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REVIEW

Refractory celiac disease: from bench to bedside

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Abstract Refractory celiac disease is defined by the persistence of symptoms of malnutrition and intestinal villous atrophy for more than 6–12 months despite strict gluten-free diet in celiac patients. Diagnosis of this rare condition is made after excluding other causes of chronic small intestinal inflammation and villous atrophy and inadvertent intake of gluten. Over the past 15 years, multidisciplinary approaches have been developed to assess the mechanism of resistance to the diet, and two distinct entities have been delineated. Type II refractory celiac disease (RCD) can be defined as a low-grade intraepithelial lymphoma. RCD II is characterised by a massive accumulation of abnormal IEL that display an aberrant hybrid NK/T cell phenotype, contain clonal T cell rearrangement(s) and can mediate a cytolytic attack of the gut epithelium. This condition has a severe prognosis, largely due to the frequent transformation of RCDII IEL into overt aggressive enteropathy-type-associated T cell lymphoma. In contrast, in type I RCD, intestinal lymphocytes have a normal phenotype, and this generally milder condition remains often difficult to differentiate from uncomplicated CD except for the resistance to gluten-free diet (GFD). Several mechanisms may underlie resistance to gluten. Herein, we review the distinctive characteristics of RCD I and RCD II, the mechanisms underlying the onset of resistance to GFD, the risk of developing high grade lymphoma and possible clues to improve their treatment.

Keywords Celiac disease · Intraepithelial lymphocytes · Colllagenous sprue · Enteropathy-type-associated lymphoma · Gluten

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Introduction

Celiac disease (CD) is an immune-mediated enteropathy induced by dietary proteins derived from wheat, barley and rye (gluten), which affects nearly 1 % genetically predisposed individuals in Europe and in the USA. Diagnosis relies on the detection of serum autoantibodies against tissue transglutaminase 2 (TG2) and the demonstration of villous atrophy with increased number of intraepithelial lymphocytes (IELs) in duodenal biopsies. Symptoms are very variable, but they are largely caused by the defective absorption of nutrients and are thus in the vast majority of cases relieved by a life-long gluten-free diet (GFD), which interrupts the immune response triggered by gluten and allows recovery of a normal villous architecture. However, in a small subgroup of CD patients, symptoms of malnutrition and villous atrophy are not corrected by the GFD (primary resistance) or relapse despite strict adherence to GFD (secondary resistance), defining refractory celiac disease (RCD) [1]. Multidisciplinary approaches have been developed to investigate the mechanism of resistance to GFD. Studies based on the analysis of the phenotype and T cell rearrangements of intestinal lymphocytes have led to differentiate type I or non-clonal RCD and type II or clonal RCD that it is now considered as an intraepithelial T lymphoma. Herein, we discuss the characteristics of RCD I and RCD II, the mechanisms underlying the onset of resistance to GFD, the risk of developing high-grade lymphoma and possible clues for therapeutic intervention in either situation.

Diagnosis of refractory celiac disease and classification in subtypes I and II

Refractory CD is defined by the persistence of symptoms of malnutrition and intestinal villous atrophy for more than 6–12 months despite strict GFD in celiac patients (Fig. 1). In approximately 50 % of cases, CD is already known, and patients have initially responded to the GFD. Since many CD patients are not strictly compliant to GFD [2], diagnosis of RCD is only made after excluding poor adherence to GFD or inadvertent intake. In case of primary resistance to GFD or if the diagnosis of CD has not been firmly demonstrated, other causes of chronic villous atrophy need to be eliminated, notably autoimmune enteropathy [3], common variable immunodeficiency [4] that can promote bacterial overgrowth and excessive local inflammation, rare cases of small intestinal T cell proliferations unrelated to CD [5], or tropical sprue if patients have been chronically exposed to intestinal infections [6]. Serum anti-TG2 antibodies are useful to ascertain the diagnosis of CD but are only detected in a minority of RCD patients [7, 8], and their presence suggests inadvertent gluten intake. Indeed, serum anti-TG2 autoantibodies disappear in uncomplicated CD after GFD. Their persistence as a consequence of on-going intestinal inflammation is, however, not formally excluded. Demonstration of HLA haplotypes encoding HLA-DQ2 or HLA-DQ8 is useful if there is a doubt on CD. Indeed, their absence excludes CD as a cause of villous atrophy (reviewed in [9]). Finally, overt lymphoma needs to be excluded before concluding to RCD as often revealed by resistance to GFD or associated with RCD, notably type II RCD (see below) (Fig. 1).

When the diagnosis of refractory CD is ascertained, the next step is to define the type of RCD by analysing the phenotype and clonality of small intestinal IEL. IELs form a

