GENE THERAPY FOR AUTOIMMUNE DISEASES: *QUO VADIS?*

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Abstract | Biological therapies using antibodies and cytokines are becoming widespread for the treatment of chronic inflammatory autoimmune diseases. However, these treatments have several limitations — such as expense, the need for repeated injections and unwanted side-effects — that can be overcome by genetic delivery. This review summarizes the ingenuity, sophistication and variety of gene-therapy approaches that have been taken in the design of therapeutic molecules and vectors, the engineering of cells and the regulation of gene expression for the targeting of disease outcome. We focus our attention on multiple sclerosis, type 1 diabetes and rheumatoid arthritis.

X-LINKED SEVERE COMBINED IMMUNODEFICIENCY (XSCID). Recessive inherited disease in which the X-chromosomally located common cytokine receptor γ-chain — the signalling component of many cytokine receptors — is mutated.

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doi:10.1038/nri1459

Somatic gene therapy is the introduction of new genetic material into the cells of an individual for therapeutic purposes. It was originally envisaged to be used only to treat inherited recessive diseases, such as X-LINKED SEVERE COMBINED IMMUNODEFICIENCY (XSCID), in which the addition of a normal gene (in this case the common cytokine receptor γ-chain) could provide a cure. However, it soon became apparent that gene therapy could be an efficient tool for the delivery of therapeutic molecules to treat a wide variety of polygenic or acquired diseases, such as autoimmune diseases (BOX 1).

Proteins that are commonly used as therapies for autoimmune diseases — such as insulin, interferons (IFNs) and tumour-necrosis factor (TNF)-specific antibodies — are expensive to produce, and many have short half-lives (particularly the cytokines), necessitating frequent administration through inefficient routes, such as subcutaneous injection. These proteins also need to be systemically administered at high concentrations to achieve local concentrations that can modify biological processes. However, because biological therapeutics cannot be locally targeted, they affect other organs and tissues, often giving rise to unacceptable side-effects, such as widespread immunosuppression[1]. In addition, when treatment stops, disease rebounds[2]. Delivery by gene therapy can overcome many of these limitations that are posed by protein administration, and it can provide long-term, safe and locally regulated gene expression (FIG. 1).

Controlling the level of therapeutic molecules is important if the therapy is to be safe and side-effects are to be reduced. Unlike protein therapies, gene therapy enables the use of transcriptionally regulated vectors that modulate gene expression in response to pharmacological agents, such as tetracycline, or to pathophysiological conditions, such as inflammation. Therefore, gene expression can be turned on during relapses and turned off during periods of remission (TABLE 1). Furthermore, the unwanted side-effects that are elicited by traditional protein treatments can be avoided by engineering molecules used for gene therapy to have short half-lives, such as the low-molecular-weight, dimeric TNF receptor (dTNFR), which blocks signalling by TNF[3]. Alternatively, therapeutic molecules could be modified to be released only at sites of disease: for example, by using engineered latent cytokines[4].

In this review, we summarize the main approaches and methods that have been developed for the treatment of autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis and type 1 diabetes, with gene therapy, and the advantages and disadvantages of these techniques.

Vector systems

Efficient gene delivery is central to the success of gene therapy. *In vivo* gene-therapy strategies require administration of the vector directly to patients; therefore, the

Box 1 | Important common features of autoimmune diseases

- Autoimmune diseases — such as type 1 diabetes, multiple sclerosis and rheumatoid arthritis — are characterized by organ-specific targeting of the immune response accompanied by tissue destruction, which can have widespread systemic complications in severe cases. These diseases comprise ~5% of the chronic diseases in the western world and therefore have an important socio-economic impact.
- These diseases are polygenic, and the strongest associations are with the expression of particular HLA haplotypes that are specific for each disease. These associations differ between ethnic backgrounds. Studies of identical twins have shown that inheritance contributes ~30% to disease susceptibility, whereas the remaining ~70% is probably due to environmental factors of unknown origin.
- Common pathological mechanisms lead to tissue destruction, such as overexpression of pro-inflammatory (T helper 1-type) cytokines, increased levels of matrix metalloproteinases and presence of free radicals. Both cellular and humoral immune mechanisms are involved.
- Age of onset varies, but the incidence of rheumatoid arthritis increases with age.
- Sex is an important factor because women are more susceptible to autoimmune diseases than men.

choice of vector is restricted by features such as its immunogenicity and the size of insert that can be cloned, and also by therapeutic requirements such as longevity of gene expression and the type and location of cells that are targeted for TRANSDUCTION (TABLE 2).

Viruses have evolved to deliver their genetic material to host cells, expressing genes required for virus replication, encapsidation and further infection. This ability has led to the development of replication-deficient viral vectors from which viral genes have been deleted and replaced by therapeutic genes. These viral vectors are assembled into infective particles by transfection of DNA into packaging cell lines that supply the essential deleted viral genes in *trans* during virus production. The resulting packaged vector can infect target cells but is defective in replication and cannot propagate, thereby providing improved safety and reducing the possibility of recombination with wild-type virus or the induction of further pathology.

In addition to viral vectors, plasmid DNA obtained from bacteria can also be used to deliver genes to cells, either in its naked form or complexed with LIPOSOMES. The important features of viral and plasmid vectors that are used for the treatment of autoimmune disease in experimental models are listed in TABLE 2; these have been reviewed elsewhere[5]. For the clinical treatment of chronic autoimmune diseases, non-immunogenic vectors are generally necessary because long-term gene expression is required. Cells that are transduced with immunogenic vectors are usually destroyed by cytotoxic lymphocytes within 2 to 3 weeks, so any beneficial effect would only be short term.

Several strategies can be used to improve the safety of these vectors[7]: the use of non-immunogenic GUTLESS ADENOVIRAL VECTORS[6]; the use of insulators that inhibit downstream effects of transcriptional units; the deletion of 3′ LONG TERMINAL REPEATS (LTRs) from RETROVIRAL VECTORS and LENTIVIRAL VECTORS; and the introduction of scaffold-attachment regions to prevent integrated viral cassettes causing INSERTIONAL EFFECTS.

TRANSDUCTION
The genetic modification of a cell using a viral vector. For retroviral vectors, this means that the cell will have a copy of the recombinant vector genome stably integrated into the host-cell chromosome.

LIPOSOMES
Lipid vesicles in which one of the components is positively charged to promote interaction with DNA. This DNA–liposome complex facilitates the cellular entry of DNA by fusing with cell membranes.

GUTLESS ADENOVIRAL VECTORS
Adenoviral vectors in which most of the viral genes have been removed to avoid immunogenicity and to provide space for the cloning of large genes.

