

# Haematopoietic Stem Cell Based Gene Therapy as a Strategy to Treat Autoimmune Disease

Frank Alderuccio<sup>1\*</sup> • James Chan<sup>2</sup> • Ban-Hock Toh<sup>2</sup>

<sup>1</sup> Department of Immunology, Monash Central and Eastern Clinical School, Commercial Road, Prahran, Victoria, Melbourne, 3181, Australia

<sup>2</sup> Centre for Inflammatory Diseases and Department of Medicine, Monash University, Victoria, 3163, Australia

Corresponding author: \* frank.alderuccio@med.monash.edu.au

## ABSTRACT

Autoimmune diseases affect 5-6% of the Caucasian population and represent a major burden on an individual's life-style and the nation's health budget. There have been no cures described with current treatment based on replacement therapy and/or broad immunosuppressive agents. A challenge for research in this field is to devise strategies aimed at specifically abrogating the immune response to self-antigens while retaining an immune system that can combat foreign pathogens. The ability of the bone marrow compartment to confer immunological tolerance to a recipient is well established and the use of allogeneic and autologous haematopoietic stem cell transplantation (HSCT) to treat autoimmune disease have been reported for a number of autoimmune diseases. Results vary between trials and diseases. A critical observation is the significant rate of relapses with autologous HSCT in humans with autoimmune disease that suggest that this may not be a curative strategy. However, the ability to easily harvest autologous haematopoietic stem cells and genetically manipulate them, opens up avenues that can be evaluated in the quest to enhance HSCT as a curative strategy. The use of gene therapy to target autoantigen to HSCs in the autoimmune diseases to establish molecular chimeras and antigen-specific tolerance is still in its infancy. Nonetheless, the few experimental studies that have directly used this approach, coupled with approaches to clear the periphery of self-reactive T cells indicates that genetically-engineered HSCT may be a feasible and viable option for the curative treatment of the autoimmune diseases.

**Keywords:** Gene Therapy, experimental autoimmunity, tolerance, haematopoietic stem cells

**Abbreviations:** AIRE, Autoimmune regulator element; APECED, Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; APS-1, Autoimmune polyendocrinopathy syndrome; EAE, Experimental autoimmune encephalomyelitis; EAG, Experimental autoimmune gastritis; EGFP, Green fluorescent protein; GAD, Glutamic acid decarboxylase; GMCSF, Granulocyte-macrophage colony stimulating factor; H/K ATPase, Hydrogen Potassium ATPase; HSCs, Haematopoietic stem cells; HSCT, Haematopoietic stem cell transplantation; HSP60, Heat shock protein 60; LCMV, Lymphocytic choriomeningitis virus; MHC, Major histocompatibility complex; MOG, Myelin oligodendrocyte glycoprotein; NOD, Non-obese diabetic mouse; PC-GMCSF, Parietal cell-GMCSF; PLP, Phospholipid protein; SLE, Systemic lupus erythematosus; T1D, Type 1 diabetes; TCR, T cell receptor; TNF, Tumour necrosis factor

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## INTRODUCTION

Collectively, autoimmune diseases represent the pathogenic consequence of an adaptive immune response that recognise and destroys self-tissue. They display a range of disorders affecting many organ systems (Diamond 2005). Autoimmune diseases are estimated to affect approximately 5-6% of the Western population (Davidson *et al.* 2001) with examples including Type 1 diabetes (T1D) (Gianani *et al.* 2005; Bach 2006), systemic sclerosis (Denton *et al.* 2006), systemic lupus erythematosus (Kang *et al.* 2006), autoimmune gastritis (Toh *et al.* 2004, 2007) and multiple sclerosis (Hafler *et al.* 2005; McQualter *et al.* 2006). For a comprehensive compilation of reviews on autoimmune diseases,

readers are directed to the recently published Autoimmune Diseases (Rose *et al.* 2006). While genetic (Morahan *et al.* 2002) and environmental factors have been associated with autoimmunity, little is known on what actually triggers disease in humans. Experimentally, autoimmune diseases can be induced in a number of ways; which may give insight into possible triggers associated with human disease. One popular hypothesis termed molecular mimicry, involves a cross-reactivity or similarity of antigenic epitopes between microbial agents and self-molecules. As a consequence, activation of a microbe-directed immune response will produce an immune response that will target and destroy the self-antigen long after the initiating microbe has been cleared (Oldstone 2005). Evidence for generalised micro-

bial involvement in autoimmune diseases is sparse but some strong associations have been reported. These include a link between rheumatic heart disease and streptococcal antigens (Guilherme *et al.* 2006), autoimmune gastritis and *Helicobacter pylori* (Amedei *et al.* 2003; D'Elisio *et al.* 2004) and reactive arthritis which involves cross reactivity between GroEL from *Salmonella typhimurium* and HSP60 (Lo *et al.* 2000). However this area still remains greatly de-bated and awaits convincing evidence (Rose *et al.* 2000; Benoist *et al.* 2001).

Autoimmune diseases are currently incurable. The ability to offer a selective treatment that will rid antigen-specific autoimmune responses without compromising the immune system as a whole remains a holy grail of immunological research. Traditional treatments for autoimmune diseases include replacement therapy such as insulin for T1D and vitamin B12 for pernicious anaemia or broad immunosuppressive agents (Rudick *et al.* 1997; Tuckermann *et al.* 2005). As our understanding of the molecular basis of these diseases develops, refined approaches are being developed to provide more selective treatments (Feldmann *et al.* 2005). A prime example of this is the development of anti-TNF treatments for patients with rheumatoid arthritis (Feldmann *et al.* 1996, 1997). However, these agents may not be suitable for all autoimmune situations, with anti-TNF blockage described for rheumatoid arthritis found to worsen disease in patients with MS (Feldmann *et al.* 2005). Thus the quest for treatments that will provide long-term remission for autoimmune diseases continues.