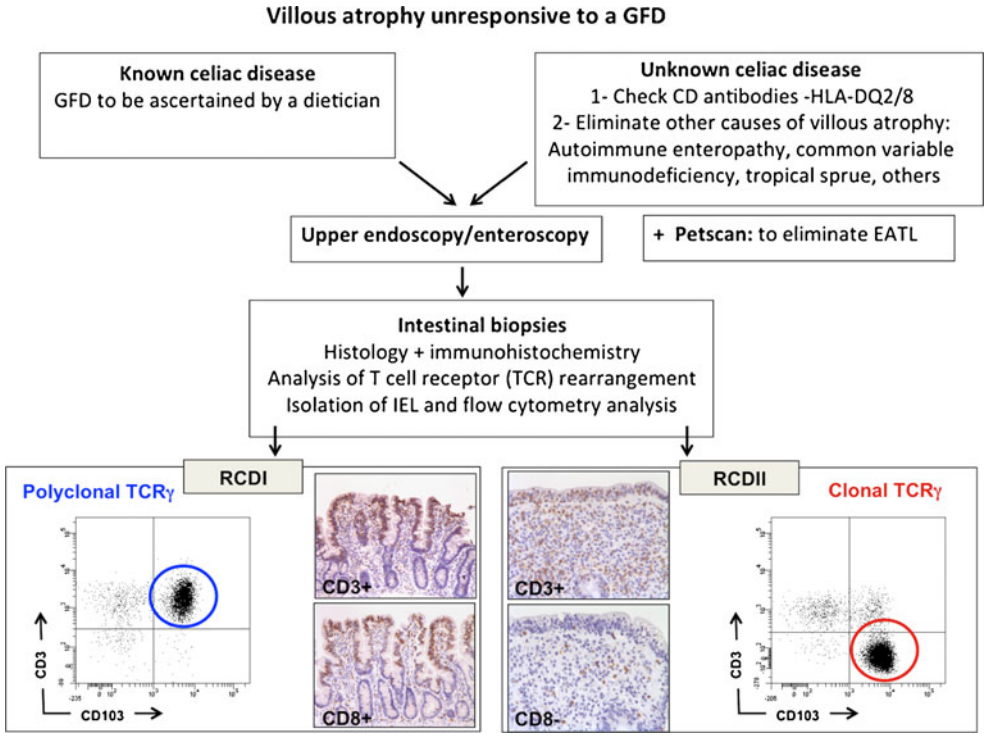

Fig. 1 Decisional guide for diagnosis of refractory celiac disease I and II (RCDI or II). In patients with known CD, diagnosis of RCD is made after eliminating any inadvertent intake of gluten. In patients with villous atrophy primary resistant to GFD, presence of celiac antibodies at diagnosis is necessary to ascertain initial diagnosis of CD; detection of HLA-DQ2 or HLA-DQ8 is also necessary to consider the diagnosis of CD; other causes of villous atrophy and malnutrition should be eliminated. Upper endoscopy and or enteroscopy allow to assess the extent of small intestinal lesions, the presence or not of ulcerative jejunitis or of a tumour and to sample intestinal biopsies. Videocapsule can help to assess intestinal lesions but is counterindicated in case of stenoses. PET scan is useful to eliminate an invasive lymphoma. Intestinal biopsies should be taken in the duodenum (generally the site of the most severe lesions) and at different levels of the intestine as indicated during endoscopy. Biopsies are processed for histology, immunohistochemistry (on formol-fixed tissues and, if possible, in frozen biopsies), analysis of T cell receptor rearrangement (TCR), isolation of IEL followed by flow cytometry phenotyping [10]. The combination of the three latter techniques allows the distinction between RCDI and RCD II. In RCDI, IEL have a normal phenotype ($CD3^+$ $CD8^+$ in tissue sections, $CD103^+$ with surface CD3 (sCD3) by flow cytometry) and there is no detectable clonal TCR rearrangement in biopsies. In contrast, in RCDII, IEL have an abnormal phenotype, generally $CD3^+$ CD8− in tissue sections, and always $CD103^+$ sCD3− by flow cytometry. The presence of intracellular CD3ε is detected after intracellular staining (not shown). Their clonal TCR rearrangement is detected in biopsies, notably by analysis of TCRγ rearrangement. In RCDII, it is useful to assess the possible dissemination of abnormal IEL in blood by flow cytometry and analysis of TCR rearrangements

large population of lymphocytes interspersed between epithelial cells that is massively expanded in the small intestine in uncomplicated CD and in RCD. Schematically, in RCDI, as in uncomplicated CD, IEL have a normal T cell phenotype, predominantly made of $CD3^+$ $CD8^+$ T cells and a polyclonal repertoire. In contrast, RCDII is characterised by a clonal expansion of IEL lacking surface CD3 and generally CD8 but containing intracellular CD3ε [7, 10, 11] (Fig. 1).

The phenotype of IEL is best studied by multicolour flow cytometry after their isolation from duodenal biopsies. In both RCDI and II as in the normal intestine, IEL express CD45, CD7 and CD103, the $\alpha_E\beta_7$ integrin that is a marker of IEL and a receptor for epithelial E-cadherin [12, 13]. In RCDI as in uncomplicated CD, over 90–95 % of IEL are $CD3^+$ T cells distributed between two subsets of $CD8^+$ αβT cells and γδT cells. The proportion of γδT cells is either normal (≅15%) or increased (up to 40 %) depending on patients. The proportion of IEL lacking surface CD3 (sCD3) is usually <5 % and thus even lower than in controls (5–20 %; mean, 8 %) [7]. No evidence of TCR clonality can be demonstrated in intestinal biopsies (Fig. 1). Up-regulation of NK receptors such as CD94 and NKG2C is frequent but more variable than in active CD. Overall, the characteristics of IEL in RCDI are not significantly different from those observed in uncomplicated CD ([7] and unpublished data).

