Testing safety of germinated rye sourdough in a celiac disease model based on the adoptive transfer of prolamin-primed memory T cells into lymphopenic mice

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Freitag TL, Loponen J, Messing M, Zevallos V, Andersson LC, Sontag-Strohm T, Saavalainen P, Schuppan D, Salovaara H, Meri S. Testing safety of germinated rye sourdough in a celiac disease model based on the adoptive transfer of prolamin-primed memory T cells into lymphopenic mice. Am J Physiol Gastrointest Liver Physiol 306: G526–G534, 2014. First published January 23, 2014; doi:10.1152/ajpgi.00136.2013.—The current treatment for celiac disease is strict gluten-free diet. Technical processing may render gluten-containing foods safe for consumption by celiac patients, but so far in vivo safety testing can only be performed on patients. We modified a celiac disease mouse model to test antigenicity and inflammatory effects of germinated rye sourdough, a food product characterized by extensive prolamin hydrolysis. Lymphopenic Rag1−/− or nude mice were injected with splenic CD4+CD62L−CD44high-memory T cells from gliadin- or secalin-immunized wild-type donor mice. We found that: 1) Rag1−/− recipients challenged with wheat or rye gluten lost more body weight and developed more severe histological duodenitis than mice on gluten-free diet. This correlated with increased secretion of IFNγ, IL-2, and IL-17 by CD4 T helper 1 (Th1) cells drive intestinal pathology in CD (23). In the mucosa, glutamine residues of gluten peptides are deamidated by tissue transglutaminase, an enzyme that is the target of CD-specific autoantibodies (3). Modified gluten peptides bind with increased affinity to HLA-DQ2 and DQ8 molecules and increase proinflammatory cytokine secretion by CD4+Th1 cells (24). CD lesions in the small intestine show mucosal infiltration with lymphocytes, crypt hyperplasia, and villus atrophy. A successful treatment of CD is gluten-free diet (GFD).

A GFD, however, is difficult to maintain, and partial or nonadherence is frequent among celiac patients. One reason for this is the inferior flavor and texture of the current industrial gluten-free food products (7). Foods derived from gluten-containing grains, after a hydrolysis of prolamins, could be an alternative to the use of naturally gluten-free cereals by celiac patients. Technically, extensive prolamin hydrolysis is achievable through the combined use of germination of grains and sourdough fermentation in food processing (17), or by the addition of fungal proteases from Aspergillus species (2). Pretreatment of gliadin (the alcohol-soluble fraction of wheat gluten) with germinating wheat proteases in vitro results in decreased reactivity with patient-derived gliadin-specific T cell clones (27). A previous study indicated that a combination of rye germination and sourdough fermentation could be used to hydrolyze secalins (the prolamins in rye flour) to levels that might be tolerated by celiac patients (17). Yet, the safety of germinated rye sourdough products for celiac patients remained to be demonstrated.

Recently, the U.S. Food and Drug Administration defined the use of the term “gluten-free” in food labeling, in agreement with the current Codex Alimentarius definition (<20 mg/kg; see Refs. 1 and 5). However, quantification of residual gluten in processed food products is technically challenging. The validity of the available analytical methods for the prediction of gluten toxicity, including the commonly used competitive R5 ELISA (21), has not been evaluated through patient trials (15). Current ELISA methods rely on the detection of specific gluten peptides by monoclonal antibodies. For gluten quantification, it is postulated that peptide content is proportional to total gluten content. It has been argued that this assumption may not generally apply to industrial cereal products processed

CELIAC DISEASE (CD) is a gluten-sensitive enteropathy that has a prevalence of 0.3–2.4% in most populations (11, 22). The disease is triggered by ingestion of gluten, the proline-rich storage proteins of wheat, barley, and rye. Ninety to ninety-five percent of CD patients carry the human leukocyte antigen (HLA) allele DQ2, and the remainder HLA-DQ8 (24), implicating HLA class II-restricted CD4+ T cells in CD pathogenesis. Dysregulated, gluten-specific CD4+ T helper 1 (Th1) cells drive intestinal pathology in CD (23). In the mucosa, glutamine residues of gluten peptides are deamidated by tissue transglutaminase, an enzyme that is the target of CD-specific autoantibodies (3). Modified gluten peptides bind with increased affinity to HLA-DQ2 and DQ8 molecules and increase proinflammatory cytokine secretion by CD4+ Th1 cells (24). CD lesions in the small intestine show mucosal infiltration with lymphocytes, crypt hyperplasia, and villus atrophy. A successful treatment of CD is gluten-free diet (GFD).

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by diverse methods (15). Furthermore, clinical safety of processed cereals as part of the GFD has only been tested for selected products in studies involving small numbers of patients (10, 20), inevitably exposing study subjects to the risk of clinical relapse. Published results were promising for the use of a combination of fungal proteases in wheat sourdough fermentation (10). Safety testing in disease models of CD could prove beneficial in identifying the most promising cereal processing techniques before trials with patients are initiated.