#### THYMIC TOLERANCE, PERIPHERAL ANTIGEN EXPRESSION AND AUTOIMMUNITY

As a system charged with the task of protecting the host from foreign pathogens, the immune system has developed the capacity to respond to any potential threat. In the adaptive immune response, this recognition is through specific antigen receptors associated with T and B cells (Goldrath *et al.* 1999; Janeway *et al.* 2005). Since these antigen receptors are generated through a process involving random recombination of defined gene elements and not in response to pathogen exposure, chances of generating receptors with self-reactivity are high. In light of this, the immune system has developed a range of mechanisms designed to reduce the generation or activation of self-reactive clones. These can involve the processes of deletion, where self-reactive cells are induced to die; or regulation, where the activation of self-reactive cells is controlled through the action of additional forces such as regulatory cells. A detailed review of immunological tolerance is beyond the scope of this review and the reader is directed to recent reviews on this subject (Walker *et al.* 2002; Goodnow *et al.* 2005; Kyewski *et al.* 2006; Zehn *et al.* 2006). The mechanism of deletion within the primary lymphoid tissues is commonly referred to as central tolerance. Within the thymus this process defines the process where developing T cells are screened for reactivity through the interaction of their T cell receptor with MHC and peptide complexes presented on thymic epithelia and dendritic cells (Gallegos *et al.* 2006). Experimentally, it was the study of Kiselow and von Boehmer who first demonstrated that the presence of a defined antigen in the thymus could induce deletion of antigen specific clones (Kiselow *et al.* 1988). Using transgenic mice that expressed a TCR specific for the male H-Y antigen, they observed that in females (lacking H-Y), anti-H-Y T cell development was normal whereas in males, these cells were absent. This has led to our current understanding that the T cell repertoire is moulded by exposure of developing T cells to self-peptides presented by thymic MHC. What governs whether a cell is deleted is dependent on TCR affinity or avidity such that thresholds exist above which cells will be signalled to die (Haribhai *et al.* 2003; Palmer 2003; Hogquist *et al.* 2005). Cells that do not reach this threshold are maintained to develop into the repertoire suitable to combat foreign antigens and also are selected to develop into regu-

latory T cells that provide a further means of subsiding the autoimmune response (Jordan *et al.* 2001).

The notion of antigen expression in the thymus and its role in self-tolerance, in particular to peripheral antigens was initially developed by the work of Hanahan and colleagues (Hanahan 1998). They observed under experimental conditions that the targeted peripheral expression of an antigen to the pancreas resulted in antigen being detected in the thymus (Jolicoeur *et al.* 1994). This concept of peripheral or tissue specific antigen expression within the thymus is now well established and has recently been cemented by studies from Derbinski and Kyewski (Derbinski *et al.* 2001; Kyewski *et al.* 2004; Derbinski *et al.* 2005; Kyewski *et al.* 2006) who estimate that this process may be responsible for the expression of several thousand genes in the thymus (Kyewski *et al.* 2004). As a mechanism of promoting tolerance to peripheral antigens, this complements the idea that exposure to antigen is a major means of tolerising autoreactive clones. While much of our understanding surrounding promiscuous gene expression and central tolerance has developed from studies involving *Aire*, it appears that promiscuous gene expression is likely to be driven by a number of yet defined transcription factors since the expression of some peripheral antigens is *Aire* independent (Kyewski *et al.* 2004, 2006).

Perhaps the most compelling evidence that natural expression of genes within the thymus can have profound effects on the T cell repertoire and autoimmunity come from recent studies involving the putative transcription factor AIRE (AutoImmune Regulator Element). For a comprehensive summary on the molecular and biochemical features of *Aire*, readers are referred to the recent review by Anderson and colleagues (Cheng *et al.* 2007). In humans, mutations in the AIRE gene result in Autoimmune polyendocrinopathy syndrome type 1 (APS-1), or APECED (Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy), characterised by autoimmune pathologies in multiple organs such as the pancreas, stomach and thyroid (Bjorses *et al.* 1998; Peterson *et al.* 1998). Analysis of various human and mouse tissues indicates the *Aire* gene is predominantly expressed in thymic medullary epithelial cells at the cortico-medullary junction and in a small proportion of dendritic cells (Heino *et al.* 1999; Zuklys *et al.* 2000; Derbinski *et al.* 2001; Kogawa *et al.* 2002; Klamp *et al.* 2006). In the mouse, targeted deletion of the *Aire* gene largely reproduces the human findings with mice developing autoimmune disorders affecting pancreas, retina, ovary, salivary glands and thyroid (Anderson *et al.* 2002). In this study, Anderson and colleagues estimate that *Aire* could be associated with the expression of up to 1200 genes indicating that a number of transcription factors are likely to be responsible for all TSA expression. This is supported by recent findings which show expression of certain peripheral antigens such as GAD67 and C-reactive protein are not influenced by *Aire* expression (Anderson *et al.* 2002). In studies that have specifically addressed the role of *Aire* in tolerance, Liston *et al.*, demonstrated that tolerance to a neoantigen driven by the insulin promoter was directly associated with expression of *Aire* such that mice deficient in *Aire* failed to delete antigen specific thymocytes in the thymus (Liston *et al.* 2003). Furthermore, the dosage of *Aire* resulted in a differential release of autoreactive cells in which heterozygous mice induced less thymic deletion and increased progression to autoimmunity (Liston *et al.* 2004). Within the thymic epithelial cell population, it seems that *Aire* expression is restricted to cells expressing the tight junction associated molecules claudin-3 and -4 which may shed clues to the origin of these cells during thymic development (Hamazaki *et al.* 2007).

