P.L., Furlan, R., Maconi, P., Glorioso, J.C., Adorini, L. and Comi, G. (2000b) Cytokine therapy in immune-mediated demyelinating diseases of the central nervous system: a novel gene therapy approach. *J.Neuroimmunol.* 107: 184-190.

Matejuk, A., Vandenbark, A.A., Burrows, G.G., Bebo Jr., B.F. and Offner, H. (2000) Reduced chemokine and chemokine receptor expression in spinal cords of TCR BV8S2 transgenic mice protected against experiment autoimmune encephalomyelitis with BV8S2 protein. *J. Immunol.* 164: 3924-3931.

Mathisen, P.M., Yu, M., Johnson, J.M., Drazba, J.A. and Tuohy, V.K. (1997) Treatment of experimental autoimmune encephalomyelitis with genetically modified memory T cells. *J. Exp. Med.* 186: 159-164.

Meehan, R., Lewis, J., Cross, S., Nan, X., Jeppesen, P. and Bird, A. (1992) Transcriptional repression by methylation of CpG. *J. Cell. Sci., Suppl.* 116: 9-14.

Muir, A., Peck, A., Clare-Salzler, M., Song, Y.-H., Cornelius, J., Luchetta, R., Krischer, J. and Maclaren, N. (1995) Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-γ transcription. *J. Clin. Invest.* 95: 628-634.

Muller-Ladner, U., Roberts, C.R., Franklin, B.N., Gay, R.E., Robbins P.D., Evans, C.H. and Gay, S. (1997) Human IL-1Ra gene transfer into human synovial fibroblasts in chrondroprotective. *J. Immunol.* 158: 3492-3498.

Nitta, Y., Tashiro, F., Tokui, M., Shimada, A., Takei, I., Tabayashi, K. and Miyazaki, J. (1998) Systemic delivery of interleukin 10 by intramuscular injection of expression plasmid DNA prevents autoimmune diabetes in nonobese diabetic mice. *Hum. Gene. Ther.* 9: 1701-1707.

Norbury, C.C., Chambers, B.J., Prescott, A.R., Ljunggren, H.-G. and Watts, C. (1997) Constitutive macropinocytosis allow TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur. J. Immunol.* 27: 280-288.

Oligino, T., Ghizzani, S.C., Wolfe, D., Lechman, E.R., Krisky, D., Mi, Z., Evan, C.H., Robbins, P.D. and Glorioso, J.C. (1999) Intra-articular delivery of a herpes simplex virus IL-1Ra gene vector reduces inflammation in a rabbit model of arthritis. *Gene Ther.* 6: 1713-1720.

Pan, R.-Y., Xiao, X., Chen, S.-L., Li, J., Lin, L.-C., Wang, H.-J. and Tsao, Y.-P. (1999) Disease-inducible transgene expression from a recombinant adeno-associated virus vector in a rat arthritis model. *J. Virol.* 73: 3410-3417.

Pap, T., Gay, R.E. and Gay, S. (2000) Gene transfer: from concept to therapy. *Curr. Opin. Rheumatol.* 12: 205-210.

Petersen, J.S., Rimvall, K., Jorgensen, P.N., Hasselager, E., Moody, A., Hejaes, K., Clausen, J.T. and Dyrberg, T. (1998) Regulation of GAD expression in rat pancreas islets and brain by γ-vinyl-GABA and glucose. Diabetologia, 41: 530-535.

Piccirillo, C., Chang, Y. and Prud'homme. G.J. (1998) Transforming growth factor beta-1 (TGF-beta-1) somatic gene therapy prevents autoimmune disease in NOD mice *J. Immunol.* 161: 3950-3956.

Porgador, A., Irvine, K.R., Iwasaki, A., Barber, B.H., Restifo, N.P. and Germain, R.N. (1998) Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J. Exp. Med.* 188: 1075-1082.

Prud'homme, G.J. and Chang, Y. (1999) Prevention of autoimmune diabetes by intramuscular gene therapy with a nonviral vector encoding an interferon-gamma receptor/IgG1 fusion protein. *Gene Ther.* 6: 771-777.

Quattrocchi, E., Walmsley, M., Browne, K., Williams, R.O., Marinova-Mutafchieva, L., Buurman,

W., Butler, D.M. and Feldmann, M. (1999) Paradoxical effects of adenovirus-mediated blockade of TNF activity in murine collagen-induced arthritis. *J Immunol.* 163: 1000-1009.

