

REVIEW

Celiac Disease: a model autoimmune disease with gene therapy applications

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Gene therapy (GT) is still at the 'experimental' stage and some recent setbacks have cooled the potential use of this therapeutic tool even in life-threatening conditions. However, this therapeutic approach has a potential, which is not limited to disease for which we have not other option. There are increasing evidence that GT will be soon used in diseases that are not life threatening. One group of diseases that can benefit from GT is the autoimmune one. Several experimental animal models have indicated the efficacy (proof of principle) of GT. In the present review, we have addressed the possibility that even extremely benign autoimmune-like

diseases such as Celiac Disease (CD) might one day profit from this type of therapy. We further point that in conditions such as CD, where the trigger is well known and the pathogenic cascade is relatively well defined, a situation not common in autoimmunity, we can even have a better situation where to explore and use GT to control disease initiation and progression. Once the risks that are still intrinsic to GT will have been reduced the therapeutic options we outline in the present review might not appear too far from reality.

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Introduction

Gene therapy (GT) has been heralded as the new strategy to treat many diseases in which no satisfactory treatment or no treatment at all exists.^{1,2} There are several examples in which GT treatment has been advocated and already implemented with different degrees of success. Typical examples we can consider are cystic fibrosis (CF)³ and X-linked immune deficiency⁴ where a single gene defect inevitably leads to death. The road to the treatment of these conditions has been scattered by many hopes, false starts, failures and complications that have hampered the path of GT. There have been, however, breakthroughs which have changed (saved) the life of patients and thus reinforced the necessity to further implement and explore the use of GT in diseases where no other therapeutic options are available. These results have also propelled the possible implementation of GT in other conditions where treatments are at best of limited value, as in cancer.⁵ Today, GT has a more ambitious implementation with the final aim not to repair a single gene, but rather to modulate a complex disease.

We have therefore moved from monogenetic (life-threatening) scenario to polygenic (not life threatening though often long-term complications might lead to death) diseases to be tackled by a GT approach, in the context of the present reviews in autoimmune diseases. The use of GT to treat autoimmune diseases has been successfully implemented in animal models paving the

road for its potential application in patients in the near future. GT, therefore, is following a path that many pioneering treatments have followed in the past. A parallel can be drawn with bone marrow transplantation (BMT), which was originally devised for life-threatening diseases⁶ and is now used for autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and others.^{7–9} With time and experience the complications of BMT, which hampered the first years of this treatment, have been overall well contained and therefore allowed the use of BMT for nonlife-threatening diseases.

Different GT strategies to treat autoimmune diseases have been considered; the first is based on the 'quantum' hypothesis or redirected magic bullet theory. In this scenario, a lymphocyte (normally a T lymphocyte) with the antigenic specificity for a self antigen, regardless of the potential significance of this autoantigen as trigger of the autoimmune disease, is located at the site of autoimmune inflammatory reaction. Examples of antigens that have been used in animal models are Collagen type II in collagen-induced arthritis, an animal model of rheumatoid arthritis,^{10,11} and myelin basic protein in EAE¹² an animal model of multiple sclerosis. In this scenario, the self-antigen specificity is used as an ideal homing device to localize cells that have been transduced to secrete powerful anti-inflammatory substances, often cytokines (such as IL-10) or cytokines receptors (such as soluble TNF receptor). Another option is to alter the function of antigen-presenting cells such as dendritic cells in order to influence the downstream activation of pathogenic T cells.^{13,14} Alternatively, it has been considered that GT could control the activation of proinflammatory signal pathway cascades, and as a

prototype the NF- κ B, a signal transduction pathway universally involved in inflammation and immune responses, has been targeted.^{15,16}

Although all these approaches to control autoimmune diseases, there is no clear picture of the involved pathogenic cascade and most of the strategies have been devised and tested in animal models, which might not always reflect the *in vivo* situation in man. There are, however, immune-mediated conditions in which the pathogenic cascade has started to become clear, thus pointing to specific and well defined key links to be targeted for therapeutic purposes. Unfortunately, in some of these diseases the therapeutic ratio (efficacy of the treatment/treatment risk) of any new treatment is quite high more so for GT, but as for BMT, we do have to consider that future applications of GT may, one day, benefit such conditions.