The lack of a truthful animal model of CD has hampered the exploration of celiac pathogenesis and the development of novel treatments (26), but also the development of safe cereal-based food products that could diversify the GFD. We recently described a model of gluten-sensitive enteropathy, based on the adoptive transfer of gliadin-sensitized memory T cells into Rag1−/− mice (6). In this in vivo model, an imbalance between regulatory and effector T cells causes an autoimmune syndrome that manifests primarily in the proximal small bowel. As a result of donor cell priming, the observed duodenitis is partially gluten-sensitive. Here, we tested in an improved model, now based on a simplified memory T cell isolation protocol, the safety for celiac patients of germinated rye sourdough, a food product under development for gluten-free baking (17). In particular, we asked whether germination and sourdough fermentation of rye would eliminate antigenicity and inflammatory effects of rye secalins in mice with gluten-sensitive enteropathy.

MATERIALS AND METHODS

Cereal protein and rye sourdough preparations. Secalin was prepared from native rye flour, as reported previously (17). Wheat gluten and gliadin preparations were purchased from Sigma-Aldrich. Sourdoughs were prepared as reported (17), using 100 g rye or rye malt flour (Laihian Mallas), 150 ml of tap water, and 20 mg Floraplan L62 dry starter (Lactobacillus brevis I-L62; Lallemand). Fermentation was for 24 h at 34°C, final pH 3.4. Approval for all animal procedures was obtained from the animal research board of the Southern Finnish State Administrative Agency (ESLH-2008–06881/Ym-23 and ESAVI/1064/04.10.03/2012).

Animals, memory CD4+ T cell preparation, and treatments. Male C57BL/6 donor mice, raised on gluten-free, standardized diet AIN-76A and maintained under specific pathogen-free conditions, were used. C57BL/6 donor mice, raised on gluten-free, standardized diet AIN-76A and maintained under specific pathogen-free conditions, were used. C57BL/6 donor mice, raised on gluten-free, standardized diet AIN-76A and maintained under specific pathogen-free conditions, were used. C57BL/6 donor mice, raised on gluten-free, standardized diet AIN-76A and maintained under specific pathogen-free conditions, were used.

To avoid the use of naive T cells, donor splenocytes (both columns from R&D Systems). Male Rag1−/− or nude mice were injected i.p. with CD4+CD62L−CD44high T cells from gliadin (or secalin-)-immunized wild-type donor mice and challenged orally with gluten (vs. gluten-free diet control [gfd]).

Prolamin analysis. Prolamin content of mouse diets was determined by competitive R5 ELISA (Ridascreen kit, catalog no. R7011; R-Biopharm) using synthetic gliadin peptides for calibration and following the manufacturer’s instructions. Measurements were repeated at least three times. Samples were extracted with 60% ethanol (vol/vol).

Histological analyses and scoring. Organ samples were fixed in formalin and embedded in paraffin, and sections (6 μm) were stained with hematoxylin and eosin. To identify proliferating cells, small bowel sections were stained using polyclonal rabbit anti-mouse Ki-67 (maximum score 7.00; see Ref. 19). Briefly, villus height/depth was measured at ×100 magnification, and villus-to-crypt ratio scores were assigned. Cellular infiltrates were scored in 3 μm sections. The severity of duodenitis was assessed in a blinded fashion at representative, well-oriented sections of maximal damage (6). Briefly, villus height/depth was measured at ×100 magnification, and villus-to-crypt ratio scores were assigned. Cellular (mainly mononuclear) infiltration of the stroma of the villi and basal infiltration with neutrophils were also assessed. Each animal was assigned a composite duodenitis score by combining the three separate parameters (maximum score 3 + 3 + 3 = 9). The severity of colitis was assessed using a histological score for T cell transfer colitis (maximum score 7.00; see Ref. 19).

Cytokine and serum antibody ELISAs. IFNγ, IL-2, IL-17 (eBioscience), and IL-10 (R&D Systems) cytokine ELISAs were performed with supernatants from in vitro cultures of splenocytes in complete RPMI 1640 medium, or from cultures of isolated memory T cells resuspended with irradiated splenocytes at a ratio of 1:4 (28 Gy). Splenocytes from individual mice or pooled memory T cells were restimulated over 72 h at 37°C, with either a combination of anti-CD3 and anti-CD28 antibodies (positive control, 3 μg and 2 μg/ml; clones 145–2C11 and 37.51; eBioscience), secalin- or endotoxin-free ovalbumin (negative control, Hyglos; both proteins 20 μg/ml), or PBS. Ratios between results for cytokine concentrations from the stimulated samples and corresponding positive controls were calculated. Serum anti-gliadin or anti-secalin IgG and IgG2c ELISAs were performed as reported previously (6). Both gliadin and secalin were coated at 1 μg/well. Serum antibody titers leading to an optical
density of 1.0 in spectrometric analysis (450 nm) were calculated from serial dilutions.

