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# Cytokine gene transfer in the therapy of autoimmune diseases

**Review Article**

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**Abbreviations:** adjuvant arthritis, (AA); adeno-associated virus, (AAV); anti-phospholipid syndrome, (APS); collagen-induced arthritis, (CIA); collagen type II, (CII); cytotoxic T lymphocytes, (CTL); dendritic cells, (DC); delayed type hypersensitivity, (DTH); experimental allergic encephalomyelitis, (EAE); experimental autoimmune uveoretinitis, (EAU); Epstein-Barr virus, (EBV); glomerulonephritis, (GN); intradermal, (i.d.); intramuscular, (i.m.); intravenous, (i.v.); multiple sclerosis, (MS); natural killer, (NK); non-obese diabetic, (NOD); nephrotoxic serum, (NTS); ovalbumin, (OVA); rheumatoid arthritis, (RA); severe combined immune deficiency, (SCID); streptococcal cell walls, (SCW); toll-like receptors, (TLR); trinitrobenzene sulphonic acid, (TNBS); virally encoded IL-10, (vIL-10)

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## Summary

**Delivery of DNA coding for disease-modifying proteins has been experimented as a therapeutic option for over 15 years. Since the first clinical trial in 1989, about 900 trials have been approved worldwide in order to demonstrate safety, feasibility, and therapeutic benefits, cancer being the most common disease indication. Systems for efficient, safe, and targeted delivery of DNA encoding therapeutic proteins are actively studied and include viral vectors (mostly retro- and adenoviruses), non-viral vectors, and delivery of naked plasmid DNA. In autoimmune diseases, classical therapies are aiming at inhibiting either lymphocyte activation (immunosuppressive agents), or the downstream inflammatory effector mechanisms responsible for organ and tissue destruction (anti-inflammatory drugs). Newer therapies are being developed targeting inflammatory and immunostimulating cytokines, with the use of the so-called "cytokine traps" (recombinant and chimeric proteins and antibodies which capture and inhibit pathological cytokines) or of recombinant regulatory cytokines able to inhibit inflammatory cytokine production. In this context, gene therapy and DNA vaccination are very promising approaches, which could avoid problems connected with life-long treatments with protein drugs and achieve optimal efficacy. In this review, two parallel approaches will be examined, delivery of genes encoding cytokine inhibitors (regulatory cytokines, cytokine inhibitors including cytokine receptors), and DNA vaccination with genes coding for the pathogenic cytokines, to trigger an endogenous anti-cytokine response with therapeutic effects.**

## I. Introduction

### A. Cytokines in chronic inflammatory and autoimmune diseases

Autoimmune diseases include a variety of chronic syndromes, characterised by anomalous and uncontrolled reaction of the host immune system against self-antigens which causes progressive tissue damage and eventual organ failure. Although the triggering causes of autoimmune reaction are not fully defined (genetic causes, environmental factors, cross-reacting infections may take part to it), autoimmunity in many cases starts as an inflammatory-type reaction with production of high amounts of inflammatory cytokines and chemokines, active in stimulating lymphocyte proliferation and inhibiting their apoptosis, and amplifying autoantibody production. Down-regulation of the innate/inflammatory reaction occurs as a consequence of inflammation (*e.g.*, inflammatory cytokines activate anti-inflammatory mechanisms in an autoregulatory circuit) and as a consequence of depletion of the triggering stimulus (*e.g.*,

when an infectious microorganism is eliminated by defence mechanisms), and re-establishes the physiological homeostatic equilibrium. In autoimmune reactions, the down-regulatory control circuits are either defective (*e.g.*, by genetic mutations), or circumvented (*e.g.*, suppressed by altered regulatory cytokines), or do not come into play (*e.g.*, because of persistence of the self triggering stimulus). The defective down-regulation of the innate/adaptive immune response amplifies and establishes the inflammatory/autoimmune reaction.

## B. Therapeutic use of cytokines and cytokine receptors/inhibitors

Cytokines are becoming important targets in new therapeutic approaches to inflammatory diseases. The role of inflammatory and regulatory cytokines in autoimmune pathogenesis and in the downstream destructive effects has been widely studied in experimental models, in particular with the use of organ/tissue specific cytokine knock-out or transgenic mice. Evidence in animal models has been in general confirmed in autoimmune patients, where enhanced levels of several cytokines (identified as pathogenic in experimental models) strictly correlate with disease severity. Inflammatory cytokines and chemokines, mostly responsible of the downstream tissue destructive effects, thus became the first target for the development of anti-cytokine therapies in autoimmunity. The pivotal role of TNF-α and IL-1β in tissue destruction in rheumatoid arthritis (RA) and in Crohn's disease has led to the development of the first biotechnological "cytokine traps" for therapeutic use. Anti-TNF-α therapy in RA is in clinical use since few years with three different drugs: infliximab, a human/murine chimeric monoclonal antibody to TNF-α (Charles et al, 1999); etanercept, a recombinant fusion protein encompassing the extracellular domain of the type II TNF-R fused to the constant portion of human IgG1 (Lyseng-Williamson and Plosker, 2004); adalimumab, a fully human anti-TNF-α neutralising monoclonal antibody (Furst et al, 2003; Toussirot and Wendling, 2004). Likewise, anakinra (the recombinant form of the cytokine IL-1Ra, the natural receptor antagonist of IL-1β) is in clinical trial for RA and Crohn's disease (Burls and Jobanputra, 2004). The drawback in the clinical use of these cytokine traps is that very frequent intravenous administrations are necessary to achieve neutralisation of the target inflammatory cytokine and clinical amelioration, which however is readily lost upon discontinuation of drug delivery. A new generation of cytokine traps with a better pharmacokinetic and pharmacodynamic profile is being developed, by engineering the extracellular portion of cytokine receptors on the constant region of IgG (Economides et al, 2003). Chimeric constructs encompassing heterodimers or in-line dimers of soluble receptor portions fused to constant IgG fragment have been prepared for IL-1 (IL-1RAcP and IL-1RI), IL-4 (IL-2Rγ and IL-4Rα), and IL-6 (IL-6Rα and gp130). These cytokine traps can potently inhibit the cytokine activity *in vivo* in experimental animals. The IL-1 trap also significantly inhibited bone erosion and arthritic symptoms in an experimental model of RA (collagen-induced arthritis, CIA, in the mouse). Thus, their development and use in autoimmune diseases may bypass many of the problems of cytokine inhibitors in chronic therapy (Dinarello , 2003).

## C. Gene therapy and cDNA immunisation - vectors, advantages, drawbacks

Gene therapy is defined as the introduction of DNA into a host for therapeutic purposes. After introduction, DNA is taken up by the hosts cells, which express the gene product encoded by the exogenous DNA. Thus, in the immune system gene therapy could be used to compensate known gene defects, to intervene in signal transduction processes, or to deliver immunomodulating factors, *e.g.* cytokines.

The first human trial of gene therapy was performed in 1989 by the group of Steve Rosenberg in cancer patients (Rosenberg et al, 1990). Since that time over 900 clinical trials of gene therapy have been approved, some of which have been completed while others are still ongoing (Edelstein et al, 2004). The delivery of DNA to the host is advantagous over the administration of peptides or attenuated pathogens since it is more stable after transfer and it is easy and cost-effective to prepare in large quantities.

In human trials today the most widely used vectors are of retroviral origin, derived from a murine retrovirus. Such vector systems target only dividing cells and transduce them with a high efficiency. However, the size of the cDNA that can be delivered with a retrovirus is very limited. Another disadvantage is the property of retrovirally delivered cDNA to stably insert into the host genome. If the cDNA insertion (which is random) alters the expression of important control genes (*e.g.*, proto-oncogenes or a tumour suppressor genes), retroviral gene therapy may also lead to serious diseases (*e.g.*, malignancies). Such theoretical risk has become reality in 2002, when two of ten SCID patients which were treated with retrovirally based gene therapy developed leukemia-like conditions due to insertional mutations (Hacein-Bey-Abina et al, 2003a, b).

The second most used vector system is adenoviral, based on serotype 5 adenoviruses, in which the regions E1a and E1b are deleted in order to prevent replication. Adenoviral vectors can carry longer cDNAs than retroviral vectors, but the size is still limited, and they can also infect non-dividing cells. In addition, they yield a high efficiency of transduction and a high level of expression. The main drawback of adenoviral vectors is their immunogenicity, with the risk of provoking severe immune and inflammatory responses (Raper et al, 2003).

In contrast to the delivery systems mentioned above, delivery of naked DNA does not imply size restrictions and has no problems of immunogenicity. However, the level of expression is lower than that achieved by viral vectors. In some trials, attempts were made to enhance the cellular uptake of the naked cDNA by the addition of cationic lipids or with mechanical treatment of the recipient tissue (Losordo et al, 2002; Fortuin et al, 2003; Taniyama et al, 2002; Wells, 2004).

## II. Cytokine and anti-cytokine therapy with gene transfer

The *in vivo* administration of cytokine genes has begun in the late 80s (Nishihara et al, 1988) and has been studied since that time in various experimental disease models. A number of cytokine genes was introduced by several techniques in a variety of models. Among the best studied genes for cytokines and cytokine receptors, in particular in autoimmune diseases, are those coding for regulatory cytokines IL-10, TGF-β, IL-1Rα, and for the soluble inhibitory cytokine receptors sTNF-R, sIL-1R, IL-18BP, and sIFN-γR. The determination of optimal route and vector system to transfer the DNA into the host cells is still a matter of discussion. Studies describing the application of naked DNA are becoming more and more abundant, but viral delivery systems, mostly adenoviral or adeno-associated vectors, are still the most common in use.

### A. Delivery systems

A number of approaches are used to deliver DNA into the host cells. These include application of naked DNA, DNA complexed with liposomes, or DNA transferred by viral vectors. DNA can be applied systemically or locally, either by injection of the DNA or by reintroduction of *ex vivo* engineered host cells at the designated site (intratumour or inflammatory sites, *e.g.* joints in RA) (Piccirillo and Prud'homme, 2003). Cells successfully used as vehicle are T cells, fibroblasts, and dendritic cells (DC) (Morita et al, 2001; Nakajima et al, 2001; Rabinovich et al, 1999).