Celiac Disease as the prototype of immune-mediated disease

We have discussed above that GT might and will likely move towards diseases in which a suitable, though not in actual fact satisfactory treatment exists, but where we have a clearer picture of the pathogenic cascade. This latter knowledge, obviously, changes the terms of the strategy we would want to follow if a GT approach has to be implemented. Indeed, a focused therapeutic approach, and particularly so for GT, would greatly benefit from a well-defined pathogenic cascade. This is still a problem in the case of autoimmune diseases, where the precise pathogenetic steps are still blurred and instead of 'magic bullet' GT approach, a more 'sledge hammer' strategy has been implemented, although GT clinical trials have already been started.

The pathogenic cascade

Celiac Disease (CD) is defined as a gluten/gliadin intolerance (though other proteins can also trigger the disease), which is characterized by pathognomonic small intestine lesions¹⁷ and to date the small intestine modifications are still considered the golden standard for diagnosis.¹⁷ CD is a common disease with an estimated prevalence of 1 in 150–300 individuals,¹⁸ it is controlled by a gluten-free diet.¹⁸ Although only a minority of CD patients present clear and pathognomonic clinical signs, in all CD patients, if untreated, this pathology poses a series of systemic complications some of which are life threatening.¹⁹ Though gluten withdrawal, as indicated above, is an efficient strategy to control the disease it does not represent a therapy, as reintroduction of gluten in the diet will trigger unequivocally again the disease and diet compliance is not followed by the patients. Sometimes the poor diet compliance is unintended as gluten is often present in 'incognito' in otherwise considered gluten-free foods. Therefore, no appropriate treatment therapy exists for this condition. This disease has also very high community costs caused particularly by the systemic complications.

CD is strongly associated with certain HLA class II alleles with 90% of the patients being HLA DQ2 and the

remaining being HLA DQ8.²⁰ Other genetic factors are, however, involved in the induction and maintenance of the disease as indicated by large genetic studies and the well-known observation that up to 35% of the 'normal' population expresses HLA DQ2. This strong HLA class II association, the strongest in an immune-mediated disease, the presence of an increased small intestine lamina propria infiltration by CD4+ T cells and the easy access, via small intestine biopsies, from patients with CD propelled many investigators to search for dominant epitopes of the gluten/gliadin sequences. Importantly, it has been possible to demonstrate that rapid and disease-specific changes were observed in small intestine organ culture of CD patients with signs of local T-cell activation in organ cultures of celiac patients.^{21,22}

Although the four key factors (HLA alleles, triggering antigen, presence of infiltrating reactive T cells and a ready availability of tissue) to perform a proper study of the T-cell adaptive immune response were at hand and well known already several years ago an essential 'ingredient' in the cooking pot of CD was missing (Figure 1). For many years it had been reported the presence of an autoantibody response in CD which was defined as antireticulin and then antiendomysium (EMA)^{23–25} to characterize an 'ill'-defined reactivity to extracellular, matrix component of the small intestine. This reactivity was not only observed in small intestine, but also in other sites and importantly in tissues from other species indicating that the 'antigen or antigens' was widely distributed and well preserved among different species.²⁶ Only a few years ago it was defined that the autoantigen recognized by these EMA autoantibodies was tissue transglutaminase tTG.²⁷ Importantly, these autoantibodies are highly specific for celiac patients with both sensitivity and specificities approaching 95%.^{28,29} With an unexpected twist tTG, the autoantigen recognized by the EMA autoantibodies, turned out to have another essential role in CD. A series of studies performed on the sequence of gliadin, one of the main constituents of gluten and a well-defined and dominant trigger of CD, suggested that peptides derived from this protein did not have the ideal fitting motif for binding to HLA DQ2. These were puzzling results as gliadin had the ability to trigger *in situ* T-cell responses, but the detection of dominant T-cell epitopes, using synthetic peptides, proved less straightforward than expected. These results implied that in some way gliadin/gluten was modified *in situ* to increase its 'tastiness' for T-cell recognition. The seminal work by Sollid³⁰ and Koning groups³¹ provided the missing link in this pathogenic cascade when they demonstrated that tTG was the enzyme able to change, via a deamidation process, some key glutamines (Q) to glutamic acid (E). This post-translational antigen modification produced a series of gliadin epitopes with an increased affinity for HLA DQ2 and DQ8 that induced a powerful T-cell response in CD4+ lamina propria T cells.^{30,31} Therefore, tTG has a dual role in CD: the autoantigen recognized by the disease-specific autoantibodies as well as the enzyme that unveils the dominant gliadin T-cell epitopes. The importance of unmasking 'cryptic' epitopes, leading to a break of tolerance, is a well-recognized factor in autoimmunity.³² It is important to stress that post-translational modification has gained a central role in other autoimmune conditions both by altering