In contrast, in RCDII, a large proportion of IEL (≥30 % and up to 98 %) lack surface expression of CD3 ($sCD3^-$) and of T cell receptors. Expression of CD8 is generally absent but can be expressed by a variable proportion of RCDII IELs in a few patients. In addition, RCDII IEL expresses a variable spectrum of NK markers such as CD94, NKG2D and NKp46 [10, 14] (Montcuquet et al., in preparation). Contrasting with the lack of surface CD3, RCDII IEL contain intracellular CD3 easily detected by intracellular staining with anti-CD3ε antibodies and display clonal rearrangements of T cell receptor chains, notably of the TCRγ chain [7, 10, 11] (Montcuquet et al., in preparation). The presence of intracellular CD3ε and of clonal TCR rearrangements differentiate RCDII IEL from the normal small subset of $sCD3^-$ IEL, only 20 % of which contain CD3ε and which do not exhibit evidence of clonal T cell rearrangement. Flow cytometry on lymphocytes isolated from biopsies is the most precise tool to identify $CD45^+$ $CD7^+$ $CD103^+$ sCD3ε–iCD3ε RCDII IEL: This technique allows defining their exact percentage in the intestinal epithelium (Fig. 1) and in other lymphoid compartments, where these cells can disseminate (see below). Yet, this technique is not available in all centres. Demonstration of phenotypically abnormal IEL can then be made in tissue sections by immunohistochemistry. Not all antibodies can be used in paraffin sections and notably antibodies available against CD103, and αβ or γδ TCR only work on frozen tissue sections. When possible, immunohistochemistry on frozen tissues will show high numbers of IEL that stained with anti-CD3ε (revealing intracellular CD3) but not with TCR antibodies, CD8 or CD4. If only paraffin sections are available, staining can demonstrate a high proportion of IEL stained with anti-CD3 but not with anti-CD8 or anti-CD4 [15] (Fig. 1). The later technique has, however, pitfalls. False positive are possible if the proportion of γδT IEL (generally negative for CD8) is very high as often in CD patients on a GFD and a cut-off of at least 50 % $CD3\varepsilon^+$ $CD8^-$ IEL is necessary to consider the diagnosis of RCDII. In all cases, ascertain the diagnosis of RCDII requires the demonstration of a clonal rearrangement of the TCR. Initial studies have shown that the clonal rearrangement is present in the sCD3-$iCD3^+$ IEL [10], but the rearrangement can be more easily detected in biopsies. In our institution, this is done using multiplex PCR and GeneScan analysis of TCRγ rearrangements on total DNA extracted from frozen biopsies, completed when positive, by checking heteroduplex formation to avoid false positive. In rare patients without detectable TCRγ rearrangements, a comparable technique can detect clonal TCRδ rearrangements. Others have suggested the detection of clonal TCRβ rearrangement [16]. Yet, *TCRB* rearrangements occur later than those of *TCRG* and *TCRD* during T cell differentiation and may not be always present in RCDII IEL (Montcuquet et al., in preparation).

Overall, these data show that the diagnosis of RCDII can be definitively authenticated by the demonstration of a clonal expansion of IEL with an aberrant and characteristic phenotype, which is now considered as an intraepithelial T cell lymphoma (see below). In most cases, diagnosis is orientated toward RCDII by clinical and biological evidence of severe malnutrition (mean body mass index inferior to 18), protein losing enteropathy (90 %) and by endoscopic demonstration of ulcerative jejunitis (UJ) (70 %) [7, 8]. UJ is defined by extensive and large mucosal ulcerations (with a diameter >1 cm) and can result in the development of single or multiple stenoses. That UJ defines a premalignant condition in CD patients has been suspected for many years before the demonstration of the abnormal IEL phenotype and the more recent definition of RCDII [17]. The diagnosis of RCDI is more difficult. Malnutrition is usually less severe than in RCDII although a few patients can be extremely sick and require total parenteral nutrition. Ulcers of the intestinal mucosa are less frequently observed (<30 %) and, when present, are small and scarce [7]. As discussed above, no phenotypical feature of intestinal lymphocytes has been described that can facilitate the distinction with uncomplicated CD. Therefore, the diagnosis of RCDI is an exclusion diagnosis made in CD patients with persistent or recurrent clinical symptoms and villous atrophy only after careful exclusion of any gluten intake (Fig. 1).

The natural history of RCD I and II: identification of RCD II as a first step toward CD-associated T cell lymphomagenesis

As CD, RCDI and II affect more often women than men: 69–78 % and 58–60 % in RCDI and RCDII, respectively [7, 8]. Their onset is observed after initial response to a GFD in 70 and 50 % of cases of RCDI and RCD II, respectively [7] and in the majority of the patients after 50 years [7, 8]. Yet, one case of primary RCDI has been reported in a young boy who was diagnosed with CD at 13 months and recovered normal villous architecture only after treatment with azathioprine [18]. In contrast, all cases of RCDII to date have been diagnosed in adults. In our series, the earliest diagnosis of RCDII was made in a 25-year-old man, who was on a GFD but was professionally exposed to wheat flour.

Few data are available concerning the exact incidence of RCDI and RCDII, but they appear as rare complications of CD. In a UK cohort, which included 713 celiac patients followed up between 1975 and 2008, West [19] observed that approximately 0.7 % of patients had or developed ulcerative jejunitis and had thus presumably RCDII. A second very recent study in a single North American referral centre suggests a cumulative incidence of 1.5 % for both RCDI and RCD II among CD patients initially diagnosed in this centre [20]. In this study, over 80 % of RCD patients were classified as type I. A higher frequency of cases of RCDI than of RCDII was also observed in two other studies from the USA [8] and from Germany [21]. In contrast, a higher frequency of RCDII over RCDI was reported in two studies from Holland [22] and from France [7]. Only the two latter studies used flow cytometry for the diagnosis of aberrant IEL, a technique that is more sensitive and more precise than immunohistochemistry as discussed above. Techniques to detect clonality may also vary in sensitivity. The proportion of RCDII cases may thus have been underestimated in some cohorts. Yet, differences in the populations of patients are also plausible. This possibility is suggested when comparing the incidence of enteropathy-type intestinal T cell lymphoma (EATL) in different cohorts and/or geographic areas. Indeed, as discussed below, RCDII is a frequent first step in the malignant lymphoid transformation of IEL that leads to the onset of EATL. Incidence of non-Hodgkin lymphoma associated with CD has been evaluated to 0.45, 3.75 and 2.7 for 1,000 patients-year in different European cohorts [23–25]. Based on a nationwide collection of data between 2000 and 2006, Verbeek et al. have reported a crude incidence of 0.10/100,000 cases of EATL in the total Dutch population with an incidence of 2.08/100,000 in over 50-year-olds [26]. In contrast, in a very recent study based on the Surveillance, Epidemiology and End Results (SEER) database of the US National Cancer Institute between 1973 and 2008 (a database that covers approximately 28 % of the US population), Sharaiha, et al. observed a much lower overall incidence of EATL of 0.016 per 100,000 (and of 0.05 per 100,000 in the population aged >60 years) [27]. These authors noted, however, an increased incidence from 0.006 per 100,000 to 0.024 per 100,000 during the study period, which coincidates with the recent increase in CD incidence in the USA [28, 29]. In the same study, it was also noted that EATL were common in men, an unexpected finding given the higher incidence of CD and RCDII in women. In addition, the highest incidence rate was in individuals of Hispanic origin with an age-adjusted incidence rate, of 0.033 per 100,000, further suggesting differences between populations of different geographical origins.