A simple way of application is the injection of suspended DNA into the host's muscle. The injected DNA is taken up by muscle cells by still undefined mechanisms. The uptake can be enhanced either chemically by treatment with cardiotoxin (Davis et al, 1993) or bupivacaine (Wells, 1993; Vitadello et al, 1994), or by electroporation of the muscle (Aihara and Miyazaki, 1998). Injection of DNA into the host's muscle is a kind of local application since the DNA is thought not to be distributed throughout the body by the blood or lymphatic system. However, plasmid DNA has been detected in plasma and in association with blood cells for several hours after intramuscular (i.m.) inoculation (Winegar et al, 1996). It was also shown that injected naked DNA is transported beyond regional lymph nodes, *e.g.*, to sites of chronic inflammation, with the contribution of antigen-presenting cells. (La Cava et al, 2000). In addition, the ablation of the injected muscle as soon as one minute after inoculation did not abrogate the induced humoral immune response (Torres et al, 1997). Thus, one has to consider that DNA applied locally to the muscle can diffuse to sites other than that of application.

The injection of suspended DNA is the most simple and cost-effective way. By far more complex is the gene-gun method since it requires some technical equipment and, in addition the the preparation of the DNA, its efficient and reproducible coating onto the carrier gold particles. On the other hand, delivery of naked DNA by gene-gun is highly efficient. In genetic immunisation studies it was shown that far less DNA has to be applied by gene-gun as compared to needle injection, to obtain the same level of expression of the exogenous gene (Barry and Johnston, 1997; Feltquate et al, 1997; Pertmer et al, 1995). The magnitude and duration of the DNA-induced immune response depends on the route of delivery. Antibody and CTL (cytotoxic T lymphocyte) responses after intradermal (i.d.) application were found to be fast and more transient, while after i.m. injection they were slower but more sustained (Ito et al, 2003). Thus, to obtain an optimal (*i.e.*, fast and sustained) immune response after genetic immunisation, DNA should be applied by gene-gun using both routes, i.m. and i.d.

The nature of the immune response also seems to depend on the application route. While needle injection induces a predominantely Th1 response, the response after gene-gun administration is mainly of the Th2 type (Barry and Johnston, 1997; Feltquate et al, 1997). A more recent study provides additional information about the influence of the form of the expressed protein on the immune response. By i.d. gene-gun immunisation, three forms (cytosolic, secreted, transmembrane) of the model protein ovalbumin (OVA) all induced a specific antibody response, while only the cytosolic and the transmembrane forms were able to generate an additional CTL reaction. In contrast, by i.m. injection only the secreted protein could generate an antibody response, whereas the CTL reaction was induced by the secreted and the transmembrane OVA forms. Thus, it could be possible to obtain different types of immune response by varying the route of DNA administration and the cellular localisation of the expressed protein (Morel et al, 2004).

In the case of autoimmune-prone hosts, i.m. injection of DNA not only induces an immune response against the expressed protein, but it can also lead to the generation of anti-DNA antibodies. Thus, in autoimmune diseases the therapeutic effect of genetic immunisation, due to response against the expressed protein, could be attenuated or even abrogated due to the concomitant acceleration of autoimmune response (increased levels of anti-dsDNA and anti-nuclear antibodies) driven by the exogenously applied DNA which mimicks the autoantigen "cellular DNA" (MacColl et al, 2001). However, although DNA administration might accelerate autoimmune disease, several studies demonstrate the significant beneficial effects of gene therapy. Among these, the administration of DNA coding for cytokines or their receptors is of particular relevance for the very promising results obtained. In the following sections we will discuss some of the main cytokine molecules used in gene therapy of autoimmunity. The most relevant studies of cytokine/receptor gene delivery in experimental models of autoimmune diseases are summarised in **Table 1**.

### B. Regulatory cytokines: IL-10

IL-10 is an anti-inflammatory and regulatory cytokine. It is produced by lymphocytes, monocytes, and (at least in mice) keratinocytes, and inhibits the synthesis of inflammatory and Th1-derived cytokines such as IL-1, TNF-α, IL-6, IL-2, and IFN-γ. A virally-encoded IL-10

**Table 1.** Cytokines/receptor gene delivery in models of autoimmune diseases

| Cytokine/Receptor | Disease Model | Outcome | Reference |
|---|---|---|---|
| **Cytokines** | | | |
| IL-10 | TNBS colitis, IL-10$^{-/-}$ colitis | ↓ loss body weight and histological score; ↓ stool IL-1β and sTNF-R2; ↓ acute phase proteins; ↓ colonic IFN-γ and IL-6; ↓ spleen TNF-α, IFN-γ, RANTES (only IL-10$^{-/-}$) | Lindsay et al, 2001, 2002 |
| | NTS nephritis in rats | ↓ glomerular crescent formation and leukocyte infiltrate; ↓ proteinuria and kidney dysfunction; ↓ glomerular expression IFN-γ, TNF-α, MCP-1 | Higuchi et al, 2003 |
| | Experimental autoimmune uveoretinitis in rats and mice | ↓ ocular pathological signs; ↓ spleen IFN-γ and IL-2 | de Kozak et al, 2002; Verwaerde et al, 2003 |
| | Diabetes in NOD mice | ↓ diabetes incidence; ↓ severe insulitis; normalisation islet insulin content; ↓ anti-insulin autoantibodies | Goudy et al, 2001; Goudy et al, 2003; Koh et al, 2000; Nitta et al, 1998; Yang et al, 2002 |
| | Islet transplantation in NOD mice | ↑ islet graft takes; normalisation glycemia; ↓ lymphocytic infiltrate; ↑ anti-oxidant enzymes | Zhang et al, 2003 |
| | SCW arthritis | ↓ erosive disease (local) both ipsilaterally and contralaterally | Miagkov et al, 2002; Whalen et al, 1999 |
| TGF-β | Lupus in MRL *lpr/lpr* mice | ↓ autoantibodies to chromatin | Raz et al, 1993 |
| | SCW arthritis | ↓ erosive disease (i.m.); ↑ cartilage matrix degradation (local) | Song et al, 1998; Zhibao et al, 2004 |
| | Diabetes in NOD mice | ↑ plasma TGF-β; ↓ pancreatic IFN-γ and IL-1, DTH, insulitis, diabetes | Piccirillo et al, 1998 |
| | Wounds in diabetic BKS.Cg-*m*$^{+/+}$ *Lepr*$^{db}$ mice | Accelerated wound healing | Chesnoy et al, 2003 |
| | EAE in mice (local) | ↓ disease score | Croxford et al, 1998 |
| IL-4 | Diabetes in NOD mice | Undetectable circulating IL-4; protection from disease development (chimera IL-4-IgG1)<br>No effect on disease development (IL-4)<br>↓ insulitis and diabetes development; induction circulating IL-4 (IL-4, epidermal delivery) | Cameron et al, 2000; Chang and Prud'homme, 1999; Goudy et al, 2001 |
| | EAE in mice (local) | ↓ disease score | Croxford et al, 1998 |
| IL-10 and IL-4 | Diabetes in NOD mice | ↓ diabetes development; ↑ number intact islets; ↓ islet lymphocytic infiltrate; ↓ glucose levels | Ko et al, 2001 |
| IL-1Ra | CIA | Protection against disease development (i.m.) | Kim et al, 2003 |
| CCL2/MCP-1 truncated form | Lupus in MRL *lpr/lpr* mice | ↓ renal damage; ↑ lifespan | Shimizu et al, 2004 |
| IL-12 p40 (inhibitory) | Lupus in MRL *lpr/lpr* mice | No effect on lupus-like symptoms | Hagiwara et al, 2000 |
| IL-2 | Lupus in MRL *lpr/lpr* mice | ↑ autoAb to chromatin | Raz et al, 1993 |
| IFN-β | EAE in mice (local) | ↓ disease score | Croxford et al, 1998 |
| IFN-γ | Diabetes in NOD mice | Acceleration diabetes | Piccirillo et al, 1998 |
| IL-12 p35/p40 | Wild type mice | Systemic Th1 responses, NK cell activation | Watanabe et al, 1999 |
| | Lupus in MRL *lpr/lpr* mice | ↑ IFN-γ, ↑ IgG2a; amelioration lupus-like symptoms | Hagiwara et al, 2000 |
| IL-18 | OVA-primed mice | ↑ IL-18; ↑ Th1 response | Kim et al, 2004; Li et al, 2004 |
| **Cytokine receptors** | | | |
| IFN-γR | Lupus in MRL *lpr/lpr* mice | ↓ disease symptoms (also when fully established) (i.m.) | Lawson et al, 2000 |
| | Diabetes in NOD mice | Protection from disease induction | Chang and Prud'homme, 1999 |
| sTNF-R1 | CIA | ↓ disease progression (local)/no effect (i.m.); ↓ disease progression (chimera with IgG; i.m.) | Mukherjee et al, 2003; Bloquel et al, 2004 |
| | EAE in mice (local) | Delay disease onset | Croxford et al, 1998 |
| sTNF-R2 | EAE in mice (local) | ↓ disease score and delay disease onset (dimeric form) | Croxford et al, 1998 |
| IL-18BP | CIA | ↓ disease symptoms | Smeets et al, 2003a |
| sIL-1RAcP | CIA (local) | ↓ local erosion; no systemic effect | Smeets et al, 2003b |

molecule (vIL-10) is very similar in structure and activity to mammalian IL-10. The therapeutic effects of IL-10 in a series of diseases has been defined in studies with administration of the recombinant protein or in genetic knock-out animal models. In addition, the DNA coding for IL-10 has been successfully used in gene therapy studies, in tumour as well as in autoimmunity models.

Autoimmunity leads to inflammatory/immune reaction against self-antigens. Autoimmune inflammation can be broadly divided into two subclasses. In the first case the reaction is directed against locally expressed autoantigens, thus inflammation occurs almost exclusively at sites where the antigen is present (plaques in the brain of multiple sclerosis -MS- patients; cartilage and bone erosion in RA joints). The second type is a systemic disease with destructive inflammation occurring in the end-organs (*e.g.*, kidney and lung), consequent to the deposition of immune complexes, abundantly generated by the potent humoral autoreaction. Autoimmune complex deposition can lead to severe organ damage and eventually death.

The beneficial effect of IL-10 gene therapy has been shown in several models using different delivery routes. In nephrotoxic serum (NTS) nephritis, an experimental rat model of crescentic glomerulonephritis (GN), hydrodynamic-based intravenous (i.v.) administration of a rather large amount of a naked plasmid coding for vIL-10 after induction of crescentic GN was able to suppress glomerular macrophage and $CD4^+$ T cells accumulation and production of IFN-γ, TNF-α, and the chemokine CCL2. This therapy ameliorated disease symptoms, which still occurred in the empty vector-treated control rats (Higuchi et al, 2003). The adenoviral-mediated transfer of IL-10 in two mouse models of Crohn's disease (TNBS-induced colitis; spontaneous colitis in $IL\text{-}10^{-/-}$ mice) also ameliorated disease by decreasing local proinflammatory cytokine production (Lindsay et al, 2002; Lindsay et al, 2001). In another animal model of autoimmunity, the experimental autoimmune uveoretinitis (EAU), systemic injection of a single dose of adenovirus coding for vIL-10 also had a beneficial effect (de Kozak et al, 2002). Additionally, these authors showed that administration of vIL-10 via *ex vivo* transduced retinal cells is also a feasible approach (Verwaerde et al, 2003).