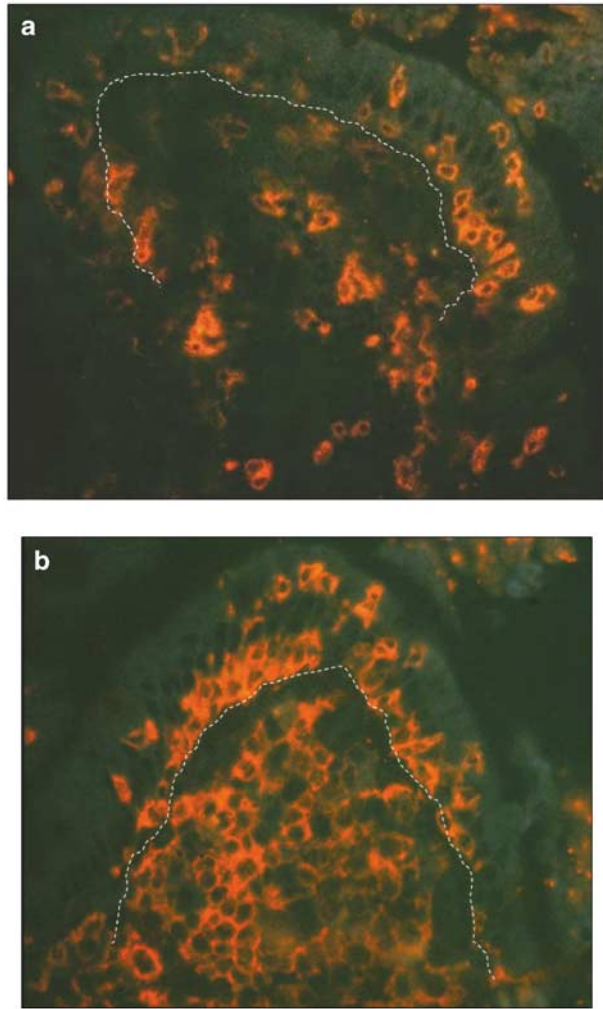


Figure 2 Redistribution of CD3⁺ cells within the duodenal mucosa of CD patients: migration of CD3⁺ cells to the subepithelial compartment (SEC) of the lamina propria after *in vitro* challenge with gliadin or cytokines. (a) Pattern of distribution of CD3⁺ cells in celiac duodenum after *in vitro* challenge with medium alone: note the low density of CD3⁺ cells (red labelled) in the subepithelial region with low intraepithelial infiltration. A similar pattern was observed with the other tested cytokines (IL-2, IL-4 and IL-7) but not IL-15. (b) Pattern of distribution of CD3⁺ cells in celiac duodenum after 24 h of *in vitro* challenge with IL-15: note the high density of CD3⁺ cells (red labelled) within the subepithelial compartment with high intraepithelial infiltration. The broken line defines the border between the epithelial and subepithelial compartments. Indirect immunofluorescence, magnification $\times 180$; C and D: the white line indicates the basal membrane of the epithelium.

biopsies.⁴⁶ IL-15 has been shown to fit perfectly the role of the Th1-inducing cytokine in CD. In CD it has been excluded a role of IL-12, the prototypical Th1 cytokine inducer, as the conductor of the small intestine Th1 response.⁴⁷ The role of others Th1-directing cytokines, such as IL-18, has not been proven, therefore, IL-15, which can influence a Th1 response,⁴⁸ could direct the gliadin-specific T-cell responses towards the Th1 path. IL-15 controls the migration and expansion of IEL, which are abnormally increased in CD,⁴⁰ but might also fit another important role in the inflamed small intestine milieu of celiac patients: allow the survival and persistence of pathogenic CD4⁺ gliadin-specific T cells.