The underlying principle supported by these studies is that the level of antigen expression within the thymus can influence the tolerance process. Altering antigen expression could influence antigen expression and presentation and the threshold required to induce deletion. In *Aire*-deficient mice, absence of antigen would thus raise the threshold required

for T cells to be deleted such that fewer potential antigen specific cells would be deleted, ultimately being released into the periphery. A similar picture is also emerging in humans. In a recent study by the Kyewski group, it was found that the expression level of tissue-restricted self-antigens correlated with the level of Aire expression in medullary epithelial cells (Taubert *et al.* 2007).

The converse of the Aire studies described above would be the consequence of adding antigen to the system and what effect this would have on tolerance. Using this as a working hypothesis we demonstrated in a transgenic model of autoimmune gastritis (EAG) that ectopic expression of a major autoantigen (H/K ATPase  $\beta$ -subunit) in MHC class II positive cells rendered mice resistant to disease induction (Alderuccio *et al.* 1993). The induction of tolerance and disease resistance has also been shown in other models including the NOD model of T1D in which ectopic expression of proinsulin II renders mice resistant to developing T1D (French *et al.* 1997; Jaekel *et al.* 2004). Similarly, ectopic expression of experimental autoimmune uveitis associated autoantigen; interphotoreceptor retinoid-binding protein (IRBP) completely protected mice from disease induction (Xu *et al.* 2000).

While transgenic expression of these autoantigens can promote tolerance, presumably through clonal deletion, examination of expression patterns within the normal thymus indicates that expression is not totally absent. Messenger RNA expression of the gastric H/K ATPase and insulin are found in the mouse thymus (Kyewski *et al.* 2002, 2004), but the fact that disease can be induced in normal mice suggests that natural expression levels are insufficient to induce complete tolerance. Parameters that may influence the degree of tolerance may include the level of expression, source of antigen expression and even the stability of antigens expressed. At closer examination, it can be seen that within the thymus, expression of tissue specific antigens is not equal amongst all cells of the thymic stroma. While medullary thymic epithelial cells seem to express all genes, this is not the case for other cells that make up the thymic stroma including cortical thymic epithelial cells, macrophages and dendritic cells (Derbinski *et al.* 2001; Kyewski *et al.* 2002). The pattern of gene expression in these cells is more restricted and the significance of this to tolerance has not been fully elucidated.

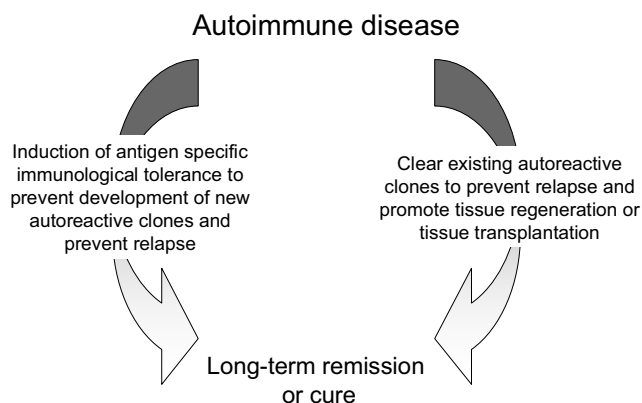
## HAEMATOPOIETIC STEM CELL BASED THERAPIES FOR AUTOIMMUNE DISEASE

This review will focus principally on the use of haematopoietic stem cells and their manipulation as a way of treating autoimmunity. However, other studies that utilise differentiated cell populations or other stem cell populations have been reported and are producing interesting results. Many studies in experimental autoimmune models have used *in vitro* manipulation of differentiated cells such as dendritic, T and B cells or fibroblasts to deliver immunosuppressive or tolerogenic signals (Siatskas *et al.* 2006). While these approaches may produce an outcome, the long-term capacity and feasibility of these treatments in a clinical setting may be compromised by the limited cellular lifespan and turnover of these differentiated cells and thus the potential for repeat treatments. The role of other stem cell populations such as embryonic and mesenchymal stem cells in biology and medicine is well recognised (Donovan *et al.* 2001; Guasch *et al.* 2005) but their use in immunology is not without major challenges and considerations such as rejection of differentiated cells by the host's immune system (Rosenthal 2003; Fairchild *et al.* 2004; Boyd *et al.* 2005; Fairchild *et al.* 2005). *In vitro*, embryonic stem cells can be directed to differentiate into HSC progenitors that are capable of engrafting in host animals and generating lymphoid cell lineages of donor origin (Burt *et al.* 2004). This finding raises the possibility of ES cells as a renewable and plentiful source of HSCs for transplantation purposes. Taking a more direct approach, Fandrich and colleagues

generated a number of rat embryonic stem cell-like lines which display embryonic stem cell characteristics such as morphology and expression of alkaline phosphatase and SSEA-1. These were injected directly into recipients and reported to induce haematopoietic chimerism and allograft acceptance (Fandrich *et al.* 2002). This is indeed an interesting finding but has yet to be reproduced.