Raz, E., Watanbe, A., Baird, S.M., Eisenberg, R.A., Parr, T.B., Lotz, M., Kipps, T.J. and Carson, D.A. (1993) Systemic immunological effects of cytokine genes injected into skeletal muscle. *PNAS* 90: 4523-4527.

Raz, E., Dudler, J., Lotz, M., Baird, S.M., Berry, C.C., Eisenburg, R.A. and Carson, D.A. (1995) Modulation of disease activity in murine systemic lupus erythematosus by cytokine gene delivery. *Lupus* 4: 286-292.

Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J.A., Roman, M., Swain, S.L., Spiegelberg, H.L. and Carson, D.A.. (1996) Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *PNAS* 93: 5141-5145.

Reynolds, J., Tam, F.W.K., Chandraker, A., Smith, J., Karkar, A.M., Cross, J., Peach, R., Sayegh, M.H. and Pusey, C.D. (2000) CD28-B7 blockade prevents the development of experimental autoimmune glomerulonephritis. *J. Clin. Invest.* 105: 643-651.

Robbins, P.D. and Ghivizzani, S.C. (1998) Viral vectors for gene therapy. *Pharmacol. Ther.* 80: 34-47.

Robert, C, Fuhlbrigge, R.C., Kieffer, J.D., Ayehunie, S., Hynes, R.O., Cheng, G., Grabbe, S., von Andrian, U.H. and Kupper, T.S. (1999) Interaction of dendritic cells with skin endothelium: a new perspective on immunosurveillance. *J. Exp. Med.* 189: 627-635.

Rothe, H., Hausmann, A., Casteels, K., Okamura, H., Kurimoto, M., Burkart, V., Mathieu, C. and Kolb, H. (1999) IL-18 inhibits diabetes development in nonobese diabetic mice by counterregulation of Th1-dependent destructive insulitis. *J. Immunol.* 163:1230-1236.

Rovere, P., Vallinoto, C., Bondanza, A., Crosti, M.C., Rescigno, M., Ricciardi-Castagnoli, P., Rugarli, C. and Manfredi, A.A. (1998) Cutting edge: bystander apoptosis trigger dendritic cells maturation and antigen-presenting function. *J. Immunol.* 161: 4467-4471.

Ruiz, P.J., Garren, H., Ruiz, I.U., Hirschberg, D.L., Nguyen, L.-V.T., Karpuj, M.V., Cooper, M.T., Mitchell, D.J., Fathman, C.G. and Steinman, L. (1999) Suppressive immunization with DNA encoding a self-peptide prevents autoimmune disease: modulation of T cell costimulation. *J. Immunol.* 162: 3336-3341.

Sallustro, F. and Lanzavecchia, A. (1999) Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. *J.Exp. Med.* 189: 611-614.

Santambrogio, L., Sato, A.K., Fischer, F.R., Dorf, M.E. and Stern, L.J. (1999) Abundant empty class II MHC molecules on the surface of immature dendritic cells. *PNAS* 96: 15050-15055.

Serreze, D.V., Fleming, S.A., Chapman, H.D. Richard, S.D., Leiter, E.H. and Tisch, R.M (1998)

B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J. Immunol.* 161: 3912-3918.

Serreze, D.V., Chapman, H.D., Post, C.M., Johnson, E.A., Suarez-Pinzon, W.L. and Rabinovitch, A. (2001) Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause, of diabetes resistance elicited by immunostimulation. *J. Immunol.* 166: 1352-1359.

Seroogy, C.M. and Fathman, C.G. (2000) The application of gene therapy in autoimmune diseases. *Gene Ther.* 7: 9-13.

Shaw, M.K., Lorens, J.B., Dhawan, A., DalCanto, R., Tse, H.Y., Tran, A.B., Bonpane, C., Eswaran, S.L., Brocke, S., Sarvetnick, N., Steinman, L., Nolan, G.P. and Fathman, C.G. (1997) Local delivery of interleukin 4 by retroviral-transduced T lymphocytes amerliorates experimental autoimmune encephalomyelitis. *J. Exp. Med.* 185: 1711-1714.