There is evidence indicating that gastrointestinal inflammation allows 'excessive' survival of lymphocytes in the mucosa of patients with inflammatory bowel disease.⁴⁹ In other words, in a normal noninflamed mucosa there is a rapid turnover, death by apoptosis of lymphocytes in the mucosa of healthy controls. This has a rationale and also an important implication in chronic inflammatory diseases as persistent and unregulated presence of activated and potentially self-reactive T cells can be extremely dangerous. In CD, we have indication that IL-15 fulfills the role of survival factor as its neutralization, in organ cultures of CD patients, allows the induction of a significant amount of T-lymphocyte apoptosis (Figure 3). IL-15 therefore, appears to have a dual role in the pathogenic cascade in CD: cofactors in the induction of epithelial death as well as in the maintenance of the pro-inflammatory response with induction of lymphocyte survival. This ability of IL-15 to allow lymphocyte survival is not limited to the lamina propria compartment, but also IEL are protected from apoptosis by IL-15 (Figure 3).

The possible role of IL-15 in the pathogenesis of CD drew the attention on the potential function of the innate immune system in the pathogenesis of CD. Indeed, IL-15 is a typical cytokine of the innate immune system, which is produced by myeloid cells, epithelial cells, but not or only in some pathological condition by lymphoid cells.⁴⁸ It has been demonstrated in the context of CD that biologically active IL-15 is rapidly made available after gliadin challenge.⁴⁴ Further studies have unraveled some of the aspects of this rapid 'release' of IL-15 after gliadin challenge, and indicated a dichotomy between the peptides that are immune dominant and recognized by resident T cells and the region of gliadin that induces this release of IL-15 (Maiuri *et al*, submitted). It has to be stressed that rather than release, we should say biological availability of IL-15. Indeed, this cytokine is mainly controlled at the post-transcriptional level⁵⁰ and often its biological activity is achieved by surface⁵⁰ rather than actual release. This has made the detection of IL-15 not an easy task as ELISA assays and mRNA studies are often inconclusive.^{48,50} In CD, this is the case as detection of IL-15 has been difficult and the only satisfactory way to prove presence and biological significance is via the specific inhibition using neutralizing monoclonal antibodies⁴⁴ or the soluble α chain of the IL-15 receptor.⁵¹ The studies on biopsies of CD patients have indicated that neutralization of IL-15 could have a potentially therapeutic value as, at least in the *in vitro* model, it has been possible to control several of the pathological signs specific of CD.⁴⁴

There is another important aspect that has to be stressed in the context of this review, which is that a small group of celiac patients can evolve into a state called refractory sprue,⁵² characterized by monoclonal expansions of IEL, which is a life threatening condition and may evolve into a frank mucosal lymphoma.⁵² In both cases, IL-15 has been described to be a key factor in the evolution and induction of these complications. Importantly, at this stage, the gluten-free diet is not anymore able to control the disease making this end point of CD an untreatable disease, which leads to death of the patients.⁵³ These patients while not responsive to gluten-free diet are celiac patients as they have the genetic (HLA DQ2), serology (anti-tTG