LONG TERMINAL REPEATS
(LTRs). DNA sequences of approximately 600–800 base pairs that are present at both ends (5′ and 3′) of the retroviral-vector genome (provirus), even after integration into the DNA of the host cell. One region of the LTR, known as the U3 region, contains strong transcriptional enhancers and a promoter that drives expression of genes that are carried by most retroviral vectors. So, the U3 region of the retroviral vector can activate expression of cellular genes that are adjacent to the site of retroviral integration.

Transcriptionally regulated gene expression. Regulated gene expression is an important consideration in the development of gene therapy for chronic non-fatal autoimmune conditions that are characterized by phases of relapse and remission, such as multiple sclerosis and rheumatoid arthritis. The regulation of gene expression also has implications for safety and for optimizing therapeutic effects. Using an ideal pharmacologically regulated system, it is possible to increase or decrease expression of the therapeutic protein through the delivery of a small inducer molecule. Removal of the inducer should prevent expression: for example, in the event of adverse effects or during a remission phase of the disease. Various pharmacologically regulated expression systems have been developed and are discussed in detail elsewhere[8].

One commonly used gene-therapy system is the tetracycline 'on' system (FIG. 2), in which gene expression under the control of a tetracycline-regulated promoter is usually induced with the tetracycline analogue doxycycline[9]; this induces a conformational change in the transactivator rtTA (reverse tetracycline-regulated transactivator) such that it can bind the promoter. Basal activity of the tetracycline-regulated promoter has been overcome through the use of a targeted eukaryotic repressor (known as the tetracycline repressor, TetR), which binds to the promoter in the absence of doxycycline but is released after exposure to doxycycline[10,11].The dynamics of regulation of the system have also been improved by development of the transactivator rtTA2^{S}-M2 (REF. 12), which is more stable than the original transactivator rtTA and activates the tetracycline-regulated promoter at a 10-fold lower concentration of doxycycline. In the absence of doxycycline, TetR is tightly bound to the tetracycline-regulated promoter, thereby inhibiting gene expression, but when doxycycline is administered, TetR is removed and the transactivator rtTA2^{S}-M2 activates transcription. The components of this system have been optimally combined in self-contained vectors in both plasmid form[13,14] and gutless adenoviruses[15]. One unresolved problem remains the immunogenicity of the system. In mice, the components do not seem to be immunogenic, but in non-human primates, there are reports of immune rejection[16,17].

In mice with COLLAGEN-INDUCED ARTHRITIS (CIA), the doxycycline-induced expression of viral interleukin-10 (IL-10) from adeno-associated virus (AAV) or plasmid vectors before disease onset was shown to decrease disease incidence and help to preserve joint structure[18]. In established CIA, doxycycline-regulated expression of dTNFR from a self-contained, regulated plasmid inhibited disease progression and had beneficial effects on paw swelling and clinical score, but it elicited no change in immunological parameters[19]. The doxycycline-regulated system has also been used in an *ex vivo* protocol in which the regulated expression of TRAIL (TNF-related apoptosis-inducing ligand) by dendritic cells (DCs) from mice with CIA was used to induce apoptosis of autoreactive T cells[20].

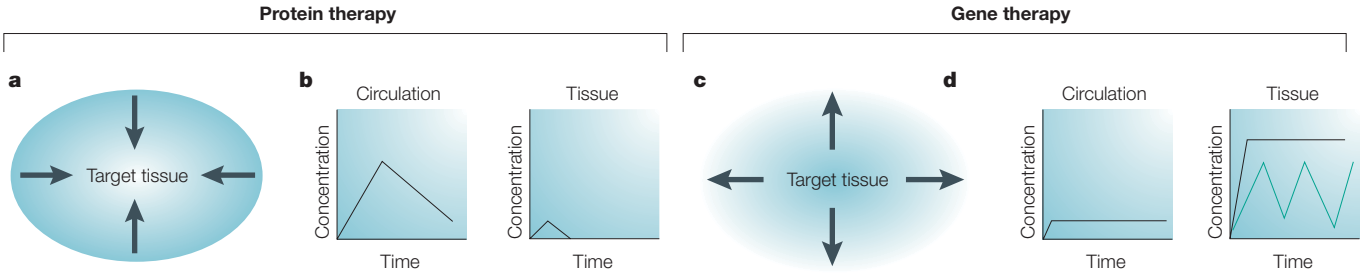

Figure 1 | **Pharmacological differences between protein and gene therapies. a** | Protein therapeutics form a concentration gradient from the blood to the tissue to be treated. **b** | Protein therapy therefore results in a high concentration in the blood that decreases quickly because of the uptake or degradation of the biological therapeutic used. The level that reaches the diseased tissue might not be sufficient for a long-lasting therapeutic effect. So, the protein needs to be frequently administered at high doses. **c** | Gene delivery can achieve long-term high concentrations locally by a single application directly to the affected tissue *in vivo*, or by using *ex vivo* engineered cells that are implanted locally or mobile cells that directly target the tissue, thereby affecting the pathological process. **d** | Therapeutic molecules expressed by the delivered genes achieve high concentrations locally and are consumed locally, so their systemic levels are low and do not affect other immune functions. In addition, transcriptional control can allow for desired expression levels only during relapse phases of disease (green line). This figure is modified with permission from REF. 138 © (1998) Springer, Heidelberg.

In addition to the tetracycline system, the pathophysiological process can also be directly harnessed to regulate therapeutic gene expression. Overexpression of pro-inflammatory cytokines, such as IL-2, is characteristic of many autoimmune diseases, and the promoters of the genes encoding these cytokines can be used to express therapeutic genes. In EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE), the *Il-2* promoter has been used to drive transcription of the gene encoding the anti-inflammatory cytokine IL-10 by encephalitogenic T cells[21]. Many genes that are induced by inflammation, such as those that encode acute-phase proteins and cytokines, have enhancer regions that respond to various transcription factors, such as nuclear factor-κB (NF-κB), activator protein 1 (AP1) and signal transducer and activator of transcription 3 (STAT3). In a recent study by van de Loo *et al.*[22], the authors combined the enhancer region of the human *IL-1β* gene with the *IL-6* promoter region to produce a hybrid promoter that has high-level responsiveness to inflammatory stimuli, both in cultured cells and in inflamed joints. Amplification of promoter activity has also been achieved by expressing the HIV transcription factor Tat (transcriptional transactivator) from the promoter of the complement component *C3* gene and then expressing a therapeutic gene from the HIV LTR, which is transactivated by Tat[23]. This system provides a higher level of therapeutic gene expression because of the overexpression of Tat, and it has been successfully applied in models of arthritis by intra-articular injection of an adenovirus expressing either the therapeutic protein IL-1 receptor antagonist (IL-1Ra) or IL-10 (REFS 24,25). Clearly, as our knowledge of transcriptional and post-transcriptional control of gene expression continues to increase, other endogenous mechanisms will be harnessed to regulate therapeutic gene expression.