All the above studies were performed in models which develop predominantly Th1-mediated autoimmune diseases. IL-10 administration leads to a decreased local production of proinflammatory cytokines such as IFN-γ and TNF-α. Thereby, the Th1 phenotype was reduced, shifting the Th1-Th2 profile towards Th2, thus ameliorating disease symptoms and outcome. Cellular mechanisms responsible for the beneficial effect of IL-10 administration were analysed in NOD (non-obese diabetic) mice, a model for autoimmune diabetes. In this model, autoimmune diabetes was suppressed by administration of either rIL-10 protein, IL-10 expression plasmid, IL-10 cDNA-transduced islet-specific T cells, or vIL-10 gene by an adeno-associated viral vector (Yang et al, 2002; and references therein). Splenocytes from vIL-10-treated mice (enriched in $CD4^+/CD25^+$ regulatory T cells as compared with controls) could block transfer of diabetes (Goudy et al, 2003). Thus, it is tempting to speculate that the beneficial effect of permanent IL-10 delivery to diabetes-prone NOD mice depends on the increased presence of $CD4^+/CD25^+$ regulatory T cells. However, the final proof of this hypothesis is still missing.

As discussed above (Section II.A.: Delivery Systems), local application of naked DNA is effective not only at the site of injection, but also at distant sites. This phenomenon is described as the contralateral effect. In models of experimental arthritis, the local transfer of viral DNA to one of the affected joints ameliorated disease both in the treated and in the untreated contralateral joint (Lechman et al, 1999; Whalen et al, 1999). The vIL-10 protein could be detected in the injected but not in the contalateral joint, indicating that systemic protection after local injection does not depend on the distribution of DNA or of the encoded protein throughout the body. The contralateral effect is antigen-specific, and not due to a generalised IL-10-dependent immunosuppression (Lechman et al, 2003). Thus, it may be possible that a population of antigen-specific $CD4^+/CD25^+$ regulatory T cells, which develops following antigen stimulation in an IL-10 enriched milieu, is responsible for the protective effect. In contrast with this hypothesis is the study by Whalen and coworkers, which demonstrates in a mouse model of DTH (delayed type hypersensitivity; where the contralateral effect of vIL-10 DNA therapy is also evident) that the effect depends on modified DC and/or macrophages which, by presenting the antigen in the context of high IL-10, generate unresponsive T cells (Whalen et al, 2001).

## C. Regulatory cytokines: IL-4

IL-4 is an anti-inflammatory cytokine produced by Th2 cells, which is involved in Th2 responses and in the regulation of inflammatory Th1 responses. In several autoimmune diseases, hyper-activation of Th1 responses has a major pathological role, thus IL-4 may have a re-balancing effect on the Th1-Th2 ratio with therapeutic effects.

In diabetic NOD mice, i.m. inoculum of a plasmid coding for a chimeric construct IL-4-IgG1 could very efficiently decrease autoimmune insulitis and diabetes, despite the lack of detectable levels of circulating IL-4 (Chang Y and Prud'homme G J, 1999). In another study, i.m. inoculum of IL-4 gene with an AAV (adeno-associated virus) vector had no effect on disease development in NOD mice (Goudy et al, 2001). However, epidermal delivery of the IL-4 gene with a EBV (Epstein-Barr virus) vector was able to induce a persistent level of circulating IL-4 and in the induction of Th2 responses, resulting in significant protection from disease development (Cameron et al, 2000).

In murine experimental allergic encephalomyelitis (EAE), a model of MS, intracerebral delivery of IL-4 naked DNA with cationic liposomes resulted in significant decrease of the disease symptoms (Croxford et al, 1998). Local delivery without liposomes and systemic administration had no effect.

## D. Regulatory cytokines: TGF-β

TGF-β, a product of both immune and non-immune cells, is another cytokine with potent anti-inflammatory and regulatory activities, which is also active in development, differentiation, tissue repair, and tumourigenesis. TGF-β has suppressive effects on the immune system since it inhibits the proliferation of T and B cells. In addition, it suppresses the IFN-γ-induced cytotoxic activity of natural killer (NK) cells, the activity of CTL, and the proliferation of lymphokine-activated killer cell precursors. TGF-β also influences the secretion of immunoglobulins by B lymphocytes, by inhibiting the synthesis of IgG and IgM while stimulating the synthesis of IgA.

The therapeutic potential of systemic administration of TGF-β in inflammation and immune-mediated pathologies has been shown in several studies, a number of them including gene transfer in autoimmune models.

In an early study using repeated inoculation of a plasmid with the gene for TGF-β, it was reported that the production of anti-DNA autoantibodies in lupus-prone MRL *lpr/lpr* mice was reduced, whereas the opposite effect (increased anti-chromatin antibodies) was obtained with IL-2 expression vector (Raz et al, 1993, 1995). In diabetic NOD mice, injection of TGF-β DNA also had therapeutic effects. Piccirillo and coworkers describe the amelioration of Th1-mediated diabetic disease in NOD mice after i.m. administration of plasmids coding for TGF-β, due to the reduced production of proinflammatory cytokines and a shift of the IFN-γ/IL-4 ratio towards IL-4, thus towards an anti-inflammatory Th2 profile (Piccirillo et al, 1998). As a control, inoculation of an IFN-γ plasmid accelerated disease development (Piccirillo et al, 1998). In addition, islet cells of NOD mice transfected with TGF-β DNA are able to delay diabetes recurrence after transplantation in NOD recipients (Suarez-Pinzon et al, 2002). In another mouse model of diabetes, i.d. injection of TGF-β DNA was able to accelerate wound healing (Chesnoy et al, 2003), indicating that TGF-β not only ameliorates the progression of inflammatory diabetic disease but also at least some of its associated effects.

TGF-β naked DNA inoculated intracerebrally with cationic liposomes could decrease disease severity in murine EAE, whereas local delivery without liposomes or i.m. administration had no effect (Croxford et al, 1998).

TGF-β plasmids have been also used in animal models of RA. In rats, a single i.m. injection of naked DNA for TGF-β suppressed the evolution of chronic erosive disease of streptococcal cell wall (SCW)-induced arthritis (Song et al, 1998). In contrast, the intra-articular injection in rabbits of an adenoviral vector expressing TGF-β resulted in enhanced cartilage matrix degradation (Zhibao et al, 2004). Besides the different animal models, reasons for these contrasting results may be route and/or time of TGF-β DNA administration, and also the expression level of the transgene due to the two different vector systems used. Experiments to clarify this issue have yet to be performed.

## E. Soluble cytokine receptors: sTNF-R

TNF-α is one of the first cytokines detectable in serum after an microbial infection, secreted by stimulated macrophages, monocytes, neutrophils, T cells, and NK cells. TNF-α is highly inflammatory and has a wide spectrum of biological activities, *e.g.*, it causes cytolysis and cytostasis of many types of tumour cells, it enhances phagocytosis and cytotoxicity by neutrophils, and it also modulates the expression of many proinflammatory cytokines, including IL-1, IL-6, IL-8, and GM-CSF. In addition, TNF-α is a potent chemoattractant for neutrophils and can induce the synthesis of a number of chemoattractants in a cell type and tissue-specific manner.

Two receptors for TNF-α have been described, a transmembrane molecule of 55 kDa (TNF-R1, CD120a, TNFRSF1A) and a second chain of 75 kDa (TNF-R2, CD120b, TNFRSF1B). TNF-R1 is expressed particularly on cells susceptible to the cytotoxic action of TNF-α, while TNF-R2 is present on many cell types, in particular those of myeloid origin. The contrasting activities of TNF-α on various cell types, *i.e.* growth-promoting *vs.* growth-inhibiting activities, are probably mediated by the differential expression and/or regulation of multiple receptors in combination with other distinct receptor-associated proteins.

In human RA, enhanced serum and synovial fluid levels of TNF-α are found, which suggests a pathological role for TNF-α in RA. Tissues expressing TNF-α and the two TNF-Rs are found within the synovial membrane and the cartilage, indicating that TNF-α may act directly on the affected site by promoting the inflammatory reaction. Mukherjee and coworkers studied the effects of TNF-α inhibition in mice with CIA (Mukherjee et al, 2003). They injected a retroviral vector carrying the gene encoding a soluble form of the TNF-R1 into the affected arthritic joint. They found that the anti-TNF treatment not only reduced joint destruction in the injected joint, but also in the contralateral and ipsilateral paws. These systemic effects were paralleled by a reduced ratio of IgG2a:IgG1 antibodies anti-collagen type II (CII) on day 7 after DNA delivery, which led the authors to speculate about a therapeutic reduction of the inflammatory Th1 response, while the Th2 response remains unaffected.

In another study, three forms of the soluble TNF-R1 have been compared in the mouse CIA model of RA. The monomeric sTNF-R1, a dimeric sTNF-R1, and sTNF-R1/IgG chimera were administered by i.m. injection of the encoding plasmid cDNA followed by electroporation. In this approach, local expression of the three variants was confirmed, but only the sTNF-R1/IgG chimera ameliorated the disease by clinical and histological parameters. The dimeric form showed, if at all, minor clinical effets, while the monomeric sTNF-R1 was inefficient (Bloquel et al, 2004).

In murine EAE, naked DNA coding for a dimeric sTNF-R2 delivered locally with cationic liposomes could significantly delay disease onset and decrease disease symptoms, whereas inoculum of sTNF-R1/IgG delayed disease onset but was rather inefficient on disease symptoms. In the same study, i.m. administration or local inoculum without liposomes had no effect, highlighting

the importance of adequate delivery systems to achieve therapeutic effects (Croxford et al, 1998).

In summary, blocking TNF-α activity by soluble receptor molecules has proven beneficial in RA, leading to the use of a recombinant sTNF-R/Fc fusion protein (Etanercept) in clinics. The studies mentioned above provide evidence that this beneficial effect could be obtained also by adminstration of the therapeutic protein by gene therapy.