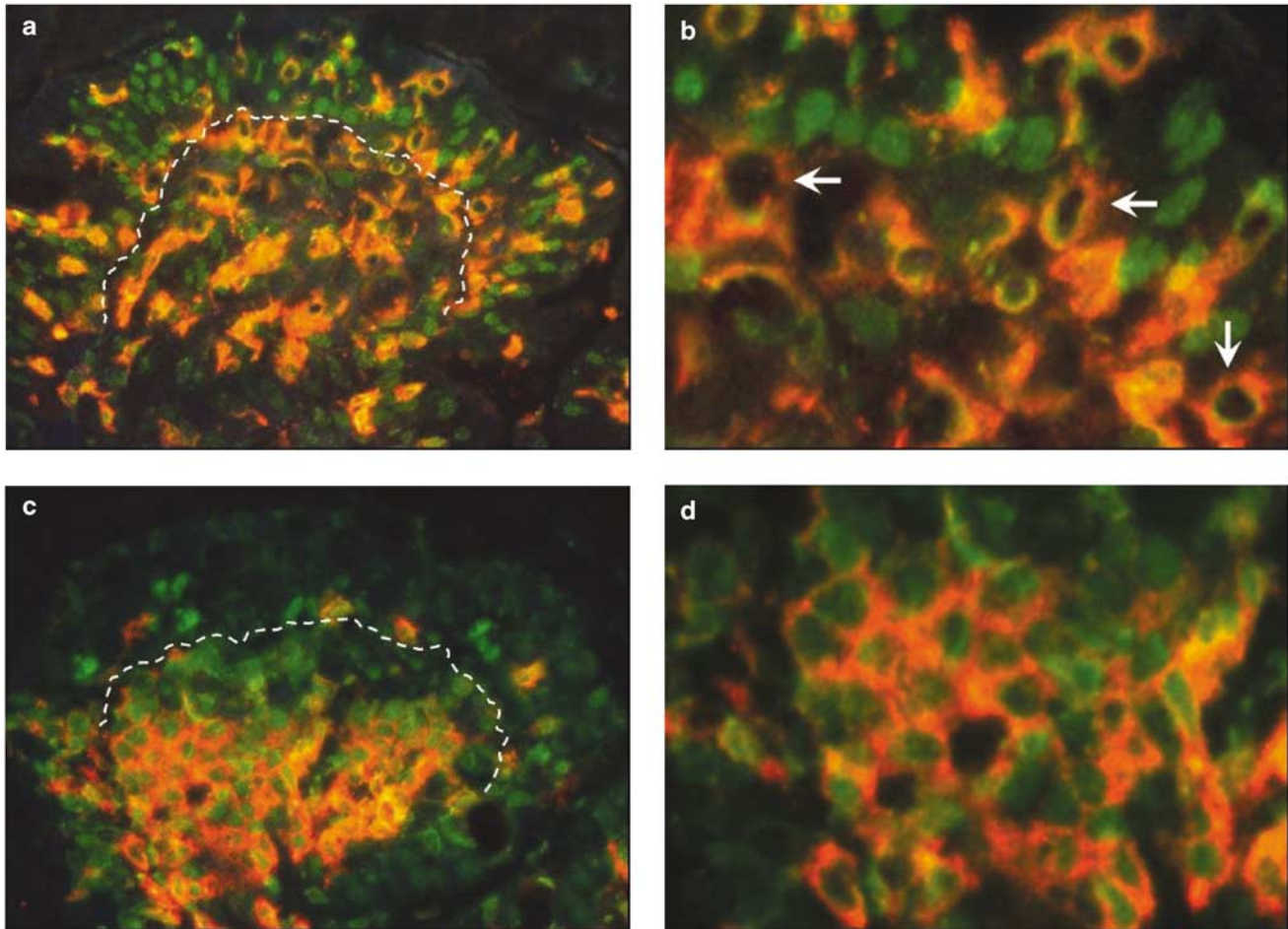


Figure 3 Selective induction of apoptosis in the SEC of the lamina propria in cultured celiac duodenum: effect of IL-15. (a and b) (high magnification of picture a): Apoptosis of enterocytes and mucosal lymphocytes in duodenal biopsies after 24 h of challenge with gliadin: immunofluorescence (two colours), TUNEL+ cells (green) and CD3+ cells (red). Many enterocytes are apoptotic (green labelled); whereas only a few CD3+ intraepithelial cells as well as CD3+ cells located in the subepithelial compartment of the lamina propria are TUNEL+. In panel (b) note, some indicated by arrowheads, the presence of nonapoptotic CD3+ cells (TUNEL-CD3+, green negative and red labelled) and the presence of many non-T apoptotic lamina propria as well as epithelial cells (TUNEL+CD3-, green positive without red colour). (c and d) (high magnification of picture c): apoptosis of enterocytes and mucosal lymphocytes after incubation with gliadin supplemented with neutralizing anti-IL15 M110 mAb. Note the significant reduction of apoptotic enterocytes (only a few cells within the epithelium are green labelled) and the noticeable increase of apoptosis in CD3+ cells in the subepithelial compartment: in this area many cells are double labelled, (TUNEL+CD3+). In panel (d) it is evident that the majority of CD3+ cells (red) are TUNEL+ (green). The broken line defines the border between the epithelial and sub-epithelial compartments. (a-c): Immunofluorescence two-colours, magnification: $\times 220$ (a and c), $\times 400$ (b and d). C and E: the white line indicates the basal membrane of the epithelium.

autoantibody) and a histopathologic pattern compatible with CD.

Targeting a GT therapy in CD

In the previous pages, we have discussed all the hard data indicating which elements are involved in the pathogenesis of CD. This cascade provides some specific target for therapy and in the present context for a GT approach. To summarize, we have a well-defined antigen gluten/gliadin,²⁰ an unequivocal central role for Th1 (IFN- γ releasing) CD4+ T cells recognizing gliadin peptides. There is a central function for tTG in changing the characteristics of gliadin peptides. An innate response is also elicited and IL-15 is a key element in this phase. In other words, we have the five W's that must be answered in any good example of investigative

journalism: **Who:** Gliadin, **Why:** genetic make-up (HLA) and lack of complete degradation of gliadin, **When:** after tTG deamidation of key glutamine (Q) to glutamic acid (E), **What:** preliminary activation of a nonadaptive immune response, **Where:** the small intestine.