In vivo gene therapy

Therapeutic genes can be delivered directly to patients (*in vivo*) or indirectly to isolated cells grown *in vitro* (that is, *ex vivo*). *In vivo*, a therapeutic gene is injected directly into the disease site (such as the central nervous system (CNS) in multiple sclerosis or the synovium in rheumatoid arthritis) or into other tissues that are easily accessible (such as the skin, muscle or liver through intravenous injection) and can function as factories to produce the therapeutic protein. Direct injection is required because most vectors, whether they are viral or non-viral, are replication deficient and cannot cross physical barriers such as endothelial cells. Several studies have used *in vivo* gene therapy in models of autoimmune disease. For example, injection of cationic-liposome–DNA complexes that encode inhibitory cytokines (such as IL-4, transforming growth factor-β (TGF-β) or IFN-β) or cytokine inhibitors (such as dTNFR) was effective in preventing the onset of EAE when these complexes were injected intracranially into the disease site[26,27].

Relatively efficient *in vivo* transfection of mouse skeletal muscle with naked plasmid DNA has been demonstrated by Wolff *et al.*[28], with transgene expression from an EPISOMALLY MAINTAINED PLASMID vector persisting for 2 months. This approach has also been used for DNA vaccination, which requires repeated administration of the plasmid DNA that encodes the immunogen. For example, the plasmid can encode an endogenous cytokine or chemokine[29], to which neutralizing antibodies develop that then block the function of the mediator. Other DNA immunogens that have been used include genes encoding T-cell receptors (TCRs) that recognize immunodominant epitopes, genes encoding the autoimmune antigen or genes encoding an MHC-class-I epitope specific for autoreactive cells[30]. This results in TOLERANCE to the autoantigen or the development of cytotoxic T cells that target the autoreactive cells. However, further investigation is required to determine the safety and long-term consequences of systemic immunosuppression using such immune structures and mediators as antigenic targets in a clinical setting, because these therapies might render patients susceptible to infections.

RETROVIRAL VECTORS
Disabled RNA viruses in which the viral genes have been replaced with engineered sequences, such as cytokine genes. The vector particles can no longer replicate in cells, but they can insert and express a therapeutic gene in appropriate target cells.

LENTIVIRAL VECTORS
Vectors that are based on slow replicating retroviruses. They have a more complex genomic structure than oncoretroviruses (which also require cell division for stable integration into the genome of a cell), and they express several accessory proteins in addition to Gag, Pol and Env. The main advantage of using these vectors for gene therapy is their relatively high efficiency of stably transducing quiescent cells.

INSERTIONAL EFFECTS
Effects on the gene expression of the host cell that are caused by the incorporation of gene-transfer vectors into the genome. These are mainly caused by retroviral and lentiviral vectors. One possible effect is gene disruption, another is gene activation, which could occur if strong enhancer sequences are present in the vector.

COLLAGEN-INDUCED ARTHRITIS (CIA). A model of rheumatoid arthritis. CIA develops in susceptible rodents and primates after immunization with cartilage-derived type II collagen.

Table 1 | **Advantages of gene therapy compared with protein therapy**

Feature	Protein therapy	Gene therapy
Administration	Multiple doses	Single dose
Effects	Systemic	Local
Cost	High	Low, plasmid as a vector is inexpensive
Regulated delivery	No	Yes
Long-term delivery	Possible, but disease rebounds when stopped	Yes, when delivered to long-lived cells in non-immunogenic vectors
Safety	Possible generalized immunosuppression; needs protein engineering to achieve targeting	Improved safety using local delivery

Electroporation increases the efficiency of intramuscular plasmid DNA transfection by 100 fold, with expression persisting for more than 1 year. This is in part because myoblasts have a slow rate of turnover[31]. This powerful non-viral gene-delivery approach has been used in various models of autoimmunity; these include arthritis treated with the gene encoding pro-opiomelanocortin[32], type 1 diabetes treated with the gene encoding prepro-insulin[33], systemic lupus erythematosus treated with a gene encoding an IFN-γ receptor–immunoglobulin fusion protein[34] and autoimmune myocarditis treated with the gene encoding IL-10 (REF. 35). However, intramuscularly electroporated plasmid DNA does, in rare cases, become integrated in the genome[36], which raises safety concerns about the clinical use of this strategy.

Intra-articular injection of vectors that express cytokines or cytokine inhibitors has been carried out in models of arthritis (discussed later); however, because arthritis affects many synovial joints, this approach does not seem to be viable in clinical settings. The use of *ex vivo*-engineered mobile cells is a better alternative because these cells can access all affected joints.

Ex vivo gene therapy

Ex vivo gene therapy involves removing cells from patients, followed by *in vitro* culturing and genetic engineering to encode a therapeutic molecule, before returning these cells to the patient. There are certain important advantages of this strategy. First, the administered cells are autologous and so immune rejection is avoided. Second, any vector system can be used for *ex vivo* manipulation, without directly exposing the patient to the vector. Clearly, *ex vivo* procedures are time consuming and expensive, but if the therapeutic benefit is shown to be long term (several years), these therapies could become the treatment of choice. This requirement for long-term therapeutic benefit influences the choice of cells that are used for this approach because the cells need to have long-term survival capabilities. The types of cell used in *ex vivo* strategies broadly fall into two categories: those that are mobile and those that are immobile.

Mobile cells as gene carriers. Mobile cells, such as T cells, B cells and DCs, have advantages because they can cross endothelial barriers to enter disease sites. T cells and B cells also proliferate after encountering cognate antigens, therefore amplifying the biological effect where it is required. In models in which the pathogenic antigen is known, it is possible to clonally expand antigen-specific $CD4^+$ T cells *ex vivo* and engineer them with a retrovirus that encodes a therapeutic protein. When returned to the patient, these $CD4^+$ T cells will localize to sites of antigen presentation (such as the affected tissue) and will proliferate in response to the presented antigen[37–42]. For example, encephalitogenic T cells that recognize myelin basic protein (MBP) have been used to deliver different cytokine genes in mouse models of multiple sclerosis (EAE). Delivery of the *Il-4* gene ameliorated EAE, whereas *Il-10* had almost no effect[43]. In addition, encephalitogenic T cells that express TGF-β ameliorated EAE development, although they were ineffective when administered after disease onset[44]. The effect of immunoregulatory T cells was potentiated when they were engineered to express IL-4, and diabetes incidence was further decreased in a T-cell transfer model[45]. However, the molecular and cellular mechanisms by which pathogenic T cells can become therapeutic by expression of an inhibitory cytokine or cytokine inhibitor are not well understood, and they require further investigation before this protocol can be attempted for the treatment of humans.