To differentiate RCDI and RCD II is important given their distinct evolution and prognosis. Three recent studies report a 5-year survival rate comprised between 80 and 96 % in patients with RCD I but of only 44 and 58 % for patients with RCDII [7, 8, 22]. The higher mortality rate in patients with RCD II is explained by the generally more severe malnutrition but also and mainly by the much higher risk to develop an overt T cell lymphoma. Thus, development of an overt T cell lymphoma is observed in 33–52 % of RCDII patients within the 5 years after diagnosis [7, 8, 22]. Onset of EATL in RCDI is much less frequent than in RCDII, with a 5-year rate of 14 % in the more pessimistic studies [7]. As indicated above, EATL are very rare non-Hodgkin T cell lymphomas. EATL have been divided into two subtypes, and only "type I EATL" (defined by negativity for the CD56 NK marker in tissue sections) have been associated with CD [30]. Depending on the studies, the overall risk of EATL is increased 8- to 30-fold in CD patients compared to the general population, indicating that this rare malignant disease is a distinctive complication of CD [31].

Many years ago, it was suggested that CD-associated EATL might derive from IEL due to their frequent epitheliotropism and to their expression of the CD103 marker [32]. The demonstration that EATL that develop in RCD II patients share the same T cell clonality as the aberrant IEL has confirmed this hypothesis [11], and it is now admitted that RCDII is an intraepithelial (or low grade) T cell lymphoma and the frequent first step of T cell lymphomagenesis in CD. In fact, RCDII IELs display features that attest their malignant transformation. In contrast with highly malignant EATL cells, they do not show evidence of active proliferation (they are $KI67^{-}$) and remain $p53^{-}$ and $CD30^{-}$. Yet, in addition to their clonal T cell rearrangements, they display chromosomal abnormalities and notably a recurrent partial trisomy 1q22-q44 [33]. Moreover, RCDII IEL can disseminate in and outside the intestine [7]. They are found in variable proportion in *lamina propria* (10–60 %) and in peripheral blood (0.5 % up to 60 %). RCDII IEL can also be found in mesenteric lymph nodes (the size of which is frequently increased in RCDII patients), in the gastric and colonic epitheliums as well as in extra-intestinal epithelial

sites such as the bronchi and the skin where their presence is revealed by infiltrated and/or ulcerated lesions [7]. Not surprisingly, a high percentage of abnormal lymphocytes in the duodenal epithelium is predictive of their presence in the peripheral blood [34]. Dissemination of RCDII IEL needs to be assessed by searching their presence in the blood, in gastrointestinal biopsies, and depending on symptoms, in bronchoalveolar fluid, bone marrow and/or in biopsies. Diagnosis combines the demonstration of the same clonal TCR rearrangement as in the intestinal biopsies and of the phenotypically aberrant population by immuno-histochemistry and/or flow cytometry. Extra-intestinal dissemination of RCDII IEL explains that EATL do not develop exclusively in the intestine. EATL may notably arise from RCDII cutaneous lesions. Their expression of CD103 and their identical TCRγ clonality [11] ascertain their origin from RCDII IEL. As a consequence of their high risk for overt EATL, RCDII patients require regular clinical follow-up combined with bi-annual thorough investigations, including notably pet-scan and enteroscopy [7]. It must, however, be stressed that EATL can arise in CD patients who do not display any evidence of RCDII. Such cases of EATL are also revealed by resistance to GFD and progressive unwanted weight loss. The association with fever and/or of intestinal obstruction is strongly suggestive of overt lymphoma [35–37]. In these patients as in EATL that complicate RCDII, the phenotype of EATL cells resembles that of aberrant RCDII IEL, although they may lose some differentiation markers, including CD3 and CD103. In addition malignant cells are KI67$^+$ and CD30$^+$. Abnormal IEL are, however, not detected outside the tumoral area and notably are not present in duodenal biopsies. The prognosis of overt EATL is poor with an overall survival currently estimated at 20 %, 5 years after the diagnosis. To define the association of EATL with RCDII may be important as our data suggest that the prognosis is better in patients without RCDII [38]. This observation needs, however, to be ascertained by further studies.

Predisposing factors for RCD I and RCD II

Whether RCD patients have distinctive genetic predisposing factors is not yet established, the small size of RCD cohorts being one major main limitation for genetic studies. Genetic susceptibility to CD is determined mainly by the HLA-DQ locus [39, 40]. The strongest association is observed with HLA-DQ2.5 [41], a lesser risk being conferred by the HLA-DQ8 haplotype [42]. It is now well established that the peptide pocket of HLA-DQ2.5 and HLA-DQ8 can electively accommodate gluten derived-peptides molecules, resulting in their presentation to CD4$^+$ intestinal T lymphocytes and the activation of an immune response against gluten (reviewed in [43]). That HLA-DQ2.5 can present a larger repertoire of gluten peptides to T cells than HLA-DQ8 is thought to explain the higher risk of CD associated with this molecule (reviewed in [43]). Moreover, disease susceptibility is associated with a dosage effect and DR3-DQ2 homozygous individuals who express more HLA-DQ2.5 molecules on antigen-presenting cells (APCs) have the highest risk for CD [44]. Interestingly, homozygoty for HLA-DQ2 might be a risk factor for T cell lymphomagenesis as observed in 44–67 % of RCDII and 53 % of EATL patients compared to 25–40 % of RCDI, 21 % of uncomplicated CD patients and 2 % of controls [7, 45]. Overall, these data suggest that the anti-gliadin CD4$^+$ T cell response that is a keystone of CD pathogenesis is important to promote the loss of homeostasis of IEL that ultimately leads to the onset of RCDII and EATL. In CD, the HLA genotype however accounts for <40 % of the genetic predisposition. Recent genome-wide association studies have identified nearly 40 non-*HLA* risk loci for CD [46–49], which overall might explain 13.7 % of the genetic variance of CD in individuals of European ancestry [49]. One potentially interesting association for RCD II concerns the *IL-2/IL21* locus shared with TID. Following results from GWAS, IL-21 was found to be markedly increased in intestinal biopsies from active CD [50] and to be produced in response to gluten peptides by specific CD4$^+$ T cells from CD patients [51]. Interestingly, IL-21 exerts strong synergistic effects with IL-15 on CD8$^+$ T cells and on NK cells [52]. IL-21 may thus act in concert with IL-15 to disrupt IEL homeostasis (see below). A second potentially relevant CD-associated variant is non-synonymous SNPs found within the coding sequence of *SH2B3* that encodes the adaptor protein LNK. This protein influences a variety of signalling pathways, notably those mediated by Janus kinases (JAK) [53]. As discussed below, JAK3 is an important relay in the signalling pathway of IL-15. Yet, it remains to be shown whether or not RCDI or RCDII are associated with known celiac susceptibility variants and more specifically with some of these variants.