So how can we envisage controlling CD with a GT approach? There are a series of options, that have to be considered. As indicated above GT for autoimmune diseases follows a couple of main strategic options and one is the quantum delivery of a packet of cytokine/inhibitor by cells that deliver the inhibitor regulatory cytokine at the site of the immune-mediated lesion, in our case the small intestine. In the past, at least for animal models, two approaches have been used to deliver inhibitory cytokines: the use of cells that do not migrate, such as fibroblasts, but can be injected where we need them to release the anti-inflammatory product cytokine or inhibitor.⁵⁴ The other is based on cells that

can migrate (lymphocytes) and that use their antigen specificity via the TCR or BCR, as a way to home where requested. As discussed above collagen type II and Myelin Basic Protein-specific T cells or genetically engineered T/B⁵⁵ cells have been used for this purpose.^{10,11} In CD, we would have a dual benefit in using antigen (gliadin)-specific T cells as these T cells tend to localize at the site of the lesion (small intestine). Moreover, since they are pathogenic, they also represent the ideal cells to be targeted. They will deliver the inhibitory signal and more importantly release the relevant therapeutic substance only upon antigenic recognition of gliadin. We will therefore combine the ability to deliver at the site of the lesion (**where**) and also have a selective delivery of the chosen inhibitor after stimulation *in situ* by gliadin peptides deamidated by tTG (**when**).

There is a third important benefit in CD as the cells that would be used for GT transfer are gliadin-specific T cells derived from the mucosa of celiac patients since only at the site of lesion true gliadin reactive and potentially pathogenic T cells are present. These mucosal T cells will also have all the gut homing potential by expressing the appropriate mucosal integrins $\alpha 4\beta 7$,^{56,57} thus, they will very likely reach the intestinal mucosa where they could affect the overall response to gliadin and therefore control disease evolution. We have therefore established that gliadin specific T cells represent an ideal tool to deliver and then release the chosen factor only upon antigenic stimulation. The next question that we will have to answer is: which gene or genes should be transduced into these CD4+ T lymphocytes?