Antigen-specific interactions of TCR with MHC have been used in another approach in which chimeric molecules that consist of the extracellular and transmembrane domains of an MHC class I molecule fused to an MBP-derived peptide (amino-acid residues 89–101), and the TCR signalling domains were expressed in transgenic T cells. When these chimeric receptors engage the TCR of MBP-specific autoreactive T cells, the receptor-modified T cells are activated and the autoreactive T cells are killed by cytolysis. Adoptive transfer of these engineered T cells to mice with EAE has been an effective therapy, persisting for more than 75 days[46].

Clearly, a limitation of these approaches is that the antigen driving the disease process must be characterized before clinical applications can be developed, and this has not been well characterized for most autoimmune conditions. Also, in each patient, the autoantigen can differ at the various stages of disease development. In the absence of a clear antigenic target in arthritic joints, type II collagen, which is the main component of cartilage, can be used as a target for mediating T-cell tropism to the joint. This has been achieved by engineering T cells with a chimeric receptor that consists of an extracellular amino-terminal single-chain Fv domain

EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE). A model of multiple sclerosis. EAE develops in susceptible rodents and primates after immunization with antigens derived from the central nervous system.

EPISOMALLY MAINTAINED PLASMID
A plasmid that persists in the nucleus of a cell independently of the chromosomal DNA.

TOLERANCE
Denotes lymphocyte non-responsiveness to antigen, but implies an active process, not simply a passive lack of response.

Table 2 | **Features of the common vectors used for gene delivery**

Vector	Host cell	Vector genome	Transgene capacity	Immuno-genicity	Genomic integration	Duration of expression	Target cell	Advantages	Disadvantages
Plasmid DNA	Bacteria	dsDNA	Unlimited	Low (can be increased by CpG motifs)	No	Up to 2 years in muscle, short term in other tissues	Dividing and quiescent	Easy and cheap to produce; can increase transfection efficiency using electroporation, gene gun or liposomes	Lacks intrinsic mechanisms for cell entry
Adeno-virus*	Human	dsDNA	5–8 kb	High	No	6 weeks	Dividing and quiescent	Highly stable; good for short-term expression *in vivo*	Does not infect lymphocytes; more than 50% of human population has pre-existing antibodies
Gutless adeno-virus	Human	dsDNA	37 kb	Low	No	At least 1 year	Dividing and quiescent	Good for long-term expression	Difficult to produce; requires helper vector to provide replicative and structural proteins in *trans* during propagation
AAV	Human	ssDNA	4 kb	Low	Rare	Up to 1 year	Dividing and quiescent	Good for long-term expression	Insert size is small; 30% of human population has pre-existing antibodies
Herpes simplex virus	Human	dsDNA	35 kb	High	No	At least 6 months	Dividing and quiescent	Can express multiple genes; mainly neurotropic *in vivo*	Induces cellular toxicity and inflammation
Retro-virus‡	Mouse	RNA	7 kb	Low	Yes	For the life of the cell	Dividing	Has *ex vivo* applications	Can cause insertional effects
Lentivirus§	Human	RNA	7 kb	Low	Yes	For the life of the cell	Dividing and quiescent	Can be produced at high titres	Can cause insertional effects

*First and second generation. ‡Mainly based on Moloney murine leukaemia virus. §Mainly based on HIV, some based on simian immunodeficiency virus. AAV, adeno-associated virus; ds, double-stranded; kb, kilobases; ss, single-stranded.

of a monoclonal antibody that recognizes type II collagen linked to the transmembrane and cytoplasmic domains of the TCR signalling chains[47,48]. T cells engineered with these chimeric receptors proliferate and secrete IFN-γ in response to type II collagen[49]. However, further studies are required to obtain therapeutic effects using these engineered T cells, through the induction of expression of endogenous genes encoding T helper 2 (T_H2) cytokines, or using BICISTRONIC VECTORS to co-express anti-inflammatory cytokines. Nevertheless, such an approach in which the unprocessed antigen is recognized in an MHC-independent manner is important because its application is universal and independent of the MHC haplotype of the patient to be treated.

DCs are the most specialized antigen-presenting cells, providing a common set of signals that initiate the clonal expansion of T cells and help to determine differentiation into either T_H1 or T_H2 cells. In autoimmune diseases in which T_H1-cytokine overexpression is dominant, strategies that skew the response towards T_H2-cytokine production have been shown to be therapeutic. DCs that are engineered to express IL-4 have proved to be powerful tools for the *ex vivo* gene therapy of experimental autoimmune disease after their *in vitro* differentiation from bone-marrow cells in the presence of autoantigen and granulocyte/macrophage colony-stimulating factor alone or together with IL-4 or TGF-β (discussed later). When re-injected into mice, these DCs were shown to migrate to the lymphoid organs where antigen presentation occurs and to affect the type of T-cell response generated[50]. The safe use of DCs for the treatment of humans will require detailed knowledge of which autoantigens need to be co-expressed with which cytokines and also a specific way of deleting engineered DCs if systemic immunosuppression develops.

BICISTRONIC VECTORS
Vectors that are designed to express two distinct genes simultaneously.