## F. IL-1 inhibitors: IL-1Ra, sIL-1R, sIL-1RAcP

Along with TNF-α, IL-1 is a key mediator of inflammation and tissue and organ damage in several chronic inflammatory pathologies. Agonist IL-1 interacts with its target cells via a receptor complex composed of the ligand binding IL-1 receptor type I (IL-1RI) and a second receptor-like molecule, the IL-1R accessory protein (IL-1RAcP), which is necessary to generate IL-1-dependent signal transduction (Wesche et al, 1997). A second IL-1 receptor (IL-1RII) also binds the IL-1 ligand and interacts with IL-1RAcP, but is unable to generate the IL-1-induced intracellular signal, thus behaves like a decoy receptor or ligand sink to regulate IL-1 responsiveness (Lang et al, 1998; Neumann et al, 2000). Two naturally occurring soluble molecules antagonising IL-1 activity *in vitro* and *in vivo* have been described. The IL-1 receptor antagonist (IL-1Ra) binds to membrane IL-1RI and displaces the agonist IL-1 ligand, but is unable to generate IL-1R-dependent signal transduction. Thus, by competition for receptor binding sites, IL-1Ra limits IL-1 bioactivity dose-dependently. The second inhbitory molecule is the soluble form of the decoy IL-1RII. Upon cell activation IL-1RII is shed from the cell surface as a soluble molecule (sIL-1RII) which still binds agonist IL-1, thereby sequestering it out of the activation circuit (Kollewe et al, 2000). Thus, both molecules, IL-1Ra and sIL-1RII, act as regulators of the inflammatory reaction by inhibiting the activity of proinflammatory IL-1.

Administration of recombinant IL-1Ra or sIL-1RII has been shown to provide beneficial effects in several experimental disease models. Recombinant IL-1Ra has been approved for the therapy of rheumatoid arthritis (Kineret®, anakinra). Administration of IL-1Ra by local gene transfer in arthritic joints has been tested experimentally in several studies, and its therapeutic effects have been confirmed in osteoarthritic models (Fernandes et al, 1999; Frisbie et al,, 2002). Two clinical trials in RA patients suggested that local IL-1Ra gene therapy is a feasible and safe option leading to intra-articular expression of the transferred IL-1Ra gene (Evans et al, 2000a, b).

Using retrovirally delivered DNA coding for IL-1Ra in knees of rabbit or mouse models of arthritis results in the previously mentioned contralatral effect. In order to verify that protection of the contralateral joint could be transferred by cells, Kim and coworkers used *ex vivo* modified autologous fibroblasts (Kim et al, 2002). These cells were stably infected to express IL-1Ra, and were injected intra-articulary into arthritic rabbit knee joints, inhibiting inflammation and providing chondroprotection in the injected joint as well in the contralateral site. Thus, local delivery of *ex vivo* genetically modified non-inflammatory autologous cells is effective in the therapy of rheumatoid diseases.

However, to achieve a protective effect the IL-1Ra gene does not require to be injected locally into the joint. In the mouse model of CIA it has been demonstrated that the i.m. injection of naked IL-1Ra cDNA is sufficient to ameliorate pathological symptoms (Kim et al, 2003) and that these effects could be enhanced by *in vivo* electroporation of the injected muscle (Jeong et al, 2004). Interestingly, IL-1Ra concentrations were found to be enhanced in sera and in the ankle joints of the treated mice, but the levels obtained were lower than those needed for protective effects in studies with administration of recombinant IL-1Ra. IL-1β expression was reduced in the ankle joints, suggesting that the protective mechanism is not directly due to systemic IL-1Ra activity. A possible mechanism is the transfection of bystander cells which reduce the inflammatory reaction at distant sites.

Maintaining constantly high levels of therapeutic agents in arthritic disease has several pharmacological disadvantages. To optimise treatment protocols, van de Loo and coworkers constructed in 2004 an adenoviral vector with disease-inducible properties. To this end, they drove expression of a reporter gene by IL-1/IL-6 enhancer/promoter elements and found the transgene being expressed only under inflammatory conditions. Such an expression system provides a very interesting and useful tool for the therapy of diseases with a spontaneous remission and exacerbation course, like RA or MS.

The signalling chain of the IL-1R complex, the accessory protein IL-1RAcP, has been identified in human serum in a soluble form, encompassing the extracellular domain of the membrane receptor. Soluble IL-1RAcP (sIL-1RAcP) can enhance the binding affinity of the inhibitory sIL-1RII for IL-1, thereby acting as a negative regulator in the IL-1 system (Smith et al, 2003). Smeets and coworkers, (2003) used a stably transfected fibroblast cell line to deliver sIL-1RAcP therapeutically into arthritic joints of mice with CIA. They found that this treatment ameliorated locally the joint destruction, but had no systemic effect. Systemic administration of the cDNA coding for sIL-1RAcP by an adenoviral system achieved an even higher rate of protection. Thus, sIL-1RAcP gene delivery can be developed for therapeutic usage in chronic inflammatory/autoimmune pathologies in which IL-1 inhibition is beneficial.

## G. Th1 cytokines and inhibitors: sIFN-γR-Fc, IL-12, IL-18, IL-18BP

Th1 inflammatory responses have a relevant role in many autoimmune pathologies. This is shown by correlation of disease severity with elevated IFN-γ levels, by amelioration of the disease upon IFN-γ neutralisation, and in mouse models with targeted deletions of the genes for IFN-γ, IFN-γ receptor (IFN-γR), IL-12, or Stat4. An efficient trap to bind circulating IFN-γ and thus inhibit its activity is the expression of a fusion protein composed of the extracellular portion of the IFN-γR fused to the

constant part of the heavy chain of murine IgG1 (IFN-γR-Fc). Interfering with the activity of Th1-associated cytokines with cDNA treatment is another very promising strategy that is actively pursued at the experimental level.

In genetically diabetic NOD mice, i.m. injection of cDNA coding for IFN-γR-Fc fusion protein protected from insulitis. In this study, administration of the IFN-γ-Fc coding vector led to detectable circulating protein levels (Chang and Prud'homme, 1999). The cDNA coding for the IFN-γR-Fc fusion protein was used in MRL *lpr/lpr* mice, a model for human lupus. In these mice, only i.m. injection followed by *in vivo* electroporation resulted in consistent expression of the fusion protein and reduced levels of circulating IFN-γ. When delivered in this way, the IFN-γR-Fc DNA ameliorated the disease even when administered to mice at an advanced stage of disease (4 months) (Lawson et al, 2000). In summary, IFN-γ plays a central role in the developmant and maintanance of several autoimmune diseases and blocking its activity by gene therapy is a feasible method to ameliorate clinical manifestations.

The expression of IFN-γ is tightly regulated. The cytokines IL-12 and IL-18 are the most potent physiological inducers of IFN-γ expression. Several studies demonstrate that blockade of their bioactivities results in reduced IFN-γ levels, thus leading to the amelioration of IFN-γ-mediated diseases. So far, a few studies used *in vivo* gene transfer to deliver IL-12 and IL-18 blocking agents.

The IL-12 gene, composed of two cDNAs coding for the two subunits p35 and p40, has been used experimentally *in vivo* as a DNA vaccine adjuvant. Intramuscular administration of the IL-12 cDNA enhanced Th1-like immune responses against the immunisation antigen (Sin et al, 1999), similarly to what obtained with *in vivo* delivery of the recombinant protein. Delivery of the IL-12 gene *per se* also resulted in systemic Th1 responses and NK cell activation (Watanabe et al, 1999). Administration of recombinant IL-12 to autoimmune MRL *lpr/lpr* mice (whose lupus-like syndrome is in large part Th1-dependent) enhanced the serum levels of IFN-γ and exacerbated pathological symptoms (Huang et al, 1996). In contrast, i.m. injection of the IL-12 gene ameliorated the lupus-like disease, despite increased serum levels of IFN-γ and increased IgG2a titers (both features of elevated Th1 reactivity). Injection of DNA coding for the p40 subunit of IL-12 only (which in homodimeric form acts as an inhibitor of IL-12 activity) had no effect on the lupus-like symptoms of MRL *lpr/lpr* mice (Hagiwara et al, 2000). Thus, despite the indication that IL-12 may indeed participate to the lupus pathology, these contrasting results indicate the need of an accurate design and validation of any IL-12-based strategies to be used therapeutically.

IFN-γ production and amplification of Th1 responses in autoimmune pathologies involve many factors besides IL-12. One of the other cytokines involved is IL-18, found at increased levels systemically and in affected tissues/organs of autoimmune patients as well as in experimental animal models of autoimmune diseases (Boraschi D and Dinarello C A, 2005). Due to its potent amplification of Th1 responses, IL-18 gene has been experimented as adjuvant similarly to the IL-12 gene. Th1-mediated immune response is induced by i.m injection of an anti-CD3scFv/IL-18 fusion DNA in antigen (OVA)-primed mice (Kim et al, 2004), thus making this approach suitable for the therapy of Th2-biased allergic disorders (Salagianni M and Kemeny D M, 2004). The induction of the Th1 immune response is dependent on the IL-18 protein and on the presence of immunostimulating CpG motifs in the DNA construct (Li et al, 2004).

Inhibition of IL-18 and of its Th1-stimulating capacity is therefore a strategy that can be pursued for the therapy of Th1-dependent autoimmune syndromes. The best known natural inhibitor of IL-18 is the IL-18 binding protein (IL-18BP), a soluble receptor-like molecule produced in inflammatory conditions to down-regulate excessive IL-18-dependent inflammation (Dinarello, 2000). The IL-18BPc gene has been delivered with adenoviral vector to mice suffering from CIA (Smeets et al, 2003a). Intra-articular overexpression of IL-18BP reduced inflammation and bone and cartilage destruction in the affected joint. Furthermore, administration of the IL-18BP encoding virus to both knees had distal and systemic protective effects on CIA. The IgG2a:IgG1 ratio of anti-CII antibodies was shifted towards IgG1, indicating that blocking IL-18 influenced the Th1/Th2 profile of the immune reaction occuring in CIA towards Th2 and anti-inflammation.

## H. Chemokines: CCL2/MCP-1

Chemokines, a family of small chemotactic cytokines, are able to attract immune cells to the site of inflammation. Thus, they are key mediators in the recruitment of leukocytes into an inflamed organ (Ward et al, 1998). MCP-1 (monocyte chemoattractant protein-1; CCL2 according to the new nomenclature) belongs to the CC subfamily of chemokines and has an important role in inflammation. CCL2 is expressed by a variety of leukocytes and other cell types (vascular endothelial cells, smooth muscle cells, glomerular mesangial cell, osteoblasts, epithelial cells) upon stimulation with inflammatory agents (*e.g.*, bacterial lipopolysaccharide, IL-1). In mesangial cells the synthesis and release of CCL2 is rapidly induced by IgG complexes. IL-1, TNF-α, PDGF, TGF-β and LIF induce the synthesis of CCL2 in human articular chondrocytes, which may thus play an active role in promoting monocyte influx and activation in synovial joints. Elevated levels of CCL2 are observed in macrophage-rich atherosclerotic plaques. Beside its chemotactic properties, CCL2 activates the tumouricidal activity of monocytes and macrophages *in vivo* and regulates the expression of proinflammatory cytokines such as IL-1 and IL-6.