There are a series of candidate genes that belong to the 'usual suspect' category with the most obvious being IL-10. CD is extremely polarized towards a Th1 phenotype, therefore CT has similarities with Crohn's disease where a strong Th1 response is also observed. Interestingly, the use of 'transgenic' IL-10 has been considered in the treatment of Crohn's disease and tested with success in an animal model of the disease.^{58,59} In another report, IL-10 gene was introduced into a bacteria which released IL-10 in the lumen.⁶⁰ In the other case, a strategy similar to the one indicated above was used, transduction of the IL-10 gene into T cells CD45RB^{high}, which normally induce a form of colitis considered to be a model of Crohn's disease.⁶¹ Clearly, only the second one of the two models has relevance for the present review. This model indicates the validity of combining cytokine therapy with delivery by GT as in this case, we should reduce the systemic effects of cytokine therapy increasing the effects where needed. It is important to stress that IL-10 cytokine therapy has been used in Crohn's disease with less than satisfactory results, probably because of the necessity of cell-cell interaction, but a GT approach might prove a more satisfactory way to deliver such inhibitory cytokine. The use of this means of delivery retrovirally transduced T cells, would be even more appropriate in CD. Indeed, it has been reported that TCR engagement increases the transcription of integrated viruses,⁶² and it has recently been shown that IL-10 release is upregulated upon lymphocytes stimulation.⁶³ In CD, therefore, we would expect to have increased release of IL-10 as an anti-inflammatory cytokine locally and increased release upon gliadin encounter. In this

case, we would obtain an ideal situation of high release of the cytokine only when the optimal biological switch required: the potential treatment only when needed. Another aspect that has to be considered is that retrovirally transduced leukocytes (among the other T cells) appear to persist for prolonged periods of time.⁶⁴⁻⁶⁶ Thus, gliadin-specific transduced T cells would remain available and active for a long period of time and at the site where they were aimed to. Obviously, we could consider other regulatory cytokines such as TGF β 1 to be transduced into gliadin-specific CD4+ T cells, but the role of this cytokine in CD is complex and its overexpression might not be ideal.⁶⁷

We have discussed above that recent studies in CD have indicated that other key links could be targeted for GT, the inhibition of tTG and the *in situ* detoxification of gliadin by a complete enzymatic digestion. This latter strategy can be envisaged using bacteria releasing the appropriate enzymes such as propyl endopeptidase from *Flavobacterium meningosepticum*, therefore using a strategy used to release the anti-inflammatory cytokine IL-10 in animal models of colitis.⁶⁰ These are options that are outside the aim and the scope of this review.

The use of gliadin-specific T cells to deliver at will the appropriate inhibitors has other potential uses. We have indicated that other pathways, besides the activation of pathogenic T cells, are involved in the pathogenesis of CD.^{21,22} In particular, there is a possible involvement of the IEL population.^{38,39,42} These IELs are increased in number and appear to be involved in the epithelial destructive phase of CD. Importantly, they are highly sensitive to the presence of IL-15, as this cytokine promotes their activation and expansion.³⁹ We have also demonstrated that IL-15 is increased in CD and its inhibition provided a clear therapeutic effect at least in the organ culture of celiac small intestine.⁴⁴ The inhibition of this cytokine in animal models of other inflammatory diseases has been shown to be efficient in controlling these pathologies.⁵¹ It can be envisaged, therefore that neutralization of IL-15 will be potentially beneficial in CD. But how to deliver the inhibitor substances at the desired site (small intestine) reduce the systemic side effects of generalized cytokine neutralization and produce it only when requested? The CD4+ gliadin-specific T cells would again be an ideal vehicle to deliver these inhibitors. IL-15 binds to a complex receptor constituted by three chains, two (β and γ) of which are shared with the IL-2 receptor complex.⁵⁰ The third chain (α -chain) is unique to either IL-2 or IL-15.⁵⁰ Therefore, it could be envisaged to generate genetically engineered CD4+ gliadin-specific T cells that express a soluble form of the IL-15 receptor α chain.