Shurin, M.R., Pandharipande, P.P., Zorina, T.D., Haluszczak, C., Subbotin, V.M., Hunter, O., Brumfield, A., Storkus, W.J., Maraskovsky, E. and Lotze, M.T. (1997) FLT3 ligand induces the generation of functionally active dendritic cells in mice. *Cell. Immunol.* 179: 174-184.

Sparwasser, T., Koch, E.-S., Vabulas, R.M., Heeg, K., Lipford, G.B., Ellwart, J.W. and Wagner, H. (1998) Bacterial DNA and immunostimulatory CpG oligodinucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* 28: 2045-2054.

Song, W., Kong, H.-L., Carpenter, H., Torii, H., Granstein, R., Rafli, S., Moore, M.A.S. and Crystal, R.G. (1997) Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J. Exp. Med.* 186: 1247-1256.

Tada, Y., Nagasawa, K., Ho., A., Morito, F., Ushiyama, O., Suzuki, N., Ohta, H. and Mak, T.W. (1999) CD28-deficient mice are highly resistant to collagen-induced arthritis. *J. Immunol.* 162: 203-208.

Takiguchi, M., Murakami, M., Nakagawa, I., Saito, I., Hashimoto, A. and Uede, T. (2000) CTLA4Ig gene delivery prevents autoantibody production and lupus nephritis in MRL/*lpr* mice. *Life Sci.* 66: 991-1001.

Tian, J., Lehmann, P.V. and Kaufman, D.L. (1997) Determinant spreading of T helper 2 (Th2) responses to pancreatic islet autoantigens. *J. Exp. Med.* 186: 2039-2043.

Tian, J., Olcott, A., Hanssen, L., Zekzer, D., and Kaufman, D.L. (1999) Antigen-based immunotherapy for autoimmune disease: from animal models to humans? *Immunol. Today* 20:190-196.

Tisch, R., Wang, B., Weaver, D.J., Liu, B., Bui, T., Arthos, J. and Serreze, D. (2001) Antigen-specific mediated suppression of β cell autoimmunity by plasmid DNA vaccination. *J. Immunol.*

166: 2122-2132.

Trembleau, S., Penna, G., Gregori, S., Chapman, H.D., Serreze, D.V., Magram, J. and Adorini, L. (1999) Pancreas-infiltrating Th1 cells and diabetes develop in IL-12-deficient nonobese diabetic mice. J. Immunol. 163: 2960-2968.

Triantaphyllopoulos, K.A., Croxford, J.L., Baker, D. and Chernajovsky, Y. (1998) Cloning and expression of murine IFNβ and a TNF antagonist for gene therapy of experimental allergic encephalomyelitis. *Gene Ther.* 5: 253-563.

Triantaphyllopoulos, K.A., Williams, R.O., Tailor, H. and Chervajovsky, Y. (1999) Amelioration of collagen-induced arthritis and suppression of interferon-γ, interleukin-12, and tumor necrosis factor α production by interferon-β gene therapy. *Arth & Rheum.* 42: 90-99.

Torres, C.A.T., Iwasaki, A., Barber, B.H. and Robinson, H.L. (1997) Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* 158: 4529-4532.

Ulmer, J.B., Deck, R.R., Dewitt, C.M., Donnelly, J.J. and Liu, M.A. (1996) Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunol.* 89: 59-67.

Vabulas, R.M., Pircher, H., Lipford, G.B., Hacker, H. and Wagner, H. (2000) CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J. Immunol.* 164: 2372-2378.

Velloso, L.A., Eizirik, D.L., Karlsson, F.A. and Kampe, O. (1994) Absence of autoantibodies against glutamate decarboxylase in the NOD mouse and low expression of GAD65 in mouse islets: differences between the animal model and human type 1 diabetes. *Clin. Exp. Immunol.* 96: 129-137.

Viney, J.L., Mowat, A.M., O'Malley, J.M., Williamson, E. and Fanger, N.A. (1998) Expanding dendritic cells *in vivo* enhances the induction of oral tolerance. *J. Immunol.* 160: 5815-5825.

von Herrath, M. and Holz, A. (1997) Pathological changes in the islet milieu precede infiltration of islets and destruction of beta-cells by autoreactive lymphocytes in a transgenic model of virus-induced IDDM. *J. Autoimmunity.* 10:231-238.