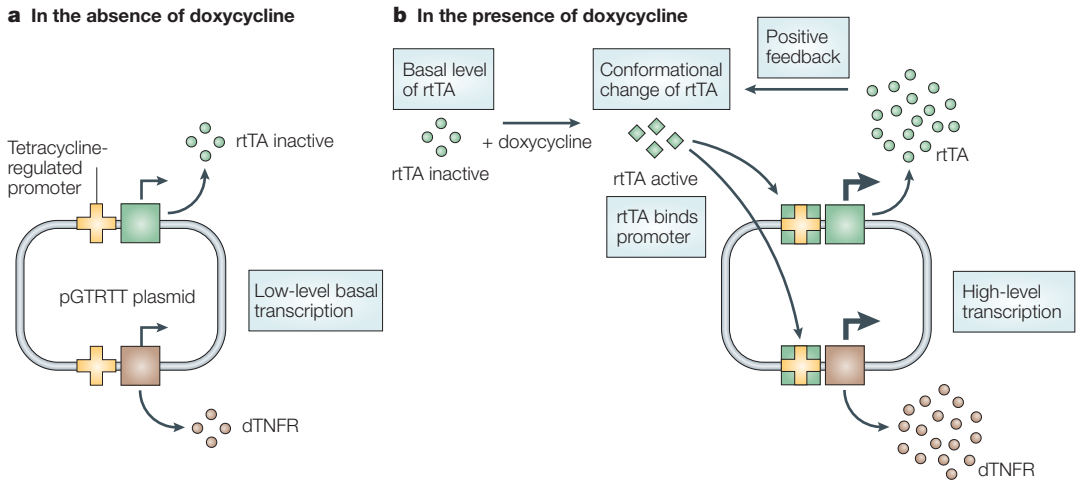

Figure 2 | **Doxycycline-regulated therapeutic gene expression.** The tetracycline 'on' system developed by Gossen *et al*.[9] has been combined in one expression vector. Here, we depict the plasmid known as pGTRTT[19], which independently expresses the transactivator rtTA (reverse tetracycline-regulated transactivator) and the therapeutic tumour-necrosis factor (TNF) antagonist the dimeric TNF receptor (dTNFR) from two tetracycline-regulated promoters. **a** | In the absence of the tetracycline analogue doxycycline, basal expression of both rtTA and dTNFR from the tetracycline-regulated promoters is minimal (thin arrows). **b** | In the presence of doxycycline, rtTA changes conformation in response to doxycycline binding and can then bind to the tetracycline-regulated promoters, increasing gene expression both of itself and of dTNFR. Once doxycycline is removed, rtTA cannot bind to its promoter and the overall levels of gene expression are reduced. As described in the main text, basal activity of the tetracycline-regulated promoters can be overcome using repressor molecules that bind the promoters in the absence of doxycycline and are released in the presence of doxycycline (not shown). This system is well suited for the treatment of autoimmune diseases that have cycles of relapse and remission, such as multiple sclerosis.

Immobile cells as gene carriers. Immobile cells that are used for gene therapy can be proliferative *in vitro* to allow for expansion of the transduced population, or they can be non-proliferative as is the case for pancreatic islets, which can be transduced, for example, by AAV or adenoviral vectors[51,52]. Immobile cells do not traffic within the body: for example, synovial fibroblasts can be obtained by ARTHROPLASTY, expanded and engineered *ex vivo* and then delivered to joints. This approach was pioneered by Evans and Robbins in Pittsburgh (United States) and has led to phase I clinical trials of gene therapy for rheumatoid arthritis[53].

Also, intracranial transplantation of retrovirally transduced fibroblasts that express IL-10 has been shown to effectively prevent the development of EAE, whereas IL-10 delivered by adenovirus or cationic-liposome–DNA complexes using the same route had no effect, indicating that the vehicle used for gene expression might influence efficacy[54]. In addition, these engineered fibroblasts also secreted other molecules *in vitro*, including the immunoregulatory cytokine TGF-β. Whether other factors are released after transplantation to the CNS is currently unknown.

Encapsulated cells are a safe, controlled, reversible and versatile alternative to immobile cells for gene therapy of autoimmune and other diseases. The capsule containing the cells that express therapeutic genes can be surgically removed or replaced at any time if required. Xenogeneic cells encapsulated in hollow fibres and engineered to express IL-4 or IL-13 have been used with therapeutic effect in models of arthritis[55]. However, a problem with cell encapsulation is that there is only limited space for population expansion, so the cells used in this system would ideally undergo contact growth inhibition or be differentiated into a non-proliferative state.

ARTHROPLASTY
A keyhole-surgery procedure to investigate joint disease.

Molecular targets

The search for molecular targets for treating autoimmune diseases is based on several strategies (FIG. 3). In general, using therapeutic targets that have a paracrine action has a more marked biological effect than using genes that target intracellular pathways. This is mainly because, in the latter case, only the cells that receive the vector express the gene and are affected (for example, by expression of a transcription-factor inhibitor or a small inhibitory RNA molecule). The degree of efficacy then depends on the number of cells that can be transferred or transduced by a particular vector. By contrast, when using secreted gene products, such as cytokines or soluble cytokine receptors, the transduced cells become factories of the therapeutic agent and can affect surrounding cells more efficiently. However, for transplanted islets, it has been shown that, although only ~35% of the cells in each islet can be successfully transfected, this quantity is sufficient to protect islets from the recurrence of autoimmunity or allo-rejection after transplantation — regardless of whether the protein product of the transfected gene is secreted or remains inside the transfected cell[56,57]. This effect, however, might depend on the purity of the β-cell preparation that is used for allogeneic transplantation[58].

Also, both vectors and therapeutic molecules should be non-immunogenic for sustained long-term therapy. The immunogenicity of particular proteins can be

modified: for example, by fusing the therapeutic protein to the poly-glycine–alanine repeats of the Epstein–Barr virus nuclear antigen 1 (EBNA1)[59]. This repeat inhibits protein degradation by the proteasome and thereby prevents peptide loading of MHC molecules and antigen presentation to cytotoxic T cells.

Reconstituting gene expression. In cases in which the absence of a protein is known to cause disease, gene therapy can be used to compensate for this absence. For example, in type 1 diabetes, restoring insulin expression to control glucose levels is paramount for achieving a potential cure[60]. Gene-therapy approaches have been used to deliver constructs that encode insulin or proteins that either facilitate glucose utilization or inhibit hepatic glucose production. For example, the insulin gene has been delivered to the liver in a form susceptible to regulation by blood glucose[60,61] or in a form engineered to be cleaved by proteases that reside in the liver, such as furin[62–64]. However, one of the main problems to