Different forms of the IL-15 receptor have been generated^{68,69} and in this case it would be required to produce a soluble α -chain receptor that retains the high-affinity binding to IL-15, but lacks the ability to integrate with the beta and gamma chains of the IL-2 receptor in order to neutralize IL-15 but avoid to deliver biologically active IL-15 to cells from which we want to remove it. Indeed, it has recently been described that 'sharing' of IL-15 α -chain receptor might allow cells that are not normally responsive to IL-15 to respond to this cytokine.⁷⁰ Thus, controlled and local IL-15 neutra-

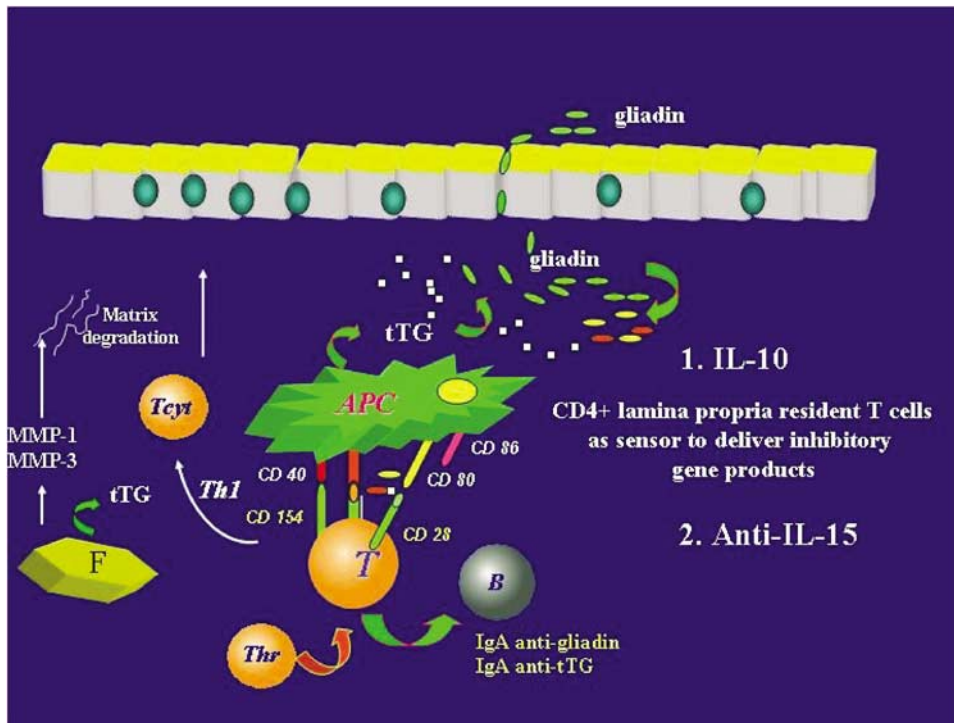


Figure 4 Example of possible therapeutic genes delivered by CD4+ gliadin-specific T cells genetically engineered to deliver the therapeutic agent upon antigen (gliadin) recognition.

lization would represent an ideal way to control IEL. There is another important aspect of IL-15 neutralization that has to be considered as shown in Figures 2 and 3. IL-15 inhibition allows the induction of apoptosis of T cells in the lamina propria and many of these cells are the CD4+ pathogenic gliadin-specific T cells. This means that upon release of soluble IL-15 α -chain a large section of mucosal CD4+ gliadin-specific pathogenic T cells would be prone to apoptosis. Another potential benefit of neutralization of IL-15 is possible in refractory sprue,⁵² characterized by an uncontrolled expansion of IEL. There is increasing evidence that IELs are sensitive to IL-15, and therefore neutralization of this cytokine via a GT approach might further benefit celiac patients (Figure 4).

Final remarks

CD is the prototype of diseases in which a clear role of antigen-specific T cells has been demonstrated and where their inhibition results in disease amelioration. The present therapeutic approach is the removal of the antigenic challenge: the gluten-free diet, which is effective if there is a strict compliance to the diet. It is, however, not always easy to follow such strict restrictions for all life and alternative approaches have to be considered. The use of GT is at the moment a remote hypothesis as CD is a relatively benign condition, with a valid therapeutic approach and GT has intrinsic risks that have been highlighted recently. The scope of this review is, however, to indicate a future application of GT when, as they will, the present limitations and intrinsic risks of GT will be overcome.

Acknowledgements

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