Mesenchymal stem cells are a rare population of cells that are predominantly found in the bone marrow and distinct from the haematopoietic stem cell and have the capacity to differentiate into a wide variety of tissues (Pittenger *et al.* 1999; Jiang *et al.* 2002). The major potential uses of mesenchymal stem cells for autoimmunity may lie in the fact that they have immunomodulatory properties and thus can potentially regulate autoimmune responses (El-Badri *et al.* 2004; van Laar *et al.* 2006; Stagg 2007). They have been reported to suppress *in vitro* responses of various immune cells including T and B cells and dendritic cells (Krampera *et al.* 2003; Jiang *et al.* 2005; Corcione *et al.* 2006). For a comprehensive overview of the immune regulatory properties of mesenchymal stem cells see the recent review by Stagg (Stagg 2007). The use of mesenchymal stem cells in autoimmunity is limited to their potential in treating human disease (El-Badri *et al.* 2004; van Laar *et al.* 2006) and experimental models. In two studies of EAE, the introduction of mesenchymal stem cells was able to ameliorate disease severity although disease development was not inhibited (Zappia *et al.* 2005; Zhang *et al.* 2005). Clearly further studies need to be performed to examine the full extent of the benefit of these cells in the treatment of autoimmunity.

The paramount example of the adult stem cell is the haematopoietic stem cell (HSC), which is the source of all cells of the lymphoid and myeloid lineages including lymphocytes, dendritic cells, macrophages and granulocytes as well as red blood cells and platelets. By far the most study on the use of stem cells to treat autoimmune disease is HSCT. This is illustrated in numerous animal studies such as EAE, SLE, collagen-induced arthritis and myasthenia gravis in which disease severity, or relapse rates are reduced following HSCT (Burt *et al.* 1998; Karussis *et al.* 1995; van Bekkum 2000; van Bekkum 2003; Pestronk *et al.* 1983). While HSCT is widely used to treat patients with severe malignancies (Shizuru *et al.* 2005) its potential in the treatment of human autoimmune disease stemmed from the observation of benefit seen in patients who happened to also have a concurrent autoimmune disease (Cooley *et al.* 1997; Snowden *et al.* 1997; Jindra *et al.* 1999). Autologous HSCT is currently being pursued in many worldwide studies targeting a range of autoimmune diseases (Popat *et al.* 2004; Hough *et al.* 2005; Tyndall *et al.* 2005; van Laar *et al.* 2006). The rationale of using HSCT to treat autoimmunity is based on the premise that the existing autoimmune response is ablated by the preconditioning regime and that the transfer of HSCs will engraft and regenerate the immune system without reactivation of the autoimmune response (Blanco *et al.* 2005). However, the outcomes are not straight forward, with mixed responses observed. While benefit can be measured in some individuals, it is also clear that the danger of relapse is real and does occur, often after many years, in a significant number of patients (Sykes *et al.* 2005; van Laar *et al.* 2006). As a process designed to simply replace the ablated immune system, a number of assumptions have to be made to believe that relapse will not occur. Firstly, the reconstituted immune system will not generate self-reactive clones capable of initiating an autoimmune response. Since antigen receptors are randomly generated and subjected to tolerance mechanisms as discussed above, it is hard to imagine that eventually self-reactive clones, similar to those involved in the initial development of autoimmunity will not be generated and thus capable of mounting an immune response. Secondly, the trigger that initiated the initial disease is no longer present. This is difficult to address since it is not clear what triggers disease in humans. Experimentally, autoimmune diseases



**Fig. 1 Critical issues to be addressed in devising curative strategies for autoimmune diseases.** Induction of antigen specific immunological tolerance to prevent the development and activation of new autoreactive clones; coupled with conditioning regimes and treatments directed at eliminating and clearing existing pathogenic clones.

can be initiated by a variety of procedures (von Herrath *et al.* 2006) but their extrapolation to human disease must be kept in perspective. And thirdly, the preconditioning regime used to prepare the recipient for HSCT has eliminated existing autoreactive clones or clones that may have been transferred with the graft. Each of these could individually result in disease relapse and it is not clear in patients that do not respond to treatment or relapse following a period of remission which of these may be responsible. Some understanding of these issues may be obtained from animal studies and this will be expanded on below. Clearly there is room for improvement and we believe that any successful curative strategy must address the two main issues of inducing immunological tolerance and clearing of pre-existing clones (Fig. 1).