In keeping with the higher frequency of HLA-DQ2 homozygoty in RCDII and EATL and therefore of a predisposing role of strong anti-gluten responses, a second factor promoting the onset of these severe complications might be long-term exposure to gluten. In a seminal study from UK, the risk to develop lymphomas was found to be 4-fold higher in patients who did not observe GFD than in compliant patients [54]. It was also reported that the risk of developing lymphomas decreased with time after CD diagnosis and thus presumably after GFD [25]. However, no significant association between poor compliance to GFD and increased risk of developing a T lymphoma was found in a recent population-based study in Sweden [55]. Biaggi and Corrazza have suggested that, not only compliance to GFD but also the amount of gluten intake might be important to influence the outcome of CD. They based their hypothesis on their observation of a more severe outcome of CD in South compared to North Europe that they suggested to be related to

a higher gluten intake [56]. However, this interesting hypothesis remains to be substantiated.

Finally, infections and more particularly viral infections may be another predisposing environmental factor favouring the onset of RCD. A role of gastrointestinal or hepatic viruses in CD onset is a long-standing hypothesis (reviewed in [57]). In adults, recurrent observations report the development of CD in patients treated with interferon alpha (IFN-α) for hepatitis C [58]. Consistent with the role of chronic viral infections in RCD, evidence of chronic hepatitis B or C was observed in our cohort in 20 % RCDI and 10 % RCDII patients at diagnosis [7]. A role for enterovirus, such as noroviruses, capable to induce persistent villous atrophy in immunodeficient patients [59] is perhaps not excluded but has not yet been investigated. The role of viruses in the pathogenesis of chronic inflammatory and autoimmune diseases is a popular hypothesis supported by a large number of studies. More than a specific virus, it is rather suspected that components of the antiviral responses and notably type I interferons might promote the onset of chonic inflammatory disorders (reviewed in [60]). Accordingly, increased secretion of type I IFN or an IFN-stimulated gene signature is observed in a spectrum of autoimmune diseases correlating with increased disease severity [61]. Type I interferon may notably stimulate the survival and proliferation of $CD8^+$ T cells and NK cells, [61] either directly or via the induction of IL-15 [62]. Virus might also stimulate IL-15 induction via stimulation of Toll-ligand receptor 3 (TLR3) [63]. Finally, chronic infection may also stimulate the production of IL-21, which, as IL-15, plays a key role in the long-term maintenance of virus-specific memory T cells [64]. The production of type I interferon and of IL-21 in RCD is, however, not yet well documented.

Pathogenesis of RCDI and RCDII

Pathogenesis of RCDI

As discussed above, immunophenotyping of intestinal lymphocytes does not allow to differentiate RCDI from uncomplicated CD. Mechanism(s) responsible for resistance to GFD remain(s) largely elusive and may vary among patients. A first subset of patients displays evidence of collagenous sprue (CS). CS is characterised by villous atrophy associated with a thickened subepithelial collagen that is visible on haematoxylin and eosin stained tissue sections but is best seen after Masson trichrome staining. In their comprehensive study of CS, Vakiani et al. [65] report that a thin subepithelial collagenous layer (<5 μm) is observed in 60 % of active CD patients with severe villous atrophy. Thickness of the collagenous layer over 5 μm, and, for most authors, over >10μm is therefore the minimal limit for the diagnosis of CS. In their series of 19 patients with CS, Vakiani et al. observed that 17 had CD. Nine were primary or secondarily resistant to GFD at time of diagnosis of CS, 8 with RCDI and one with RCDII. Resistance to the diet was observed in patients with the thickest subepithelial fibrotic layer. Fibrosis and degrees of villous atrophy decreased in parallel with the symptoms in CD patients responsive to GFD and in some but not all RCDI patients after immunomodulatory treatment by corticosteroids associated or not with azathioprine. Yet, several RCD patients required total parenteral nutrition. As also noted by other authors, CS is not specific of CD and is observed in some rarer disorders also associated with chronic intestinal inflammation [65, 66]. Overall, these data indicate that subepithelial accumulation of collagen favoured by chronic inflammation can result in the onset of resistance to GFD in a subset of CD patients. Daum et al. [67] have provided evidence of increased collagen synthesis in the absence of adequate fibrolysis, but how the chronic immune process results in such imbalance is not yet elucidated.