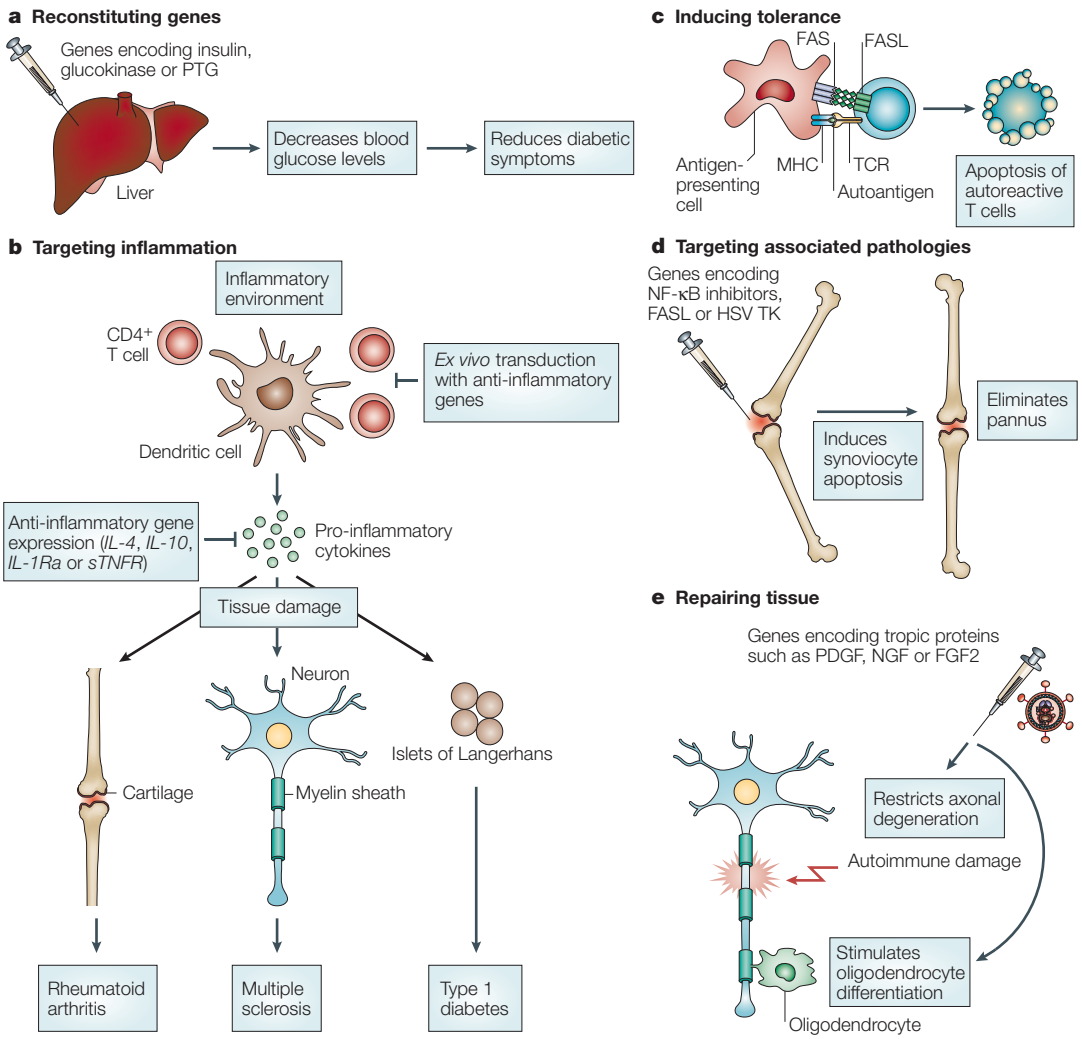

Figure 3 | **Molecular targets in autoimmune diseases. a** | Reconstituting genes. This approach is characteristic for type 1 diabetes and is based on the direct injection of insulin or the engineering of insulin expression by the liver, which is aided by providing glucose-regulating genes. PTG, protein targeting to glycogen. **b** | Targeting inflammation. Most autoimmune diseases are characterized by a T helper 1 (T_H1)-cell-type autoreactive inflammatory response. This can be counteracted by the expression of genes that provide an anti-inflammatory milieu, such as interleukin-1 (IL-1) receptor antagonist (*IL-1Ra*), *IL-4*, *IL-10*, soluble tumour-necrosis-factor receptor (*sTNFR*) and others that can induce a T_H2-type regulatory immune response. The therapeutic genes can be delivered *in vivo* or *ex vivo* through antigen-specific T cells or dendritic cells (DCs). **c** | Inducing tolerance. The elimination of autoreactive T cells can be achieved by expressing autoantigenic peptides that associate with MHC molecules of DCs in a T_H2-type environment, by vaccination against genes that encode the chains of the specific T-cell receptor (TCR) or by inhibition of the interaction between antigen-presenting cells and T cells. The example shown involves the expression of FAS ligand (FASL; also known as CD95L) or TRAIL (TNF-related apoptosis-inducing ligand) by DCs, which induces the apoptosis of autoreactive T cells. **d** | Targeting associated pathologies. The reduction of pannus growth in arthritis, mediated by apoptosis of synoviocytes, can be induced by direct injection into the joint of genes encoding nuclear factor-κB (NF-κB) inhibitors, FASL or suicide proteins such as herpes simplex virus (HSV) thymidine kinase (TK). **e** | Repairing tissues. This can be achieved by gene transfer of tropic genes — such as those encoding platelet-derived growth factor (PDGF), nerve growth factor (NGF) and fibroblast growth factor 2 (FGF2) — to stimulate tissue repair *in situ*, or the avoidance of rejection after allogeneic transplantation in type 1 diabetes.

be overcome in the treatment of type 1 diabetes is the slow response to changing glucose levels: whereas pancreatic β-cells react to hyperglycaemia by releasing insulin in less than 1 minute, current gene-therapy approaches show a lag time of at least 1–2 hours before a rise in glucose levels causes a release of insulin[65,66].

An alternative strategy for the treatment of type 1 diabetes is to lower glucose levels by modulating the metabolic pathways of glucose utilization. Adenovirus-mediated gene transfer of glucokinase has been used as a combination therapy together with insulin gene replacement[67]. Another approach is to deliver the gene encoding the natural protein PTG (protein targeting to glycogen) to stimulate glycogenesis in the liver, thereby reducing blood glucose levels[68].

Inhibiting inflammation and targeting the cytokine imbalance. Our increased knowledge of the pathological mechanisms involved in autoimmune diseases and other inflammatory diseases such as cancer[69,70] has allowed us to identify novel targets for therapy that could modify the immunological processes leading to disease development and progression. A common feature of all autoimmune diseases is the breaking of tolerance and the activation of pro-inflammatory cytokine signalling cascades. Therefore, manipulation of cytokine activities or their intracellular pathways has become an important target for the therapy of autoimmune diseases. In many models of autoimmune diseases, it has been shown that regulation of immune responses and inhibition of disease progression could be achieved by targeting the reactive immune cells or other mediators of pathogenesis. Successful strategies have included targeting $CD4^+$ T cells with monoclonal antibodies that are specific for CD4 and targeting the overexpression of pro-inflammatory cytokines using anti-inflammatory cytokines (such as IL-4, IL-10, TGF-β and IL-13), cytokine inhibitors (such as IL-1Ra), cytokine-specific antibodies or soluble receptors (such as TNFR)[26,55,71–73].

The main targets in rheumatoid arthritis are TNF and IL-1, and the gene-therapy approach consists of delivering molecules that block the actions of these cytokines. TNF has been targeted using a soluble TNFR, which blocks the binding of TNF to the cell-surface receptor, thereby antagonizing its activity[37,74]. Similarly, to target IL-1, its natural antagonist IL-1Ra has been successfully delivered by engineered synoviocytes both in animal models of arthritis and in a phase I clinical trial[75]. High levels of IL-1Ra expression were achieved in patients, which is important because the inhibition of IL-1 activity requires a large molar excess of IL-1Ra, which is not generally achieved using IL-1Ra protein therapy. The blocking of IL-1 by IL-1Ra is also effective for the treatment of type 1 diabetes[71,76].