## GENE THERAPY INDUCED IMMUNOLOGICAL TOLERANCE

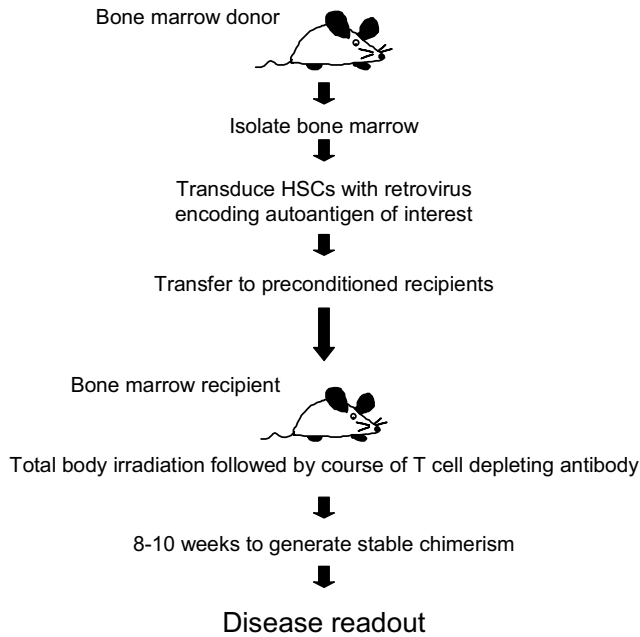
The central role of the thymus in T cell development and tolerance is outlined above, but the link between bone marrow and thymus may not yet have been fully appreciated. It has been known since the studies of Medawar and colleagues that immune tolerance is achieved when animals share a blood supply during development and this is achieved through the bone marrow compartment. Non-identical twin calves that share their circulation display mixed chimerism and readily accept skin graft from each other (Billingham *et al.* 1952) and the transfer of allogeneic bone marrow in neonatal mice will result in acceptance of donor-matched derived grafts (Billingham *et al.* 1953). In short, bone marrow derived cells such as dendritic cells are integral components of the thymic stroma and play a critical role in the process of thymic tolerance (Brocker *et al.* 1997; Palmer 2003; Gallegos *et al.* 2006). Therefore the question arises whether genetic manipulation of the system can be used to promote antigen specific tolerance. As mentioned above, in transgenic whole animal models the answer is yes.

A major question that arises from these observations is whether this can be translated to the clinic. The use of gene therapy to treat haematopoietic diseases is not new and is currently used in the clinic in a range of haematological disorders (Verma *et al.* 2005; Herzog *et al.* 2006). Therefore much of the technical requirements for this strategy are already in place. However, the field has had serious issues to deal with such those associated with the development of T cell leukemia in 2 boys with X-linked severe combined immunodeficiency (Hacein-Bey-Abina *et al.* 2003) treated using gene therapy to introduce the  $\gamma$  chain and repair development of T and NK cell compartments (Hacein-Bey-Abina *et al.* 2002). While ongoing caution and care is prudent, it may appear this unfortunate event was due to the oncogenic properties of the introduced transgene and its

positioning upstream of the LMO2 gene which was activated to induce the leukemia (Berns 2004). Overall, it seems there have been little adverse effects associated with gene therapy (Berns 2004) and we should thus continue to examine and explore ways in which this powerful technology can be utilised.

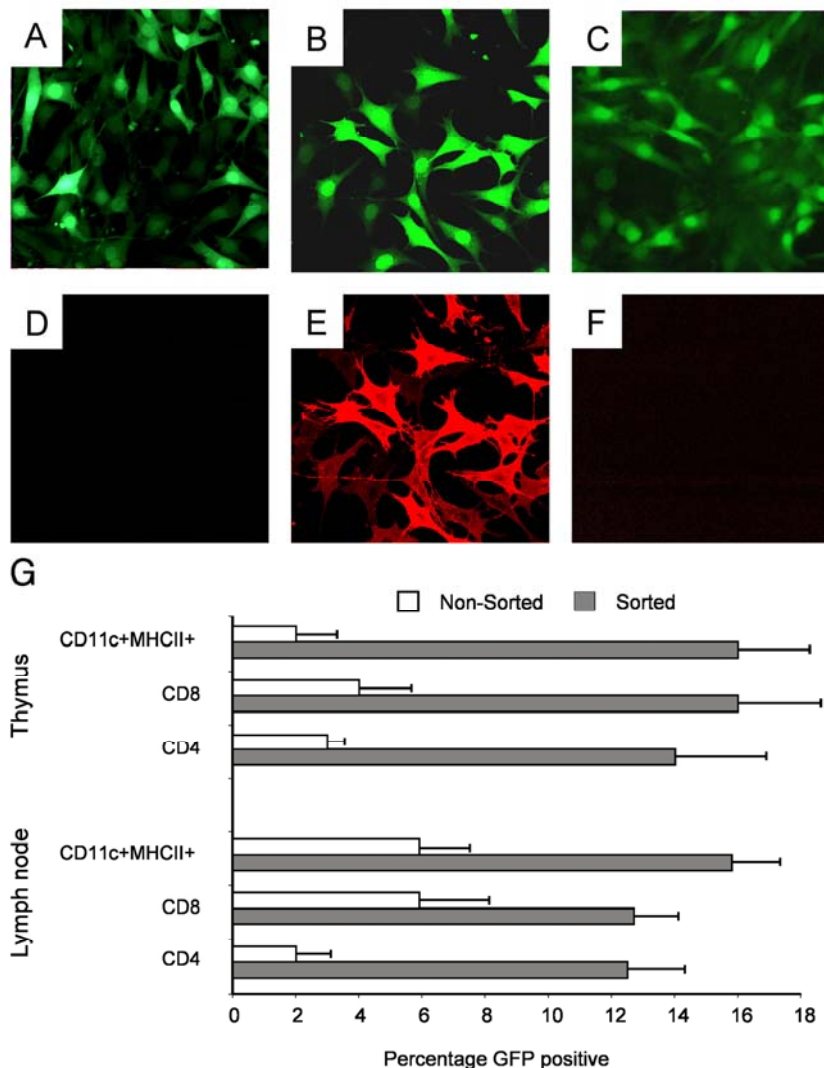
The targeting of HSCs through gene therapy to induce antigen-specific immunological tolerance is still new and has not been attempted in the clinic. Even experimental studies are limited but the studies to date are promising and indicate that the autoimmune repertoire can be influenced in this way to enhance immunological tolerance. Studies which have not focused on defined autoantigens but use neoantigens or alloantigens have been useful in establishing the validity of the concept. The first example used a neo-antigen model of T1D that involved the transgenic expression of LCMV glycoprotein (gp) in the pancreas and gp-specific T cells (gp TCR). Under normal situations double transgenic mice remained disease-free but following immunisation with LCMV, double transgenic mice developed diabetes (Ally *et al.* 1995). However, if bone marrow from these mice was transduced with retrovirus encoding the LCMV gp gene and used to generate bone marrow chimeras, deletion of gp-specific T cells occurred within the thymus, rendering mice deficient in peripheral gp-specific T cells and resistant to T1D induction (Ally *et al.* 1995). Similar strategies have been used to induce tolerance to MHC alloantigens. Following transfer of bone marrow cells transduced with retrovirus encoding the allogeneic MHC class I gene, H-2K<sup>b</sup> gene, expression of the alloantigen was observed in multiple haematopoietic cell lineages, and this was coupled with reduction of the T cell response to the K<sup>b</sup> alloantigen and acceptance of K<sup>b</sup> skin grafts (Sykes *et al.* 1993; Fraser *et al.* 1995). This has also been demonstrated for class II alloantigens (Wong *et al.* 2003) with prolonged survival of kidney allografts expressing identical class II transgene and disparate to recipient MHC (Sonntag *et al.* 2001). The use of MHC alloantigens has been used to treat experimental autoimmune diseases. It is known that in the NOD mouse that disease susceptibility is associated with the H2<sup>S7</sup> MHC haplotype (K<sup>d</sup>, A<sup>g7</sup>, E<sup>null</sup>, D<sup>b</sup>) and that disease can be inhibited in a dominant manner by the heterozygous expression of other MHC haplotypes (Prochazka *et al.* 1989). Furthermore, resistance can be transferred via the bone marrow compartment (Serreze *et al.* 2004) where bone marrow derived dendritic cells are likely responsible for promoting clonal deletion (Chen *et al.* 2007). The recent studies by Iacomini and colleagues have introduced a true gene therapy approach to this concept. In studies using retroviral transduction and transfer of HSCs, tolerance to MHC alloantigens can be induced (Bagley *et al.* 2002) which can promote alloantigen specific central deletion (Kang *et al.* 2002) or even the generation of CD4+CD25+ T regulatory cells (Forman *et al.* 2006). A pertinent finding that has come from these studies that is highly relevant to any potential clinical translation is the finding that persistence of antigen is required to maintain tolerance (Bonilla *et al.* 2006; Tian *et al.* 2006). For autoimmunity, we suggest that this is critical to any strategy aimed at promoting long-term tolerance and disease remission and argues against strategies that target differentiated cells and thus limit the presence of the antigen to the life of the cell. By targeting stem cells in the first instance, the potential for promoting life-long expression of antigen in bone marrow derived cells involved in tolerance induction is built into the strategy (Alderuccio *et al.* 2003, 2004).