In a recent study, the gene for a N-terminal truncated CCL2 mutant, which acts as a competitive inhibitor by blocking the interaction of CCL2 with its receptor CCR2, was delivered i.m. by electroporation in 16 week-old MRL *lpr/lpr* mice (which have already developed the spontaneous progressive lupus-like syndrome that will culminate in fatal kidney failure). Repeated administration resulted in protection from renal injury, due to reduced

leucocytic infiltration, and in a prolonged lifespan of the mice (Shimizu et al, 2004).

## III. Anti-cytokine therapy with gene transfer: cDNA vaccination

By varying gene delivery systems, dosage and routes of administration, it is possible to generate a significant immune response of the host against the gene product encoded by the introduced DNA. A key feature of cDNA vaccination is the fact that neutralising immune responses (mostly production of neutralising antibodies) can be generated against self proteins. Overcoming tolerance to self antigens may be due on one side to the fact that the majority of experimental evidence has been obtained in autoimmune mice (where tolerance mechanisms are already compromised). However, significant production of autoantibodies could be obtained also in immunocompetent mice (Youssef et al, 2000). Another reason for efficient induction of antibodies could be the presence in the DNA carrier plasmid of adjuvant sequences (*e.g.*, CpG sequences in bacterial DNA) which could amplify the immune response against the encoded protein. Also, protein expression in anomalous cell compartments and tissue locations (*e.g.*, in the muscle instead of lymphoid organs) may induce uptake of newly synthesized recombinant proteins by antigen-presenting cells. Although the mechanisms by which cDNA immunisation is effective are not fully defined, the approach is already in use in experimental models of autoimmunity. The induction of "beneficial autoimmunity" against pathological endogenous cytokines is the primary goal of this vaccination strategy (Wildbaum et al, 2003). The overall goal is that of achieving prolonged inhibition of inflammatory cytokines involved in the pathogenesis and/or in the downstream destructive effects of the disease (Karin, 2004). The drawback of the approach is that cytokine inhibition could eventually results in defective defence reactions and increased susceptibility to infections (immunosuppression). A summary of the most relevant experimental results obtained so far is reported in the **Table 2**.

### A. Chemokines (chemotactic cytokines)

The *in vivo* inhibition of chemokine activity by induction of neutralising antibodies through cDNA vaccination was studied in great detail by the group of Nathan Karin. Two animal models of autoimmune syndromes, experimental allergic encephalomylitis (EAE) and adjuvant-induced arthritis (AA), have been used to study the pathological involvement of the chemokines CCL3/MIP-1α, CCL2/MCP-1, CCL4/MIP-1β, CCL5/ RANTES, and CXCL10/IP-10. Multiple injections of chemokine cDNA could induce significant titres of specific antibodies with neutralising capacity (Youssef et al, 1998, 1999, 2000; Salomon et al, 2002; Wildbaum et al, 2002). In the rat EAE model, vaccination with

**Table 2.** DNA vaccination against cytokines/cytokine receptors in models of autoimmune diseases

| Cytokine/Receptor | Disease Model | Outcome | Reference |
|---|---|---|---|
| CCL2/MCP-1 | EAE, AA | neutralising antibodies; prevention disease induction; inhibition full-blown disease (AA) | Youssef S et al., 1998; Youssef S et al., 1999; Youssef S et al., 2000 |
| CCL3/MIP-1α | EAE, AA | neutralising antibodies; prevention disease induction; inhibition full-blown disease (AA) | Youssef S et al., 1998; Youssef S et al., 1999; Youssef S et al., 2000 |
| CCL4/MIP-1β | EAE | neutralising antibodies; exacerbation disease induction | Youssef S et al., 1998 |
| | AA | neutralising antibodies; prevention disease induction; inhibition full-blown disease | Youssef S et al., 2000 |
| CCL5/RANTES | AA | neutralising antibodies; prevention disease induction; inhibition full-blown disease | Youssef S et al., 2000 |
| | EAE | neutralising antibodies; no effect on disease induction | Youssef S et al., 1998 |
| CXCL10/IP-10 | EAE, AA | neutralising antibodies; prevention disease induction; inhibition full-blown disease | Salomon I et al., 2002; Wildbaum G et al., 2002 |
| TNF-α | EAE, AA, APS | neutralising antibodies, memory T; resistance to disease induction (EAE, AA), reduction disease symptoms (AA, APS) | Blank M et al., 2003; Wildbaum G et al., 2000; Wildbaum G and Karin N, 1999 |
| CpG/TLR9 | Diabetes in NOD mice | ↓ IFN-γ, ↑ IL-10; inhibition diabetogenic process | Quintana F J et al., 2000 |
| | EAE in rat | ↑ IFN-γ; resistance to disease induction | Boccaccio G L et al., 1999 |
| | EAE in SJL mice | ↑ Th1, ↑ IL-12; breaking tolerance induced by peptide immunisation, flare-up disease | Ichikawa H T et al., 2002 |
| IL-2Rα (CD25) | AA | no antibodies, anti-ergotypic T cell response, ↑ IL-10; protection from disease development | Mimran A et al., 2004 |
| IL-18 | Lupus in MRL *lpr/lpr* mice | neutralising antibodies; decrease IFN-γ, lymphadenopathy, kidney damage; increase life span | Bossù P et al., 2003 |
| IL-12/IL-18 | Lupus in MRL *lpr/lpr* mice | decrease IFN-γ, lymphadenopathy, kidney and lung damage | Neumann D et al., 2005 |

CCL3/MIP-1α, CCL2/MCP-1, or CXCL10/IP-10 could prevent the disease, even when EAE was induced two month after vaccination (Youssef et al, 1998, 1999, 2000; Wildbaum et al, 2002). In addition, vaccination with CXCL10/IP-10 cDNA in the EAE model was effective also when applied therapeutically in the full-blown disease (Wildbaum et al, 2002). In contrast, administration of CCL4/MIP-1β cDNA exacerbated the outcome of EAE, while vaccination with CCL5/RANTES cDNA had no effect (Youssef et al, 1998, 1999). Vaccination in the AA model was effective with all four chemokine cDNAs, with generation of neutralising antibodies followed by protective immunity and immunological memory to the vaccine (Salomon et al, 2002; Youssef et al, 2000). Vaccination could be performed after disease onset, and still inhibited development and progression of the disease (Youssef et al, 2000; Salomon et al, 2002). The neutralising antibodies induced by the CXCL10/IP-10 cDNA vaccination could block leukocyte migration and alter the Th1/Th2 balance of autoantigen (myelin basic protein)-specific T cells towards lower inflammation, and could adoptively transfer the disease suppressive effect (Salomon et al, 2002).

## B. TNF-α

Because of the major involvement of TNF-α in the pathological consequences of autoimmune chronic inflammatory diseases such as RA, several approaches of anti-TNF-α therapies have been attempted. Therapies with neutralising antibodies or engineered soluble receptors have indeed shown a significant beneficial effect in patients with RA. Thus, vaccination with TNF-α DNA is being developed as a possibly more effective approach to inhibition of TNF-α. In an experimental rat EAE model, a high titer of neutralising antibodies to TNF-α could be induced by immunisation with TNF-α naked DNA vaccine, in parallel to resistance to EAE induction. These vaccine-induced antibodies could transfer resistance to EAE in naive rats (Wildbaum and Karin, 1999). Vaccination with TNF-α naked DNA vaccine induced immunological memory and production of anti-TNF-α neutralising antibodies also in BALB/c mice with experimental anti-phospholipid syndrome (APS; an autoimmune syndrome often associated with lupus and responsible of recurrent abortions). Anti-TNF-α vaccination early during disease development could significantly reduce the titres of anti-phospholipid autoantibodies, consequently decreasing foetal loss and normalising platelet counts and the prolonged activated partial thromboplastin time (one of the features of APS). Vaccination in mice with already established APS had less pronounced effects (Blank et al, 2003). In another experimental autoimmune arthritis model, AA in rats, administration of the TNF-α naked DNA vaccine before induction of the disease also resulted in the generation of immunological memory and high titers of neutralising anti-TNF-α antibodies, in parallel to inhibition of disease development. These antibodies could transfer disease inhibition to non-vaccinated animals. Vaccination in rats with full-blown disease resulted in block of the disease with rapid and long-lasting normalisation of the arthritis score (Wildbaum et al, 2000).

## C. TLR9 agonists

Among the receptors of the TLR/IL-1R family (which include both the receptors for the cytokines IL-1 and IL-18 and the toll-like receptors TLR responsible for initiation of innate defence), a particular interest is being devoted to activation of TLR9, a receptor expressed on the plasma membrane of leukocytes which is triggered by interaction with unmethylated CpG sequences mainly present in bacterial and viral DNA.

In cDNA vaccination approaches in which cytokine gene delivery occurs through inoculation of a bacterial-derived expression plasmid, the contribution of plasmid-dependent triggering of TLR9 is evident and should be duly considered.

In diabetic NOD mice, cytokine production by spleen cells is biased towards Th1 (high IFN-γ, low IL-10 in response to the polyclonal stimulus ConA). However, triggering of TLR9 with CpG oligonucleotides apparently re-directs the response towards a more anti-inflammatory functional phenotype (low IFN-γ, high IL-10) with the induction of protective IgG2b antibodies against HSP60 and p277 (Quintana et al, 2000). In the rat EAE model, administration of CpG-containing non-coding plasmids induced IFN-γ production *in vivo* and potently suppressed the induction of autoimmune encephalomyelitis (Boccaccio et al, 1999). However, stimulation of TLR9 with CpG may break tolerance to autoantigens and be responsible of the disease flare-up in EAE of SJL mice (Ichikawa et al, 2002) and may be a key step in triggering autoantigen presentation and autoantibody production leading to autoimmune diseases (Leadbetter et al, 2002; Rui et al, 2003; Viglianti et al, 2003; Darabi et al, 2004; Iliev et al, 2004; Marshak-Rothstein et al, 2004a,b; Waldner et al, 2004; Wang and Krieg, 2004; Means et al, 2005). Thus, although the use of CpG-containing plasmids or DNA sequences apparently has a re-equilibrating effect in altered immune response (being able to shift excessive Th1 responses towards Th2 and vice-versa), the final outcome may vary depending on the microenvironmental situation, and TLR9 triggering may be detrimental, resulting in induction or exacerbation of autoimmunity.

Indeed, TLR triggering (through bacterial or viral infection, after conventional vaccination, or consequent to traumatic events) may be among the events promoting loss of tolerance and contributing to the development of autoimmunity (Toubi and Schoenfeld, 2004). In this view, DNA vaccination approaches in autoimmune disease should carefully consider the impact of the vaccine formulation (e.g. the type of plasmid) on the disease itself.