Another anti-inflammatory cytokine, IL-4, has been shown to be beneficial in various animal models of autoimmune disease. IL-4-encoding vectors were effective when delivered *in vivo* — either systemically or locally (to the joint) — or *ex vivo* by genetically modified fibroblasts, macrophages, B cells or DCs[77–80]. More recently, IL-4-encoding DNA was also found to be therapeutic after either GENE-GUN DELIVERY or intradermal injection[81]. However, the route of administration might have an important role, because systemic administration of IL-4 suppressed active arthritis in a mouse model, whereas intra-articular administration had no effect[82,83]. However, both intravenous and peri-articular transfer of the *Il-4* gene reduced the severity of established arthritis in mice[84].

A single intramuscular injection of plasmid DNA can also be used for the long-term systemic expression of therapeutic proteins. Injection of plasmid DNA encoding IL-4 had no therapeutic effect in EAE. However, the use of an IL-4–immunoglobulin fusion protein or TGF-β, which have longer half-lives than IL-4, was effective in reducing the clinical and histopathological signs of EAE, indicating that the pharmacokinetic characteristics of the injected molecule can have important implications for therapeutic efficacy[85]. Similarly, injection of mice with plasmid or AAV vectors that express IL-10, or with DCs or diabetogenic T cells that express IL-4, prevented or ameliorated insulitis and reduced the incidence of diabetes[45,86–89]. Other cytokines that have been targeted using gene-therapy techniques include IL-18, which was inhibited using the IL-18-binding protein[90], and IL-13 (REF. 91).

The development of latent cytokines has provided a novel method to modify the pharmacokinetic properties of cytokines and to ensure their specific targeting to sites of disease, thereby avoiding unwanted side-effects[4]. Latent cytokines are fusion proteins that are encapsulated by a disulphide-bonded shell, provided by the latency-associated peptide of TGF-β, that is covalently linked to the cytokine by a MATRIX METALLOPROTEINASE (MMP)-cleavable linker. This structure gives the cytokine an increased half-life and therapeutic index. Because cytokine release is highly dependent on the extent of MMP activity at the site of disease, this maintains the latency of cytokines in normal tissues and fluids[4]. Latent cytokines can be safely delivered either *in vivo* or *ex vivo*. This approach has the additional advantage that potent therapeutic molecules that have been rejected for human use because of their systemic side-effects could be reassessed in a clinical setting.

Inducing tolerance. A common strategy that is effective in different models of autoimmune disease is the induction of tolerance by suppressing CO-STIMULATORY SIGNALS during T-cell activation. For example, expression of a cytotoxic T lymphocyte antigen 4 (CTLA4)–immunoglobulin fusion protein has been successfully used to block the interaction between CD80/CD86 and CD28, and consequently to inhibit the development of EAE and arthritis[92,93]. Also, the expression by DCs of immunosuppressive cytokines, such as IL-4 and viral IL-10, has been used to treat diabetes and arthritis, respectively[86,94].

A new strategy for inducing gene-therapy-mediated tolerance combines gene transfer of an encephalitogenic epitope with the use of a carrier cell, such as B cells. Adoptive transfer of B cells that express either a proteolipid protein (PLP)-derived peptide (amino-acid

GENE-GUN DELIVERY
High-speed delivery of gold particles coated with DNA.

MATRIX METALLOPROTEINASE
Member of a family of tightly controlled zinc-dependent enzymes. These enzymes degrade the extracellular matrix in processes of cell migration that occur during embryogenesis, wound healing, inflammation and tumour dissemination.

CO-STIMULATORY SIGNALS
Signals to a T cell (provided by interaction with either a soluble or a membrane-bound molecule) that have little or no effect alone, but either enhance or modify the physiological effect of the primary signal, which is mediated by engagement of the T-cell receptor.

residues 139–151)[95] or a chimeric MBP–IgG construct rendered most mice tolerant to EAE induction and, in the latter case, was also effective in reducing the severity of established disease[96]. The clinical effect requires the expression of FAS ligand (FASL; also known as CD95L) by the B cells for the deletion of the autoreactive cells.

In other studies, the combination of DNA vaccines encoding encephalitogenic determinants with *IL-4* gene transfer has successfully ameliorated ongoing disease[97]. However, the failure of a clinical trial that aimed to induce tolerance in patients with multiple sclerosis using an ALTERED PEPTIDE LIGAND[98,99] highlights the complexities of targeting peptide–MHC–TCR interactions in humans, which differ greatly from those of inbred animals that express a single MHC haplotype.

Targeting apoptosis. The inhibition of proliferation of cells of the immune system or the synovial outgrowth is an alternative strategy to control inflammation in arthritis. Apoptosis of inflammatory cells can be induced using gene therapy: for example, the elimination of autoreactive T cells and neutrophils in established arthritis has been achieved by inducing the expression of the pro-apoptotic ligands FASL or TRAIL by DCs[20,100–102]. Similarly, the mammalian lectin galectin-1 has been used to promote antigen-specific cellular apoptosis. Galectin-1 binds to β-galactoside-containing proteins at the cell surface, thereby modifying TCR signalling and inducing cell death. Gene expression of galectin-1 by autologous fibroblasts was effective for the treatment of established CIA by increasing apoptosis of pathogenic T cells and promoting a shift to a therapeutic T_H2-type immune response[103].

Apoptosis of synoviocytes in inflamed joints has been achieved by intra-articular transfer of the thymidine kinase (*tk*) gene of herpes simplex virus (HSV) followed by administration of the nucleoside analogue gancyclovir. Gancyclovir is phosphorylated by HSV TK, resulting in inhibition of endogenous DNA polymerase activity and cell death. This success in animal models[104,105] has led to a clinical trial being set up. Apoptosis of synovial cells was also achieved by blocking NF-κB translocation to the nucleus through the expression of the α-subunit of inhibitor of NF-κB (IκBα)[106] or using double-stranded DNA oligonucleotide decoys[107]. The NF-κB pathway has also been targeted in pancreatic islets *in vitro*[108] using an adenovirus that expresses a mutated super-repressor IκBα. Inhibition of NF-κB prevented nitric-oxide production and protected islet cells from IL-1-mediated toxicity. It is important to note that in a pro-inflammatory environment, inhibition of the NF-κB pathway has protective effects on islets but deleterious effects on synoviocytes.

Targeting other associated pathologies. Strategies that target non-immune processes include inhibition of MMPs, restoration of cell-cycle control, reduction of free radicals, inhibition of angiogenesis and amelioration of the debilitating effects of pain.

The tumour-suppressor gene *TP53* has been shown to be mutated in rheumatoid arthritis. Reconstitution of cell-cycle control by delivering wild-type p53 prevents the synovial lining becoming hyperplastic; it achieves this by promoting apoptosis, and so preventing invasion by this highly aggressive tissue, which destroys cartilage and bone[109,110]. Other molecules with functions similar to that of p53 in cell-cycle regulation have also been delivered by gene therapy, including the cyclin-dependent kinase (CDK) inhibitors p16 and p21 (REFS 111,112). The *RAS* oncogene has also been targeted using dominant-negative constructs, which were found to be effective in suppressing bone destruction[113].