Our own study in this area uses a number of experimental disease models including autoimmune gastritis, type 1 diabetes and EAE. The overall strategy is similar across models and is outlined in Fig. 2. Following the isolation of bone marrow from 5-fluorouracil treated mice, the HSCs are cultured with IL-6 and SCF and transduced with MMLV based retrovirus (Kitamura *et al.* 2003) encoding autoantigen of interest and eGFP to allow easy enrichment of transduced cells prior to transfer and also tracking and enumerating



**Fig. 2 Experimental gene therapy strategy used to induce immunological tolerance to defined autoantigens.** Harvested haematopoietic stem cells are transduced with retrovirus encoding autoantigen of interest and EGFP for enrichment and *in vivo* tracking. Transduced HSCs are transferred to irradiated recipients and allowed to generate stable molecular chimeras. The development of autoimmune disease or immunological features are examined in spontaneous models or following disease induction.

tion of cell populations *in vivo*. Using this we have demonstrated the specific production and predominant secretion of proinsulin in cells transduced with retrovirus encoding proinsulin2 (Chan *et al.* 2006) and the expression of MOG following transduction with retrovirus encoding MOG (Fig. 3). Recipient mice are preconditioned with total body irradiation prior to transfer and given a 3-week course of anti-CD4 T cell depleting antibody following transfer. We have found the ability to purify EGFP<sup>+</sup> cells prior to transfer enhances the chimerism achieved from transduced cells (Fig. 3G) and have obtained high levels of molecular chimerism with as few as 3000 cells. As an extension of previous studies that demonstrated that bone marrow from transgenic mice expressing proinsulin2 prevented the development of T1D in NOD mice (French *et al.* 1997; Steptoe *et al.* 2003), we generated chimeric mice in which HSCs were transduced with retrovirus encoding proinsulin 2. Chimerism was generated in a range of lymphoid and myeloid cell populations such as CD4 and CD8 T cells, B cells and CD11c+MHCII+ dendritic cells (Chan *et al.* 2006) (Fig. 3G). Of importance to our hypothesis that bone marrow derived dendritic cells are involved, immunohistochemical analysis of thymus confirmed the presence of GFP expressing dendritic cells located predominantly in the cortico-medullary junction (Chan *et al.* 2006). This initial study focused on the effect of this procedure on the level of insulinitis associated with the early stages of T1D in the NOD model. We found that both the incidence and degree of insulinitis was reduced in mice that received HSCs transduced with proinsulin2 compared to mice that received HSCs transduced with control autoantigen (gastric H/K ATPase  $\beta$ -subunit) or normal bone marrow. This initial finding supports the concept that immunological tolerance to key auto-