## D. IL-2Rα (CD25)

Antibodies against the IL-2R α chain (CD25) is a therapeutic strategy used since the mid-1980s to achieve immunosuppression by inhibiting the activity of the cytokine IL-2, *e.g.*, in allograft rejection. Suppression of IL-2 through inhibition of CD25 is being developed for treating autoimmune syndromes, using a DNA vaccination approach. Vaccination with cDNA coding for the soluble

form of CD25 was performed in rats before induction of AA. Vaccination with cDNA coding for the soluble form of CD25 was performed in rats before induction of AA. Vaccination with cDNA coding for the soluble form of CD25 was performed in rats before induction of AA. Vaccination completely inhibited disease development and skewed cytokine production from a Th1 inflammatory profile (high IFN-γ, low IL-10) to a Th2 anti-inflammatory profile (low IFN-γ, high IL-10). No antibodies against CD25 were produced, but an anti-ergotypic (down-regulatory) T cell response was generated against the activation marker CD25. Indeed, anti-ergotypic T cells could proliferate and produce IL-10 reacting to the presence of CD25-expressing activated T cells, and could transfer resistance to disease induction to non-vaccinated rats (Mimran et al, 2004).

### E. IL-18

Vaccination against mature murine IL-18 was performed in young MRL *lpr/lpr* mice, *i.e.*, before disease onset (Bossù et al, 2003). Vaccinated mice had significant expression of IL-18 mRNA in the muscle (inoculation site) and increased circulating IL-18 levels. Circulating IL-18 was found mostly bound to neutralising antibodies, absent in non-immunised control mice. Upon repeated vaccination, mice showed a significant decrease in IFN-γ production, lymphadenopathy, kidney inflammation and damage (including presence of immune complexes) and of consequent proteinuria, and survived significantly longer that non-vaccinated animals. Despite the decreased presence of immune complexes (damage by immune complexes is the major cause of kidney failure and fatal outcome), no decrease of anti-DNA and anti-phospholipid autoantibodies was evident. This is in agreement with previous evidence suggesting lack of correlation between autoantibody levels and renal damage/early death (Yasuda et al, 2001). Indeed, it has been reported that the increased amount of immune complexes in MRL *lpr/lpr* kidney does not depend on increased deposition (a consequence of increased autoantibody levels) but rather to decreased clearance (Cruse et al, 2000).

On the basis of these results of anti-IL-18 vaccination, and following the notion that IL-12 acts in synergy with IL-12 in inducing Th1 inflammatory responses and has a central role in several autoimmune syndromes, another study was carried out in MRL *lpr/lpr* mice, by vaccinating animals with cDNA of both IL-12 and IL-18 (Neumann et al, 2005). Concomitant administration of cDNA coding for the two cytokines induced a potent inhibition of lymphadenopathy and splenomegaly (the most striking characteristics of the disease), and reduced almost to zero the kidney damage and proteinuria. In addition, the strong inflammatory infiltrate evident in lungs of *lpr/lpr* mice was absent in IL-18/IL-12 vaccinated animals.

## IV. Conclusions and perspectives

Therapy of chronic, life-long pathologies such as autoimmune diseases is a crucial issue that is being addressed from different viewpoints. The chronic pathology depends on immune tolerance breaks that trigger a misdirected inflammatory and immune reaction against self-antigens, a reaction that includes a number of inflammatory cytokines and chemokines. A radical therapy of autoimmunity, which could reconduct the immune response to correct recognition, is still far from reality. Current therapies are therefore directed at inhibiting the pathological effects of the misdirected immune response with anti-inflammatory and immunosuppressive strategies. Anti-cytokine drugs, in particular recombinant cytokine inhibitors like soluble receptors for TNF and IL-1, are being tested in clinical situations with good results. However, the chronicity of disease implies that drugs, to be effective, are administered repeatedly and continuously, with consequent side effects of toxicity, loss of efficacy, triggering of neutralising antibodies.

On these grounds, autoimmune diseases are ideal candidates for gene-based therapies, where stable delivery of genes encoding therapeutic cytokines/cytokine inhibitors could ensure long-lasting release of the beneficial molecule. Likewise, gene-based vaccination against pathogenic endogenous cytokines could provide long-lasting neutralisation of the exceeding cytokine. Data collected within a large body of studies in different experimental animal models suggest that the hypothesis of cytokine gene transfer in autoimmunity is a feasible option which should achieve long-term efficacy in the absence of the collateral effects caused by recombinant drugs. However, it is clear that the vector used for delivery and the route and modalities of DNA administration are crucial for the successful outcome of the therapy. In addition, careful analysis of possible long-term consequences of the treatment is necessary, in order to avoid the risk of iatrogenic immunosuppression and imbalanced immunoregulation.

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## References

Aihara H and Miyazaki J **(1998)** Gene transfer into muscle by electroporation in vivo. **Nat Biotechnol** 16, 867-870.

Barry MA and Johnston SA **(1997)** Biological features of genetic immunization. **Vaccine** 15, 788-791.

Blank M, Krause I, Wildbaum G, Karin N and Shoenfeld Y **(2003)** TNFα DNA vaccination prevents clinical manifestations of experimental antiphospholipid syndrome. **Lupus** 12, 546-549.

Bloquel C, Bessis N, Boissier M C, Scherman D and Bigey P **(2004)** Gene therapy of collagen-induced arthritis by electrotransfer of human tumor necrosis factor-α soluble receptor I variants. **Hum Gene Ther** 15, 189-201.

Boccaccio GL, Mor F and Steinman L **(1999)** Non-coding plasmid DNA induces IFN-γ in vivo and suppresses autoimmune encephalomyelitis. **Int Immunol** 11, 289-296.

Boraschi D and Dinarello CA **(2005)** IL-18 in autoimmunity. **Eur Cytokine Netw** 16, in press.

Bossù P, Neumann D, Del Giudice E, Ciaramella A, Gloaguen I, Fantuzzi G, Dinarello CA, Di Carlo E, Musiani P, Meroni PL, Caselli G, Ruggiero P and Boraschi D **(2003)** IL-18 cDNA vaccination protects mice from spontaneous lupus-like autoimmune disease. **Proc Natl Acad Sci USA** 100, 14181-14186.

Burls A and Jobanputra P **(2004)** The trials of anakinra. **Lancet** 364, 827-828.

Cameron MJ, Arreaza GA, Waldhauser L, Gauldie J and Delovitch TL **(2000)** Immunotherapy of spontaneous type 1 diabetes in nonobese diabetic mice by systemic interleukin-4 treatment employing adenovirus vector-mediated gene transfer. **Gene Ther** 7, 1840-1846.

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

Charles P, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C, Breedveld FC, Smolen JS, Eberl G, deWoody K, Feldmann M and Maini RN **(1999)** Regulation of cytokines, cytokine inhibitors and acute-phase proteins following anti-TNF-α therapy in rheumatoid arthritis. **J Immunol** 163, 1521-1528.

Chesnoy S, Lee PY and Huang L **(2003)** Intradermal injection of transforming growth factor-β1 gene enhances wound healing in genetically diabetic mice. **Pharm Res** 20, 345-350.

Croxford JL, Triantaphyllopoulos K, Podhajcer OL, Feldmann M, Baker D and Chernajovsky Y **(1998)** 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.

Cruse JM, Lewis RE and Dilioglou S **(2000)** Fate of immune complexes, glomerulonephritis and cell-mediated vasculitis in lupus-prone MRL/Mp l*pr/lpr* mice. **Exp Mol Pathol** 69, 211-222.

Darabi K, Karulin AY, Boehm BO, Hofstetter HH, Fabry Z, LaManna JC, Chavez JC, Tary-Lehmann M and Lehmann PV **(2004)** The third signal in T cell-mediated autoimmune disease? **J Immunol** 173, 92-99.

Davis HL, Whalen RG and Demeneix BA **(1993)** Direct gene transfer into skeletal muscle in vivo: factors affecting efficiency of transfer and stability of expression. **Hum Gene Ther** 4, 151-159.

de Kozak Y, Thillaye-Goldenberg B, Naud MC, Da Costa AV, Auriault C and Verwaerde C **(2002)** Inhibition of experimental autoimmune uveoretinitis by systemic and subconjunctival adenovirus-mediated transfer of the viral IL-10 gene. **Clin Exp Immunol** 130, 212-223.

Dinarello CA **(2000)** Targeting interleukin 18 with interleukin 18 binding protein. **Ann Rheum Dis** 59 Suppl 1, 17-20.

Dinarello CA **(2003)** Setting the cytokine trap for autoimmunity. **Nat Med** 9, 20-22.

Economides AN, Carpenter LR, Rudge JS, Wong V, Koehler-Stec EM, Hartnett C, Pyles EA, Xu X, Daly TJ, Young MR, Fandl JP, Lee F, Carver S, McNay J, Bailey K, Ramakanth S, Hutabarat R, Huang TT, Radziejewski C, Yancopoulos GD and Stahl N **(2003)** Cytokine traps: multi-component, high-affinity blockers of cytokine action. **Nat Med** 9, 47-52.

Edelstein ML, Abedi MR, Wixon J and Edelstein RM **(2004)** Gene therapy clinical trials worldwide 1989-2004-an overview. **J Gene Med** 6, 597-602.

Evans CH, Ghivizzani SC, Herndon JH, Wasko MC, Reinecke J, Wehling P and Robbins PD **(2000a)** Clinical trials in the gene therapy of arthritis. **Clin Orthop** S300-S307.

Evans CH, Ghivizzani SC, Oligino TJ and Robbins PD **(2000b)** Gene therapy for autoimmune disorders. **J Clin Immunol** 20, 334-346.

Feltquate DM, Heaney S, Webster RG and Robinson HL **(1997)** Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. **J Immunol** 158, 2278-2284.

Fernandes J, Tardif G, Martel-Pelletier J, Lascau-Coman V, Dupuis M, Moldovan F, Sheppard M, Krishnan BR and Pelletier JP **(1999)** In vivo transfer of interleukin-1 receptor antagonist gene in osteoarthritic rabbit knee joints: prevention of osteoarthritis progression. **Am J Pathol** 154, 1159-1169.

Fortuin FD, Vale P, Losordo DW, Symes J, DeLaria GA, Tyner JJ, Schaer GL, March R, Snell RJ, Henry TD, Van Camp J, Lopez JJ, Richenbacher W, Isner JM and Schatz RA **(2003)** One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. **Am J Cardiol** 92, 436-439.