In chronic inflammatory diseases, the formation of free radicals, such as reactive-oxygen species, has been proposed to have an important pathological role. Expression of free-radical-scavenging enzymes, such as extracellular superoxide dismutase and catalase, by *ex vivo* transfection of synoviocytes has been shown to be anti-inflammatory and to inhibit MMP activity in arthritis[114], and expression of manganese superoxide dismutase has been shown to extend islet-graft survival in a model of diabetes[115]. In arthritis, inhibition of extracellular-matrix degradation by MMPs can also be achieved by delivery of tissue inhibitors of MMPs (TIMPs) or TGF-β[42,116–118]. Interestingly, the inhibition of angiogenesis by endostatin, angiostatin or soluble vascular endothelial growth factor (VEGF)-receptor gene transfer has also been successfully used in arthritis[119–121].

In arthritis, pain is one of the most debilitating and severe effects that is associated with cartilage erosion. Chronic pain is a new target in patients with arthritis, and expression of the pain-relieving endorphin precursor prepro-encephalin A from an HSV vector promoted mobility and reduced hyperalgesia[122]. Similarly, intramuscular injection of naked plasmid DNA that encodes pro-opiomelanocortin reduced hyperalgesia and paw swelling, which was mediated in part by increased secretion of endorphins and pro-opiomelanocortin-derived adrenocorticotropic hormone[32]. Limb ischaemia caused by diabetes has also been targeted successfully using tissue kallikrein[123], and limb neuropathy was shown to be reversed using VEGF[124] or by the expression of nerve growth factor (NGF) delivered by an HSV vector[125].

Tissue regeneration and repair

Restoration of normal tissue function is a major problem in autoimmune diseases. Strategies to treat type 1 diabetes include transplantation of genetically engineered islets of Langerhans, pancreatic stem cells and non-pancreatic progenitors such as surrogate β-cells[126]. The lack of available autologous pancreatic β-cells strengthened the impetus for the development of allogeneic transplantation; however, this type of therapy must be accompanied by immunosuppression.

An interesting new approach is to promote islet neogenesis in the liver. Adenovirus-mediated transfer of the genes encoding the β-cell differentiation-inducing transcription factors neurogenic differentiation 1 (NeuroD1)

ALTERED PEPTIDE LIGAND
Peptide that has an altered autoantigen-derived sequence and modifies T-cell function when presented in the appropriate MHC context.

TP53
Tumour-suppressor oncogene that encodes p53. The mutation of the *TP53* gene in cancer and rheumatoid arthritis can lead to lack of cell-cycle control and apoptosis.

and pancreatic and duodenal homeobox protein 1 to streptozotocin-induced diabetic mice was shown to ameliorate hyperglycaemia and to reverse the symptoms of diabetes[127,128]. Transfer of *NeuroD1* together with the islet growth factor betacellulin was able to completely reverse diabetes owing to islet neogenesis. In addition, apoptosis of β-cells has also been prevented by *in vitro* and *in vivo* adenoviral transfer of the anti-apoptotic molecules BCL-2 (B-cell lymphoma 2) and A20 respectively[129,130].

A successful therapeutic approach for multiple sclerosis should include both the restriction of axonal degeneration and the stimulation of oligodendrocyte differentiation. Current strategies for the treatment of multiple sclerosis involve transplantation of oligodendrocytes or oligodendrocyte precursors in specific affected regions. However, this approach is inadequate for the treatment of disseminated disease, in which multifocal treatment is required. A recent positive result has been achieved through the treatment of EAE with neurospheres derived from adult stem cells[131]. Adoptive transfer of encephalitogenic T cells genetically modified to produce either the α-subunit of platelet-derived growth factor or NGF resulted in the amelioration or even suppression of ongoing EAE[132,133]. Moreover, intrathecal delivery of HSV1 vectors carrying fibroblast growth factor 2 induced the differentiation and migration of oligodendrocytes into areas of demyelination[134]. In addition, another area of active research is the use of mesenchymal stem cells to repair cartilage in arthritis[135,136].

Concluding remarks

It is important that the ethics and science of gene therapy for autoimmune diseases develop together, because these diseases are mostly non-fatal and the benefit to patients must outweigh any potential risk. Therefore, safety issues — such as generalized immunosuppression, immunogenicity of transgenes and vectors, and fate of engineered cells — need to be assessed in long-term studies.

A current limitation of most preclinical studies of treatments for autoimmune disease is that immunogenic vectors are often used as proof of concept in animal models. Progress has also been restricted because many studies are short term (in part due to the vector) or have used an acute model of disease, which does not truly reflect the chronic nature of autoimmune diseases in humans. However, using numerous targets, these studies have provided strong evidence that local or systemic gene therapy could be a potent method of treatment and so warrants further investigation.

Advances in the areas of vector design, safety, methods of reducing the immune response to transgenes and engineering of novel therapeutics have provided researchers with better tools to combat autoimmune diseases. Using combinations of technologies, as well as therapeutic targets, is to be encouraged, and this should provide safe and efficient methods for the treatment of autoimmune diseases. In the next few years, we expect that new clinical trials of gene therapies for autoimmune diseases will be set up. We predict that the use of injected plasmid DNA combined with non-immunogenic transcriptionally regulated promoters that allow the expression of latent cytokines will be an important approach. In addition, if autologous T cells are to be used, it will be important to include suicide genes to remove the genetically engineered cells in the case of adverse events[37].

The use of combination therapy also needs to be investigated. A recent clinical study in patients with rheumatoid arthritis showed that using recombinant TNF-specific antibodies and recombinant IL-1Ra protein did not induce synergistic effects[137]. In part, this result is not surprising as both targets are part of the same pathological pathway that leads to cartilage degradation, and it is known that TNF-specific antibodies can directly downregulate the expression of IL-1. However, it is unlikely that one biological agent will be sufficient to stop complex autoimmune pathological processes that have superimposed phases of acute and chronic inflammation. So, we suggest the local use of agents that target different stages of the pathological process: for example, cytokine imbalance and free-radical formation, or cellular extravasation and cellular apoptosis, or angiogenesis and immunosuppression.

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Acknowledgements

The authors are grateful for research support from the Arthritis Research Campaign (United Kingdom) and The Wellcome Trust (United Kingdom). We are also grateful to L. Layward for careful reviewing and editing of the manuscript. We apologize to our colleagues who have made important contributions to the field but could not be cited due to space constraints.

Competing interests statement

The authors declare competing financial interests: see Web version for details.

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