**Fig. 3 Retroviral induced autoantigen expression and *in vivo* molecular chimerism.** An MMLV based retrovirus encoding autoantigen and EGFP can be used to transduce *in vitro* culture cells lines (A-F) or promote *in vivo* chimerism following transfer of transduced haematopoietic stem cells (G). Mouse 3T3 cells transduced with retrovirus encoding myelin oligodendrocyte (MOG) (A, B, D, E) or proinsulin2 (C, F). Transduced cells are identified by coexpression of EGFP (A-C). Specific expression of MOG is detected by indirect immunofluorescence staining with MOG specific mAb (E), with negative reactivity seen with control secondary antibody (D) or staining of irrelevant antigen (F). Images were captured with  $\times 20$  objective. Chimerism is achieved in cells of myeloid (CD11c+MHCII+ dendritic cells) and lymphoid (CD4 and CD8 T lymphocytes) origin in thymus and lymph node following transfer with transduced HSCs (G). Recipient mice preconditioned with total body irradiation are transferred with HSCs and analysed 8-10 weeks later. Elevated chimerism levels are obtained in mice that received transduced (sorted for EGFP expression) HSCs (n=4) prior to transfer compared to mice which received non-sorted cells (n=3). Chimerism levels were analysed by Mann-Whitney unpaired test and is data presented as mean  $\pm$  standard error of the mean.

immune disease associated autoantigens can influence the development of disease. We are currently extending these studies to assess the development of diabetes in transferred mice.

An earlier study by Peters and colleagues used a retroviral system to direct expression of myelin basic protein (MBP) under control of the proximal *lck* promoter and found this treatment did not reduce the incidence of EAE following MPB challenge (Peters *et al.* 2000). Unfortunately it is difficult to interpret these data since little information was presented on chimerism levels or even evidence of antigen expression *in vivo*. By targeting thymocytes with the proximal *lck* promoter, it may also be indicating that simple expression of antigen in the thymus may not be sufficient to induce tolerance and that antigen must be presented by the appropriate cell population such as dendritic cells (Brocker *et al.* 1997; Palmer 2003). More recently however, Scott and colleagues have shown in the SJL model of EAE that HSCs transduced with retrovirus encoding phospholipid protein (PLP) and transferred to irradiated hosts are resistant to EAE induction following challenge with PLP<sub>139-151</sub> peptide (Xu *et al.* 2006). In a more pre-clinical setting which treated mice 12 days after PLP<sub>139-151</sub> immunisation, a beneficial but not absolute effect was also observed. We have observed a similar effect in the C57BL/6 model of primary progressive EAE that is induced by immunisation with MOG<sub>35-55</sub> peptide (Mendel *et al.* 1995). Chimeric mice transferred with HSCs transduced with retrovirus encoding MOG are completely resistant to MOG<sub>35-55</sub> induced EAE (J. Chan, manuscript in preparation). Taken together, these findings support the underlying basis that immunological tolerance to a targeted autoantigen can be achieved through genetically manipulated HSCT and can prevent the development of disease. While existing data supports central deletion as the main mechanism, it is not clear whether this strategy can also generate regulatory cells. With this in mind, it still remains to be determined whether the induction of tolerance to one antigen will also lead to promote tolerance to other associated autoantigens. The major challenge of course is in treating and remitting established disease. Experimentally can we treat animals with established autoimmunity to reverse disease and promote long-term remission without the need for long-term immunosuppression?

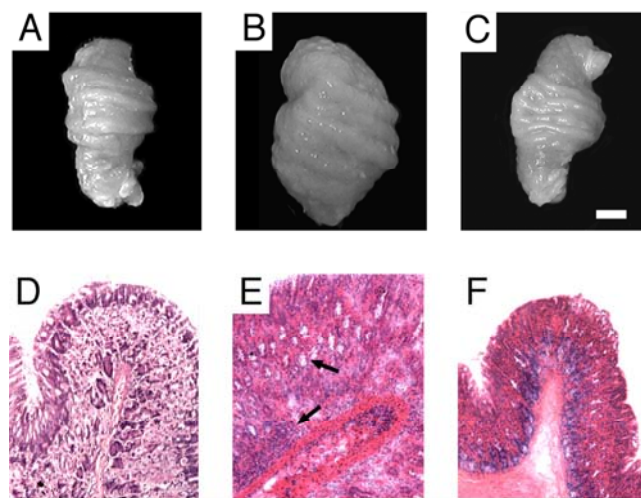
The issue of chimerism levels required to achieve tolerance is an important consideration. The general consensus across a number of these studies is that only relatively low levels of chimerism are required to achieve tolerance. Levels of 10-15% are suggested for transplantation tolerance (Wekerle *et al.* 2001) and recently microchimerism levels as low as 1% were reported to be sufficient to induce tolerance of CD8 T cells in an allogeneic setting (Bonilla *et al.* 2006). In another study that involved skin allograft survival across an MHC class II barrier, Tian and colleagues reported that chimerism levels of approximately 1%, generated by retroviral transduction of HSCs were sufficient to promote graft survival (Tian *et al.* 2006). In our own studies, chimerism levels varied widely between cell populations and ranged between 2-50% (Chan *et al.* 2006), while Steptoe and colleagues generating levels of 5% chimerism following HSCT could prevent the development of T1D in the NOD mouse (Steptoe *et al.* 2003). The level of chimerism required to induce self-tolerance will ultimately have a major impact on strategies used to establish chimerism since the degree of toxicity associated with conditioning regimens must be weighted up against the potential benefit. We are very interested in this and aspects of our current studies using experimental models are geared at assessing the relationship between low toxic regimens, level of chimerism and tolerance.