Frisbie DD, Ghivizzani SC, Robbins PD, Evans CH and McIlwraith CW **(2002)** Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. **Gene Ther** 9, 12-20.

Furst DE, Schiff MH, Fleischmann RM, Strand V, Birbara CA, Compagnone D, Fischkoff SA and Chartash EK **(2003)** Adalimumab, a fully human anti tumor necrosis factor-α monoclonal antibody and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid Arthritis). **J Rheumatol** 30, 2563-2571.

Goudy K, Song S, Wasserfall C, Zhang YC, Kapturczak M, Muir A, Powers M, Scott-Jorgensen M, Campbell-Thompson M, Crawford JM, Ellis TM, Flotte TR and Atkinson MA **(2001)** Adeno-associated virus vector-mediated IL-10 gene delivery prevents type 1 diabetes in NOD mice. **Proc Natl Acad Sci USA** 98, 13913-13918.

Goudy KS, Burkhardt BR, Wasserfall C, Song S, Campbell-Thompson ML, Brusko T, Powers MA, Clare-Salzler MJ, Sobel ES, Ellis TM, Flotte TR and Atkinson MA **(2003)** Systemic overexpression of IL-10 induces CD4+CD25+ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. **J Immunol** 171, 2270-2278.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M and Fischer A **(2003a)** A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. **N Engl J Med** 348, 255-256.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen J I, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva L E, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A and Cavazzana-Calvo M **(2003b)** LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. **Science** 302, 415-419.

Hagiwara E, Okubo T, Aoki I, Ohno S, Tsuji T, Ihata A, Ueda A, Shirai A, Okuda K, Miyazaki J and Ishigatsubo Y **(2000)** IL-12-encoding plasmid has a beneficial effect on spontaneous autoimmune disease in MRL/MP-*lpr/lpr* mice. **Cytokine** 12, 1035-1041.

Higuchi N, Maruyama H, Kuroda T, Kameda S, Iino N, Kawachi H, Nishikawa Y, Hanawa H, Tahara H, Miyazaki J and Gejyo F **(2003)** Hydrodynamics-based delivery of the viral interleukin-10 gene suppresses experimental crescentic glomerulonephritis in Wistar-Kyoto rats. **Gene Ther** 10, 1297-1310.

Huang FP, Feng GJ, Lindop G, Stott DI and Liew FY **(1996)** The role of interleukin 12 and nitric oxide in the development of spontaneous autoimmune disease in MRL/MP-*lpr/lpr* mice. **J Exp Med** 183, 1447-1459.

Ichikawa HT, Williams LP and Segal BM **(2002)** Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. **J Immunol** 169, 2781-2787.

Iliev AI, Stringaris AK, Nau R and Neumann H **(2004)** Neuronal injury mediated via stimulation of microglial toll-like receptor-9 (TLR9). **FASEB J** 18, 412-414.

Ito K, Ito K, Shinohara N and Kato S **(2003)** DNA immunization via intramuscular and intradermal routes using a gene gun provides different magnitudes and durations on immune response. **Mol Immunol** 39, 847-854.

Jeong JG, Kim JM, Ho SH, Hahn W, Yu SS and Kim S **(2004)** Electrotransfer of human IL-1Ra into skeletal muscles reduces the incidence of murine collagen-induced arthritis. **J Gene Med** 6, 1125-1133.

Karin N **(2004)** Induction of protective therapy for autoimmune diseases by targeted DNA vaccines encoding pro-inflammatory cytokines and chemokines. **Curr Opin Mol Ther** 6, 27-33.

Kim EJ, Cho D and Kim TS **(2004)** Efficient induction of T helper type 1-mediated immune responses in antigen-primed mice by anti-CD3 single-chain Fv/interleukin-18 fusion DNA. **Immunology** 111, 27-34.

Kim JM, Jeong JG, Ho SH, Hahn W, Park EJ, Kim S, Yu SS, Lee YW and Kim S **(2003)** Protection against collagen-induced arthritis by intramuscular gene therapy with an expression plasmid for the interleukin-1 receptor antagonist. **Gene Ther** 10, 1543-1550.

Kim SH, Lechman ER, Kim S, Nash J, Oligino TJ and Robbins PD **(2002)** Ex vivo gene delivery of IL-1Ra and soluble TNF receptor confers a distal synergistic therapeutic effect in antigen-induced arthritis. **Mol Ther** 6, 591-600.

Ko KS, Lee M, Koh JJ and Kim SW **(2001)** Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. **Mol Ther** 4, 313-316.

Koh JJ, Ko KS, Lee M, Han S, Park JS and Kim SW **(2000)** Degradable polymeric carrier for the delivery of IL-10 plasmid DNA to prevent autoimmune insulitis of NOD mice. **Gene Ther** 7, 2099-2104.

Kollewe C, Neumann D and Martin MU **(2000)** The first two N-terminal immunoglobulin-like domains of soluble human IL-1 receptor type II are sufficient to bind and neutralize IL-1β. **FEBS Lett** 487, 189-193.

La Cava A, Billetta R, Gaietta G, Bonnin DB, Baird SM and Albani S **(2000)** Cell-mediated DNA transport between distant inflammatory sites following intradermal DNA immunization in the presence of adjuvant. **J Immunol** 164, 1340-1345.

Lang D, Knop J, Wesche H, Raffetseder U, Kurrle R, Boraschi D and Martin MU **(1998)** The type II IL-1 receptor interacts with the IL-1 receptor accessory protein: a novel mechanism of regulation of IL-1 responsiveness. **J Immunol** 161, 6871-6877.

Lawson BR, Prud'homme GJ, Chang Y, Gardner HA, Kuan J, Kono DH and Theofilopoulos AN **(2000)** Treatment of murine lupus with cDNA encoding IFN-gammaR/Fc. **J Clin Invest** 106, 207-215.

Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ and Marshak-Rothstein A **(2002)** Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. **Nature** 416, 603-607.

Lechman ER, Jaffurs D, Ghivizzani SC, Gambotto A, Kovesdi I, Mi Z, Evans C H and Robbins PD **(1999)** Direct adenoviral gene transfer of viral IL-10 to rabbit knees with experimental arthritis ameliorates disease in both injected and contralateral control knees. **J Immunol** 163, 2202-2208.

Lechman ER, Keravala A, Nash J, Kim SH, Mi Z and Robbins PD **(2003)** The contralateral effect conferred by intra-articular adenovirus-mediated gene transfer of viral IL-10 is specific to the immunizing antigen. **Gene Ther** 10, 2029-2035.

Li Y, Ishii K, Hisaeda H, Hamano S, Zhang M, Nakanishi K, Yoshimoto T, Hemmi H, Takeda K, Akira S, Iwakura Y and Himeno K **(2004)** IL-18 gene therapy develops Th1-type immune responses in Leishmania major-infected BALB/c mice: is the effect mediated by the CpG signaling TLR9? **Gene Ther** 11, 941-948.

Lindsay J, Van Montfrans C, Brennan F, Van Deventer S, Drillenburg P, Hodgson H, Te Velde A and Sol Rodriguez Pena M **(2002)** IL-10 gene therapy prevents TNBS-induced colitis. **Gene Ther** 9, 1715-1721.

Lindsay JO, Ciesielski CJ, Scheinin T, Hodgson HJ and Brennan FM **(2001)** The prevention and treatment of murine colitis using gene therapy with adenoviral vectors encoding IL-10. **J Immunol** 166, 7625-7633.

Losordo DW, Vale PR, Hendel RC, Milliken CE, Fortuin FD, Cummings N, Schatz RA, Asahara T, Isner JM and Kuntz RE **(2002)** Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. **Circulation** 105, 2012-2018.

Lyseng-Williamson KA and Plosker GL **(2004)** Etanercept: a pharmacoeconomic review of its use in rheumatoid arthritis. **Pharmacoeconomics** 22, 1071-1095.

MacColl G, Bunn C, Goldspink G, Bouloux P and Gorecki DC **(2001)** Intramuscular plasmid DNA injection can accelerate autoimmune responses. **Gene Ther** 8, 1354-1356.

Marshak-Rothstein A, Busconi L, Lau M, Tabor A S, Leadbetter EA, Akira S, Krieg A M, Lipford GB, Viglianti GA and Rifkin IR **(2004a)** Comparison of CpG s-ODNs, chromatin immune complexes and dsDNA fragment immune complexes in the TLR9-dependent activation of rheumatoid factor B cells. **J Endotoxin Res** 10, 247-251.

Marshak-Rothstein A, Busconi L, Rifkin IR and Viglianti GA **(2004b)** The stimulation of Toll-like receptors by nuclear antigens: a link between apoptosis and autoimmunity. **Rheum Dis Clin North Am** 30, 559-74, ix.

Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT and Luster AD **(2005)** Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. **J Clin.Invest** 115, 407-417.

Miagkov AV, Varley AW, Munford RS and Makarov SS **(2002)** Endogenous regulation of a therapeutic transgene restores homeostasis in arthritic joints. **J Clin Invest** 109, 1223-1229.

Mimran A, Mor F, Carmi P, Quintana FJ, Rotter V and Cohen IR **(2004)** DNA vaccination with CD25 protects rats from adjuvant arthritis and induces an antiergotypic response. **J Clin Invest** 113, 924-932.

Morel PA, Falkner D, Plowey J, Larregina AT and Falo Jr LD **(2004)** DNA immunisation: altering the cellular localisation of expressed protein and the immunisation route allows manipulation of the immune response. **Vaccine** 22, 447-456.

Morita Y, Yang J, Gupta R, Shimizu K, Shelden EA, Endres J, Mule JJ, McDonagh KT and Fox DA **(2001)** Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. **J Clin Invest** 107, 1275-1284.

Mukherjee P, Wu B, Mayton L, Kim SH, Robbins PD and Wooley PH **(2003)** TNF receptor gene therapy results in suppression of IgG2a anticollagen antibody in collagen induced arthritis. **Ann Rheum Dis** 62, 707-714.

Nakajima A, Seroogy CM, Sandora MR, Tarner IH, Costa GL, Taylor-Edwards C, Bachmann MH, Contag CH and Fathman CG **(2001)** Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. **J Clin Invest** 107, 1293-1301.

Neumann D, Kollewe C, Martin MU and Boraschi D **(2000)** The membrane form of the type II IL-1 receptor accounts for inhibitory function. **J Immunol** 165, 3350-3357.

Neumann D, Tschernig T, Popa D, Schmeidl A, de Lema GP, Resch K and Martin MU **(2005)** Intramuscular administration of plasmids encoding IL-12 and IL-18 synergistically ameliorate the autoimmune pathology of MRL/Mp *lpr/lpr* mice. Submitted for publication.