CS is not the only cause of resistance to GFD in RCDI. In a small series of 20 patients with RCDI, we have observed evidence of collagenous sprue only in five cases (25 %). One plausible hypothesis is that the intestinal immune reaction initially induced by gluten has evolved into an autoimmune reaction. Consistent with this hypothesis, most RCDI patients improve under immunosuppressive treatments [7, 22]. CD share many features with bona fide autoimmune diseases, notably type I diabetes. Thus, there is a large overlap between predisposing genetic factors [49], and extraintestinal autoimmunity develops with age in CD patients, notably in those that are not compliant to GFD [68]. In one recent study, a higher frequency of autoimmune disorders was observed in RCD patients (29.4 %) compared to a whole cohort of CD patients (19 %), although the difference did not reach significance [20]. Finally, the mechanisms that lead to tissue damage in CD share similarities with those invoked in type I diabetes. In uncomplicated CD, epithelial damage is thought to be largely dependent on cytototoxic $CD8^+$ αβT IEL, which express activating NK receptors. They notably express NKG2D that can license the killing of enterocytes, which have up-regulated expression of NKG2D ligands [69]. A comparable mechanism was shown to participate in the destruction of pancreatic β-islets in a mouse model of type I diabetes [70]. That intestinal lymphocytes become autoreactive in RCDI remains, however, to be firmly established and the mechanism(s) that convert(s) anti-gliadin into autoimmune responses need(s) to be identified. A role of IL-15, a cytokine produced in excess in the intestinal mucosa of patients with active CD is possible [71, 72]. In active CD, this cytokine is thought to orchestrate a cytolytic attack of the epithelium by $CD8^+$ αβ T IEL. IL-15 can notably induce the production of cytolytic proteins in IEL, enhance expression and signalling via NKG2D [69] and likely participate in the up-regulation of NKG2D ligands on enterocytes [14]. In addition, this cytokine can interfere with two

mechanisms that play a key role to establish and maintain tolerance to both dietary proteins and self-antigens. Thus, IL-15 impairs TGF-β signalling by activating the phosphorylation of the transcription factor c-jun, which translocates into the nucleus and prevents Smad3-dependent transcription of TGF-β target genes [73]. TGF-β is one important factor to limit T cell activation, and severe autoimmunity develops in mice with T cells lacking a functional TGF-β receptor [74, 75]. IL-15 can also interfere with the function of FoxP3$^+$ CD4$^+$CD25$^+$ regulatory T cells (Tregs). The later cells play a central role in the control of autoimmunity as indicated by the development of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome in boys with mutations inactivating FOXP3, a transcription factor indispensable for the generation of functional Tregs [76]. By stimulating the activation of the phosphoinositide-3 kinase, IL-15 can render effector T cells and notably CD8$^+$ T cells unresponsive to the suppressive effects of Tregs cells. This mechanism likely operates in vivo in CD as intestinal and peripheral T cells isolated from patients with active CD show resistance to Tregs immunoregulation [77, 78]. In mice, IL-15 might also interfere with the generation of Tregs, although it is as yet unclear whether this mechanism operates in CD [79]. Therefore, it is tempting to suggest that, due to the presence of IL-15, autoreactive T cells, notably CD8$^+$, may escape retrocontrol by TGF-β and Tregs, progressively accumulate and ultimately sustain an intestinal immune response that becomes independent of gluten intake. This scenario remains, however, to be formally demonstrated and the up-regulation of IL-15 to be ascertained in RCDI. Our unpublished data suggest that some but not all RCDI patients have markedly increased serum levels of IL-15.

Pathogenesis of RCDII

As discussed above, RCDII is characterised by a massive infiltration of clonal IEL which invariably display a CD103$^+$ sCD3-iCD3$^+$ phenotype (Fig. 2). The presence of chromosomal abnormalities contrasts with their low in situ proliferation and suggest that their progressive accumulation results from their impaired elimination by apoptosis. Moreover, RCDII patients exhibit severe epithelial lesions, suggesting that RCDII IEL can, alike CD8$^+$ αβ T IEL in active CD cells, exert cytotoxicity against enterocytes. Accordingly and alike the latter cells, RCDII IEL express several activating NK receptors, notably NKG2D and, in vitro, they can spontaneously kill enterocyte lines that express NKG2D ligands [14]. If the role of IL-15 remains to be demonstrated in RCDI, our work indicates that this cytokine is up-regulated in *lamina propria* mononuclear and in enterocytes of RCDII patients [71] and plays a central role in the pathogenesis of RCDII (Fig. 2).

IL-15 is a cytokine structurally and functionally related to IL-2. In contrast with IL-2 that is predominantly produced