#### A FURTHER CONSIDERATION: DEALING WITH THE EXISTING AUTOREACTIVITY

An absolute requirement associated with any curative stra-

tegy for autoimmune disease is that it must also deal with the existing disease. Not only will immunological tolerance to culprit autoantigen(s) be required, but existing autoreactive clones must also be eliminated (Fig. 1). Failure to achieve this may not lead to a successful treatment. We have observed evidence of this in our study of experimental autoimmune gastritis (EAG) but there has been very little research published in this area. In this study we utilised two transgenic mouse lines; PC-GMCSF tg mice which spontaneously develop EAG (PC-GMCSF tg) due to expression of the proinflammatory cytokine GM-CSF in stomach parietal cells (Biondo *et al.* 2001), and IE-H/K $\beta$  tg mice which express the target autoantigen (H/K ATPase  $\beta$ -subunit) under the control of the MHC class II promoter and are resistant to disease development (Alderuccio *et al.* 1993). When crossed, double transgenic mice do not develop autoimmunity, confirming the dominant nature of tolerance associated with IE-H/K $\beta$  tg mice (Murphy *et al.* 2003). However in experiments in which bone marrow from IE-H/K $\beta$  tg mice is transferred to irradiated diseased PC-GMCSF mice, we observed a lack of T cell reactivity in peripheral lymphocytes but within the gastric tissue, inflammatory infiltrate and pathology was still evident (Murphy *et al.* 2003). From this we concluded that the ability to induce T cell tolerance in an established disease setting may not be sufficient to promote disease remission.

We have started to address this issue of existing pathology in autoimmune disease. Many autoimmune diseases are treated with broad immunosuppressants such as the corticosteroid prednisolone. In patients with pernicious anaemia (the clinical endpoint of autoimmune gastritis), it was reported in the 1960s that treatment with prednisolone resulted in disease remission and recovery of gastric function (Jeffries *et al.* 1966; Wall *et al.* 1968). However this was not a curative treatment with disease symptoms relapsing following prednisolone withdrawal. We have recently reproduced this study in the EAG mouse model by treating EAG mice with prednisolone (Biondo *et al.* 2006). We observed that not only did prednisolone treatment clear the inflammatory infiltrate within the gastric mucosa, but that the gastric mucosa recovered to a normal morphology (Fig. 4). Upon withdrawal of prednisolone we also observed disease relapse in mice with an intact thymus; however in mice in which EAG was induced by neonatal thymectomy (Alderuccio *et al.* 2002) and thus lacked a thymus, we noted that over 40% did not relapse (Biondo *et al.* 2006). This finding



**Fig. 4 Autoimmune disease remission induced by prednisolone.** Gross morphology (A-C) and hematoxylin and eosin staining (D-E) of stomachs from normal mice (A, D), mice with established autoimmune gastritis (B, E) and mice with EAG and treated with prednisolone (C, F). Areas of cellular destruction and inflammatory infiltrate in panel E are indicated by arrows. Gross morphology images (panels A-C) shown at same magnification. (bar = 2 mm) Histology images (panels D-E) were captured with  $\times 10$  objective.

implies that in a situation where existing pathogenic clones are cleared and development of new autoreactive T cells is prevented, long-term remission may be possible (Biondo *et al.* 2006). We now wish to extend this observation to other experimental autoimmune models such as EAE. Ultimately we wish to determine whether diseased mice treated with prednisolone together with transfer of genetically manipulated HSCs can result in a state of long-term remission or cure without the need to ongoing immunosuppression.

## CONCLUSION

While progress is being achieved to suggest that gene therapy involving HSCT can be utilised to address the treatment of the autoimmune diseases, there are still many questions to be answered. Data from neoantigen and alloantigen studies suggest that clonal deletion is the main form of tolerance induction but whether this is the case for autoantigens introduced through HSC transduction and transfer or whether the generation of regulatory cells is the mechanism is not known. One can envisage that both mechanisms may be employed with the defining factor varying from one TCR to another.

The ability to specifically dampen an autoimmune response while maintaining a functioning immune system poses a major challenge for basic and clinical research. Our current position on treatments involving generalised or semi-targeted suppression of immune related features still leave the patients with side effects and increased susceptibility to infections. Clearly, there is a pressing need for innovative strategies to improve the current status. The use of autologous HSCT is being trailed in a number of autoimmune diseases as a means of “resetting” the immune system to a pre-autoimmune state. The potential for relapse with this strategy is realised and indicates that alone, this is not a curative process. Gene therapy is a powerful tool to manipulate the genetic signature of targeted cells. It is commonly thought of as a strategy used to introduce a missing function to cells associated with various diseases such as those that affect the haematopoietic system. However it could also be used to introduce or increase the expression of molecules in other settings that may be beneficial. We suggest that gene therapy associated with HSCT is a viable strategy to promote antigen specific tolerance in the autoimmune diseases. While many issues still need to be addressed before clinical implementation, there is mounting and strong experimental data to support continued pursuance of this strategy.

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