Nishihara K, Miyatake S, Sakata T, Yamashita J, Kikuchi H, Kawade Y, Zu Y, Namba Y, Hanaoka M and Watanabe Y **(1988)** Augmentation of tumor targeting in a line of glioma-specific mouse cytotoxic T-lymphocytes by retroviral expression of mouse gamma-interferon complementary DNA. **Cancer Res** 48, 4730-4735.

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.

Pertmer TM, Eisenbraun MD, McCabe D, Prayaga SK, Fuller DH and Haynes JR **(1995)** Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. **Vaccine** 13, 1427-1430.

Piccirillo CA, Chang Y and Prud'homme GJ **(1998)** TGF-β1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. **J Immunol** 161, 3950-3956.

Piccirillo CA and Prud'homme GJ **(2003)** Immune modulation by plasmid DNA-mediated cytokine gene transfer. **Curr Pharm Des** 9, 83-94.

Quintana FJ, Rotem A, Carmi P and Cohen IR **(2000)** Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. **J Immunol** 165, 6148-6155.

Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J and Chernajovsky Y **(1999)** Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. **J Exp Med** 190, 385-398.

Raper SE, Chirmule N, Lee S, Wivel NA, Bagg A, Gao GP, Wilson JM and Batshaw ML **(2003)** Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. **Mol Genet Metab** 80, 148-158.

Raz E, Dudler J, Lotz M, Baird SM, Berry CC, Eisenberg RA and Carson DA **(1995)** Modulation of disease activity in murine systemic lupus erythematosus by cytokine gene delivery. **Lupus** 4, 286-292.

Raz E, Watanabe A, Baird SM, Eisenberg RA, Parr TB, Lotz M, Kipps TJ and Carson DA **(1993)** Systemic immunological effects of cytokine genes injected into skeletal muscle. **Proc Natl Acad Sci USA** 90, 4523-4527.

Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, *et al*. **(1990)** Gene transfer into humans-immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. **N Engl J Med** 323, 570-578.

Rui L, Vinuesa CG, Blasioli J and Goodnow CC **(2003)** Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. **Nat Immunol** 4, 594-600.

Salagianni M and Kemeny DM **(2004)** Anti-CD3 sFv/IL-18 fusion DNA for allergy therapy. **Immunology** 111, 16-18.

Salomon I, Netzer N, Wildbaum G, Schif-Zuck S, Maor G and Karin N **(2002)** Targeting the function of IFN-γ-inducible protein 10 suppresses ongoing adjuvant arthritis. **J Immunol** 169, 2685-2693.

Shimizu S, Nakashima H, Masutani K, Inoue Y, Miyake K, Akahoshi M, Tanaka Y, Egashira K, Hirakata H, Otsuka T and Harada M **(2004)** Anti-monocyte chemoattractant protein-1 gene therapy attenuates nephritis in MRL/lpr mice. **Rheumatology (Oxford)**

Sin JI, Kim JJ, Arnold RL, Shroff KE, McCallus D, Pachuk C, McElhiney SP, Wolf MW, Pompa-de Bruin SJ, Higgins TJ, Ciccarelli RB and Weiner DB **(1999)** IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell-mediated protective immunity against herpes simplex virus-2 challenge. **J Immunol** 162, 2912-2921.

Smeets RL, van De Loo FA, Arntz OJ, Bennink MB, Joosten LA and van Den Berg WB **(2003a)** Adenoviral delivery of IL-18 binding protein C ameliorates collagen-induced arthritis in mice. **Gene Ther** 10, 1004-1011.

Smeets RL, van De Loo FA, Joosten LA, Arntz OJ, Bennink MB, Loesberg WA, Dmitriev IP, Curiel DT, Martin MU and van Den Berg WB **(2003b)** Effectiveness of the soluble form of the interleukin-1 receptor accessory protein as an inhibitor of interleukin-1 in collagen-induced arthritis. **Arthritis Rheum** 48, 2949-2958.

Smith DE, Hanna R, Friend D, Moore H, Chen H, Farese AM, MacVittie TJ, Virca GD and Sims JE **(2003)** The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. **Immunity** 18, 87-96.

Song XY, Gu M, Jin WW, Klinman DM and Wahl SM **(1998)** Plasmid DNA encoding transforming growth factor-β1 suppresses chronic disease in a streptococcal cell wall-induced arthritis model. **J Clin Invest** 101, 2615-2621.

Suarez-Pinzon WL, Marcoux Y, Ghahary A and Rabinovitch A **(2002)** Gene transfection and expression of transforming growth factor-β1 in nonobese diabetic mouse islets protects-β-cells in syngeneic islet grafts from autoimmune destruction. **Cell Transplant** 11, 519-528.

Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, Nakamura T, Ogihara T, Kaneda Y and Morishita R **(2002)** Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. **Gene Ther** 9, 372-380.

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

Toubi E and Schoenfeld Y **(2004)** Toll-like receptors and their role in the development of autoimmune diseases. **Autoimmunity** 37, 183-188.

Toussirot E and Wendling D **(2004)** The use of TNF-α blocking agents in rheumatoid arthritis: an overview. **Expert Opin Pharmacother** 5, 581-594.

van De Loo FA, de Hooge AS, Smeets RL, Bakker AC, Bennink MB, Arntz OJ, Joosten LA, van Beuningen HM, van der Kraan PK, Varley AW and van Den Berg WB **(2004)** An inflammation-inducible adenoviral expression system for local treatment of the arthritic joint. **Gene Ther** 11, 581-590.

Verwaerde C, Naud MC, Delanoye A, Wood M, Thillaye-Goldenberg B, Auriault C and de Kozak Y **(2003)** Ocular transfer of retinal glial cells transduced ex vivo with adenovirus expressing viral IL-10 or CTLA4-Ig inhibits experimental autoimmune uveoretinitis. **Gene Ther** 10, 1970-1981.

Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ and Marshak-Rothstein A **(2003)** Activation of autoreactive B cells by CpG dsDNA. **Immunity** 19, 837-847.

Vitadello M, Schiaffino MV, Picard A, Scarpa M and Schiaffino S **(1994)** Gene transfer in regenerating muscle. **Hum Gene Ther** 5, 11-18.

Waldner H, Collins M and Kuchroo VK **(2004)** Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. **J Clin Invest** 113, 990-997.

Wang Y and Krieg AM **(2004)** Induction of autoantibody production but not autoimmune disease in HEL transgenic mice vaccinated with HEL in combination with CpG or control oligodeoxynucleotides. **Vaccine** 22, 2641-2650.

Ward SG, Bacon K and Westwick J **(1998)** Chemokines and T lymphocytes: more than an attraction. **Immunity** 9, 1-11.

Watanabe M, Fenton R G, Wigginton J M, McCormick KL, Volker KM, Fogler WE, Roessler PG and Wiltrout RH **(1999)** Intradermal delivery of IL-12 naked DNA induces systemic NK cell activation and Th1 response in vivo that is independent of endogenous IL-12 production. **J Immunol**163, 1943-1950.

Wells DJ **(1993)** Improved gene transfer by direct plasmid injection associated with regeneration in mouse skeletal muscle. **FEBS Lett** 332, 179-182.

Wells DJ **(2004)** Gene therapy progress and prospects: electroporation and other physical methods. **Gene Ther** 11, 1363-1369.

Wesche H, Korherr C, Kracht M, Falk W, Resch K and Martin MU **(1997)** The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). **J Biol Chem** 272, 7727-7731.

Whalen JD, Lechman EL, Carlos CA, Weiss K, Kovesdi I, Glorioso JC, Robbins PD and Evans CH **(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.

Whalen JD, Thomson AW, Lu L, Robbins PD and Evans CH **(2001)** Viral IL-10 gene transfer inhibits DTH responses to soluble antigens: evidence for involvement of genetically modified dendritic cells and macrophages. **Mol Ther** 4, 543-550.

Wildbaum G and Karin N **(1999)** Augmentation of natural immunity to a pro-inflammatory cytokine (TNF-α) by targeted DNA vaccine confers long-lasting resistance to experimental autoimmune encephalomyelitis. **Gene Ther** 6, 1128-1138.

Wildbaum G, Nahir MA and Karin N **(2003)** Beneficial autoimmunity to proinflammatory mediators restrains the consequences of self-destructive immunity. **Immunity** 19, 679-688.

Wildbaum G, Netzer N and Karin N **(2002)** Plasmid DNA encoding IFN-γ-inducible protein 10 redirects antigen-specific T cell polarization and suppresses experimental autoimmune encephalomyelitis. **J Immunol** 168, 5885-5892.

Wildbaum G, Youssef S and Karin N **(2000)** A targeted DNA vaccine augments the natural immune response to self TNF-α and suppresses ongoing adjuvant arthritis. **J Immunol** 165, 5860-5866.

Winegar RA, Monforte JA, Suing KD, O'Loughlin KG, Rudd CJ and Macgregor JT **(1996)** Determination of tissue distribution of an intramuscular plasmid vaccine using PCR and in situ DNA hybridization. **Hum Gene Ther** 7, 2185-2194.

Yang Z, Chen M, Wu R, Fialkow LB, Bromberg JS, McDuffie M, Naji A and Nadler JL **(2002)** Suppression of autoimmune diabetes by viral IL-10 gene transfer. **J Immunol** 168, 6479-6485.

Yasuda T, Yoshimoto T, Tsubura A and Matsuzawa A **(2001)** Clear suppression of Th1 responses but marginal amelioration of autoimmune manifestations by IL-12p40 transgene in MRL-FAS(lprcg)/FAS(lprcg) mice. **Cell Immunol** 210, 77-86.

Youssef S, Maor G, Wildbaum G, Grabie N, Gour-Lavie A and Karin N **(2000)** C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. **J Clin Invest** 106, 361-371.

Youssef S, Wildbaum G and Karin N **(1999)** Prevention of experimental autoimmune encephalomyelitis by MIP-1α and MCP-1 naked DNA vaccines. **J Autoimmun** 13, 21-29.

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 YC, Pileggi A, Agarwal A, Molano RD, Powers M, Brusko T, Wasserfall C, Goudy K, Zahr E, Poggioli R, Scott-Jorgensen M, Campbell-Thompson M, Crawford JM, Nick H, Flotte T, Ellis TM, Ricordi C, Inverardi L and Atkinson MA **(2003)** Adeno-associated virus-mediated IL-10 gene therapy inhibits diabetes recurrence in syngeneic islet cell transplantation of NOD mice. **Diabetes** 52, 708-716.

Zhibao M, Ghivizzani SC, Lechman E, Glorioso JC, Evans CH and Robbins PD **(2004)** Adverse effects of adenovirus-mediated gene transfer of human transforming growth factor-β 1 into rabbit knees. **Arthirtis Res Ther** 5, R132-R139.

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