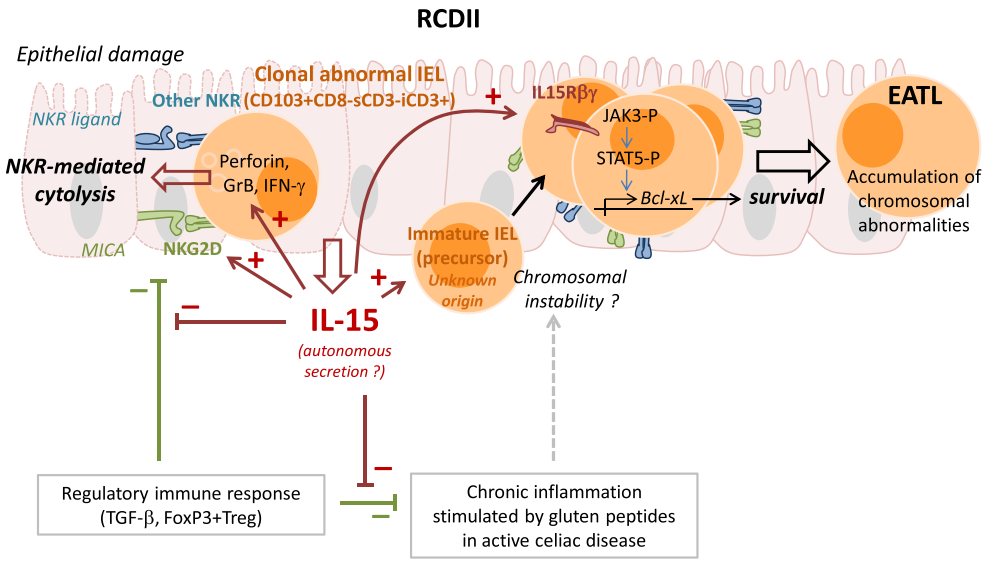

Fig. 2 Pathogenesis of type II refractory celiac disease (RCDII): hypothetical scheme. RCDII is characterised by the progressive intraepithelial expansion of a clone of lymphocytes (IEL) with an abnormal hybrid T/NK phenotype. The clone of abnormal IEL does not proliferate actively but accumulates progressively despite chromosomal abnormalities and replaces normal T IEL. The abnormal IEL clone can disseminate toward *lamina propria* and in and outside the gut but also transform into enteropathy-type T cell lymphoma (EATL). IL-15 plays a central role in the pathogenesis of RCDII. IL-15 is produced by many cell types and notably by enterocytes. IL-15 activates the NK-like cytotoxicity of RCDII IEL against epithelial cells via the interaction between NK receptors expressed on RCDII IEL and their ligands expressed epithelial cells (see text), resulting in severe epithelial damage. IL-15 rescues RCDII IEL from apoptosis via a pathway involving JAK3, STAT5 and the anti-apoptotic factor BCL-xL. IL-15 can block regulatory pathways and thus sustain chronic inflammation, which promotes chromosome instability. IL-15 might also initiate the process by acting on an abnormal immature lymphoid precursor. The mechanisms stimulating IL-15 overexpression remain, however, to be elucidated (see text)

by activated T cells, IL-15 can be synthesised by many cell types and notably by the enterocytes. IL-15 shares the β (CD122) and γ (CD132) chains of the IL-2 receptor, and the two chains associate to form an autonomous signalling module preferentially expressed on NK cells, $CD8^+$ T cells and on some subsets of IELs. A third chain, IL-15Rα is, as IL-15 expressed by many cell types and has a high avidity for IL-15 but no signalling function. IL-15Rα can associate with the β and γ chain on lymphocytes to form a receptor of very high affinity for IL-15. In addition and more importantly, IL-15Rα can bind IL-15 and form at the cell membrane long-lived complexes [80] that are presented to adjacent lymphocytes (reviewed in [81]). Such complexes likely form at the surface of enterocytes in RCDII patients since IL-15 can be detected by flow cytometry on enterocytes extracted from RCDII biopsies [71]. IL-15 bound to IL-15Rα at the enterocyte surface can in turn activate RCDII IEL. Indeed, the later cells strongly express the β/γ signalling module and, accordingly, respond in vitro to very low concentrations of IL-15 [71, 82]. Our data indicate that IL-15 induces the expression of cytotoxic proteins in RCDII IEL and stimulates their production of IFN-γ and their NKG2D-dependent cytotoxicity against enterocyte lines [14, 71]. As MICA, one NKG2D ligand, is strongly upregulated at the epithelial surface of enterocytes in RCDII patients, RCDII IEL activated by enterocyte-derived IL-15 might exert their cytotoxicity against epithelial cells in vivo and be responsible for the severe enteropathy observed in RCDII patients. IL-15 is also mandatory for the survival of RCDII IEL. In vitro, very low concentrations of IL-15 can maintain long-term survival of RCDII IEL and the use of specific inhibitors and/or shRNA showed that the anti-apoptotic properties of IL-15 depend on a signalling cascade, which, downstream IL-15Rβγ, involves the Janus kinase 3, the transcription factor Stat5 and the anti-apoptotic molecule Bcl-xL. Using duodenal organ cultures, it was possible to demonstrate that this pathway is activated in situ in the intestine of active CD and RCDII patients and can be blocked by an antibody against IL-15 [82]. The latter results suggest that IL-15 protects normal IEL in active CD and aberrant IEL in RCD II from apoptosis and allow their progressive accumulation (Fig. 2). This hypothesis is in keeping with the observation of a massive accumulation of $KI67^-$ (non proliferating) IEL in the intestine of mice overexpressing IL-15 in the gut epithelium [82, 83]. The potent anti-apoptotic effect of IL-15 may thus prevent the elimination of transformed RCDII lymphocytes. These cells may then acquire new mutations or chromosomal abnormalities and ultimately convert into overt aggressive EATL, a scenario likely facilitated by persistent inflammation that can promote chromosomal instability (reviewed in [33]). The fostering role of IL-15 on T cel lymphomagenesis is supported by observation in transgenic mice with ubiquitous overexpression of IL-15 as over 30 % of these mice develop lymphomas or leukemias with a hybrid T CD8/NK phenotype [84].

Several questions remain however unsolved. The first concerns the origin of RCDII IEL. Their stereotypical phenotype evokes a common origin and/or pathway of transformation. On the one hand, the clonal rearrangements of the T cell receptor and the presence of intracellular CD3 suggest a T cell lineage [85] (Montcuquet et al., in preparation). On the other hand, RCDII IELs display NK-like features. As discussed above, they express a spectrum of activating NK receptors and exert a strong spontaneous NK-like cytoxicity [14]. Such NK-like properties are partially shared by the normal polyclonal $CD8^+$ αβT IELs that expand in uncomplicated CD. It was therefore suggested that CD IEL that are chronically activated in the presence of IL-15 can undergo reprogramming into NK cells and ultimately loose T cell surface expression [69, 85]. Our recent data rather suggest that RCDII IEL arise from immature T cell precursors with hybrid features of NK cells [85] (Montcuquet et al., in preparation). A second unsolved question concerns the mechanism(s) that drive(s) exaggerated expression of IL-15 in active CD and in RCD II. Due to its binding to IL-15Rα and to a generally low level of expression, precise quantisation of IL-15 is difficult, and its regulation in CD remains poorly understood. Our data point out to post-transcriptional regulation [71]. Studies in organ cultures suggest that some gluten peptides may, via unknown relays, induce the production of IL-15 [14, 71, 86]. Accordingly, IL-15 expression decreases in CD patients after GFD [71], and strict adherence to a GFD diet (even if not sufficient) is absolutely required to improve symptoms and villous atrophy in RCDII patients. In RCDII patients on a strict diet, other factors than gluten must, however, sustain IL-15 expression. One such factor might be IFN-α, which can induce the production of IL-15 during viral infection [62]. As discussed above, chronic infections by hepatitis B or C viruses are present at diagnosis in 20 % of RCDII patients of our cohort and might perhaps predispose to RCDII by promoting upregulation of IL-15. Finally, IL-15 production might also be self-sustained by tissue damage, as suggested by the induction of IL-15 in thermal or oxidising stress-activated dendritic cells [87]. However, further studies are needed to assess these hypotheses and delineate IL-15 regulation in CD and RCD.