Whalen, J.D., Lechman, E.L., Carlos, C.A., Weiss, K., Kovesdi, I., Glorioso, J.C., Robbins, P.D. and Evans, C.H. (1999) Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. *J Immunol.* 162: 3625-3632.

Whiteacre, C.C. and Campbell, K.A. (2000) Oral tolerance as a treatment modality for autoimmune diseases. In: Fathman, C.G. (Ed.), *Current Directions in Autoimmunity*, Vol. 2. Karger, New York. pp. 1-23.

Wildbaum, G., Westermann, J., Maor, G. and Karin, N. (2000) A targeted DNA vaccine encoding Fas ligand defines its dual role in the regulation of experimental autoimmune encephalomyelitis. *J. Clin. Invest.* 106: 671-679.

Williamson, E., O'Malley, J.M. and Viney, J.L. (1999) Visualizing the T-cell response elicited by oral administration of soluble protein antigen. *Immunology* 97: 565-572.

Wiest-Ladenburger, U., Fortnagel, A., Richter, W., Reimann, J. and Boehm, B.O. (1998) DNA vaccination with glutamic acid decarboxylase (GAD) generates a strong humoral response in BALB/c, C57BL/6 and in diabetes-prone NOD mice. *Horm. Metab. Res.* 30: 605-609.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P.L. (1990) Direct gene transfer into muscle in vivo. *Science* 247: 1465-1468.

Wolff, J.A., Ludtke, J.J., Acsadi, G., Williams, P. and Jani, A. (1992) Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mole. Gene.* 1: 363-369.

Woods, J.M., Tokuhira, M., Berry, J.C., Katchke, K.J., Kurata, H., Damergis Jr., J.A., Arai, K.-I. and Koch, A.E. (1999) Interleukin-4 adenoviral gene therapy reduces production of inflammatory cytokines and prostaglandin E2 by rheumatoid arthritis synovium ex vivo. *J. Investig. Med.* 47: 285-292

Woods, J.M., Katschke Jr., K.J., Tokuhira, M., Kurata, H., Arai, K.-I., Campbell, P.L.,and Koch, A.E. (2000) Reduction of inflammatory cytokines and prostaglandin E2 by IL-13 gene therapy in rheumatoid arthritis synovium. *J. Immunol.* 165: 2755-2763.

Xu, L.Y., Huang, Y.M., Yang, J.S., Van Der Meide, P.H., Link, H. and Xiao, B.G. (2000) Suppression of ongoing experimental allergic encephalomyelitis (EAE) in Lewis rats: synergistic effects of myelin basic protein (MBP) peptide 68-86 and IL-4. *Clin. & Exp. Immunol.* 120: 526-531.

Yew, N.S., Wang, K.X., Przybylska, M., Bagley, R.G., Stedman, M., Marshall, J., Scheule, R.K. and Cheng, S.H. (1999) Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid:pDNA complexes. *Hum. Gene Ther.* 10: 223-234.

Yew, N.S., Zhao, H., Wu, I.-H., Song, A., Tousignant, J.D., Przybylska, M. and Cheng, S.H. (2000) Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mole. Ther.* 1: 255-262.

Young, D.A., Lowe, L.D., Booth, S.S., Whitter, M.J., Nicholson, L., Kuchroo, V.K. and Collins, M. (2000) IL-4, IL-10, IL-13 and TGF-β from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. *J. Immunol.* 164: 3563-3572.

Youssef, S., Wildbaum, G., Maor, G., Lanir, N., Gour-Lavie, A., Grabie, N. and Karin, N. (1998) Long-lasting protective immunity to experimental autoimmune encephalomyelitis following

vaccination with naked DNA encoding C-C chemokines. *J. Immunol.* 161: 3870-3879.

Zhang, H., Yang, Y., Horton, J.L., Samoilova, E.B., Judge, T.A., Turka, L.A., Wilson, J.M. and Chen, Y. (1997) Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas) ligand gene transfer. *J. Clin. Invest.* 100:1951-1957.

Zhu. J., Deng, G.-M., Levi, M., Wahren, B., Diab, A., van der Meide, P.H. and Link, H. (1998) Prevention of experimental autoimmune neuritis by nasal administration of P2 protein peptide 57-81. *J. Neuropath. and Exp. Neur.* 57: 291-301.