Treatment of RCD: a need for new strategies

To date, there is no curative treatment for RCDI or RCDII and management of the patients relies on a combination of nutritional support and immunosuppressive or ablative treatments. As RCDI and RCDII are both rare diseases and have only been recently individualised as distinctive entities, there is no standardised therapeutic strategy. The choice of specific treatments is presently guided by observational experiences in small cohorts

of patients followed up in expert centres [88]. Their respective indications in RCDI and RCDII are not yet well delineated.

In all patients, nutritional deficiencies, metabolic disorders and hypoproteinemia should be corrected. Total parenteral nutrition is necessary in patients with severe malnutrition and can be useful to ascertain resistance to GFD in a few patients. Even if not sufficient to induce remission, a strict GFD must be initiated or maintained to prevent gluten-induced lymphocyte activation and to reduce local inflammation. In RCDII, however, the clonal population persists and can evolve toward overt EATL [7].

Steroids, notably budesonide preferred for its topical action and low rate of side effects on short-term use, improve clinical symptoms in most patients whatever the type of RCD, but histological responses are observed only in 30–40 % of cases [7, 8]. Moreover, dependence or resistance to corticosteroids is usual in both RCDI and RCD II, and corticosteroids have no significant effect on the numbers of RCDII IEL. Other immunosuppressive drugs have been used, notably azathioprine, or more rarely cyclosporine or anti-TNF-α. Transient clinical response can be observed, but histological improvement is rare [7, 8, 88]. Moreover, the later drugs are unable to reduce the numbers of RCDII IEL. On the contrary, azathioprine might enhance the risk or accelerate the onset of overt lymphoma [7, 89].

Ablative treatments aiming at the destruction of abnormal IEL have been attempted in RCDII. In contrast with highly proliferative EATL cells that can be destroyed at least transiently by anti-proliferative chemotherapeutic agents, RCDII IEL have a very low proliferative rate and are therefore very difficult to eradicate by such drugs [7]. RCDII IEL might thus represent a reservoir of transformed cells that promote relapse after chemotherapy for EATL. Depletion of RCDII IEL by anti-CD52 antibody or by purine analogues such as pentostatine or cladribine (2 CDA) has therefore been suggested [90, 91]. Anti-CD52 treatment could reduce the numbers of abnormal IEL but, in our experience, was associated with the rapid onset of overt lymphoma, probably as a consequence of the profound T cell depletion and immunosuppression induced by this antibody [7]. Similarly, treatment with 2CDA could induce some clinical, histological and haematological responses [90], but the risk of transformation into overt lymphoma was increased in a series of 17 RCD II patients [91], and we observed the explosive onset of overt lymphoma within 3–8 weeks after treatment in two patients [7]. A distinct strategy based on autologous haematopoietic stem cells transplantation has been attempted in a series of 13 patients. Clinical and histological responses were observed, but the numbers of abnormal IEL were only reduced in some patients and the long-term outcome of this treatment, notably to prevent the onset of EATL is not yet established [92, 93]. Administration of an ablative treatment before autologous haematopoietic stem cells transplantation may perhaps improve the haematological response. We are currently evaluating this strategy in a prospective phase II trial.

Overall, these data indicate that the treatment of RCD, and notably of RCDII, remains a serious challenge and that new strategies must be designed. They are notably necessary to prevent the onset of overt EATL, which has a very poor prognosis with only 20 % of patients alive 5 years after diagnosis [35–37]. One attractive possibility might be to target the anti-apoptotic pathway activated by IL-15 [82]. Consistent with this hypothesis, injection of a humanised antibody blocking IL-15 in transgenic mice overexpressing human IL-15 in the gut epithelium induced the rapid apoptosis of intestinal lymphocytes and a drastic decrease in the intestinal lymphocyte infiltration. Since this antibody is not currently available for clinical use, an alternative might be to use pharmacological antagonists able to block the anti-apoptotic cascade elicited by IL-15 in RCDII IEL. Interesting candidate are the JAK3 inhibitors that are currently under development of clinical use [94]. These drugs might be used alone or perhaps in combination with more conventional chemotherapy agents to eliminate RCDII IEL.

Conclusion

Over the past 15 years, a combination of multidisciplinary approaches has allowed to precisely define refractory celiac disease and to delineate at least two distinct entities. Type II RCD can be defined as a low-grade intraepithelial lymphoma. Its diagnosis relies on precise diagnosis criteria. Resistance to GFD is due to IL-15-driven accumulation of a clone of transformed IEL that display an aberrant hybrid NK/T cell phenotype and can mediate a cytolytic attack of the gut epithelium. This condition has a severe prognosis, notably due to the frequent transformation of RCDII IEL into overt aggressive EATL and efficient treatments able to eradicate RCDII IEL and to block this evolution remain to be designed. In contrast, RCDI is generally a milder disease that remains difficult to differentiate from uncomplicated CD except for the resistance to GFD. In these patients, on-going inflammation proceeds independently of gluten but the mechanism(s) of villous atrophy is not well explained and may differ between patients. Clinical symptoms are usually alleviated by immunosuppressive drugs and the risk of developing lymphomas is much less than in RCDII. However, long-term immunosuppression has adverse side effects. It only rarely achieves histological recovery and patients can relapse or remain with permanent or intermittent symptoms. New treatments are therefore desirable to improve the quality of life and long-term outcome of RCDI patients.

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