**FACS analysis.** Postsort FACS analysis of T cell populations was performed on a BD FACScan cytometer (BD Biosciences) using fluorescence-labeled antibodies for CD4, NK1.1, CD19 (clones RM4–5, PK136, eBio1D3; eBioscience), CD8, CD62L, and CD44 (clones 53–6.7, MEL-14, IM7; Biologend), gated on lymphocytes.

**Statistical analyses.** Data were analyzed with Prism 5 software (GraphPad). Statistical comparisons were performed using unpaired Student’s t-tests. Mann-Whitney U-tests were performed for nonparametric data, and one-way ANOVA and Neuman-Keuls tests were used for comparisons between multiple groups. Means, SE, medians, or interquartile ranges were calculated.

**RESULTS**

**CD4⁺CD62L⁻CD44high-memory T cell grafts.** Postcolumn sort FACS analysis of splenic CD4⁺CD62L⁻CD44high-memory T cell grafts confirmed depletion of CD19⁺ B cells (<1%) and CD8⁺ T cells (<2%). There remained a fraction of NK1.1⁺ NK cells (5%). Purity of CD4⁺ T cells was 80%, of which >99% were CD62L⁻ cells, and 84% were CD4⁺ CD62L⁻CD44high-memory T cells. Restimulation of CD4⁺ CD62L⁻CD44high-memory T cells from secalin-immunized donors demonstrated that this memory population contained secalin-reactive T cells that responded with the secretion of IFN-γ in the supernatant [cytokine secretion ratio 0.035 ± 0.014 (SE) vs. 0.001 ± 0.000 for negative control, P < 0.001, IFN-γ ELISA assay; results not shown].

Pathological changes after adoptive transfer of gliadin-sensitized CD4⁺CD62L⁻CD44high-memory T cells. To determine whether CD4⁺CD62L⁻CD44high-memory T cells were effective inducers of gluten-sensitive enteropathy, two groups of Rag1⁻/¹ mice (n = 14) were injected with gliadin-sensitized CD4⁺CD62L⁻CD44high T cells and thereafter challenged with oral gluten (vs. the gluten-free control diet). Figure 1 depicts the in vivo experimental protocol. After 8 wk posttransfer, gluten-challenged mice had lost more body weight (P < 0.01, Fig. 2A) and suffered from more severe histological duodenitis (score of 8.00 median, 6.88–9.00 interquartile range vs. 6.50, 5.25–8.00, P < 0.05, Fig. 2B) than mice on the GFD. These results were similar to those obtained previously in recipients of CD4⁺CD45RBlowCD25⁻ memory T cells (6). There was a trend for increased small bowel weight in gluten-challenged mice, consistent with more severe inflammation [2.12 ± 0.11 (SE) vs. 1.84 ± 0.10 g, P = 0.08; data not shown]. Duodenal pathology was characterized by mononuclear cell infiltration of the basal and villus lamina propria, villus atrophy, and crypt hyperplasia (Fig. 3). Also, neutrophilic infiltration of the basa lamina propria was observed, leading to cryptitis and crypt abscesses. Multinucleated giant cells were found in moderate to severe duodenitis. These features were observed both in recipients on gluten-free or gluten-containing diet but were more severe in the group on gluten-containing diet (Fig. 2B). Additional organ involvement (pneumonitis, colitis, pancreatitis) of variable severity was frequent, but duodenitis remained the predominant autoimmune organ manifestation. Histological colitis was scored and did not differ between recipients on gluten-containing diet vs. those on GFD (score of 3.00 median, 2.00–5.00 interquartile range vs. 4.00, 1.75–5.25; P = 0.90). Taken together, pathological changes were indistinguishable from the disease observed after transfer of CD4⁺CD45RBlowCD25⁻ memory T cells (6), although in this study the degree of intestinal inflammation was generally more severe. The findings demonstrated that gliadin-sensitized CD4⁺CD62L⁻CD44high T cells were effective inducers of gluten-sensitive enteropathy in Rag1⁻/¹ mice.

**Effects of secalin-primed CD4⁺CD62L⁻CD44high-memory T cells.** In preparation for testing the immunostimulatory and inflammatory effects of germinated rye sourdough, we had to ascertain that rye secalin could replace gliadin/wheat gluten in both donor priming and oral challenges in the mouse disease model. Two groups of Rag1⁻/¹ recipients (n = 9) were therefore challenged with a diet containing 2.5 g secalin/kg vs. the GFD, after adoptive transfer of secalin-sensitized CD4⁺CD62L⁻CD44high T cells. After 9.5 wk, secalin-challenged recipient mice suffered from exacerbation of duodenitis (score of 9.00 median, 8.25–9.00 interquartile range vs. 6.50, 3.50–8.00, P < 0.01, Fig. 4A) and had lost more body weight than mice on the GFD [percentage of starting weight 93.6 ± 1.8 (SE) vs. 105.0 ± 4.3%, Fig. 4B]. These results were similar to those after adoptive transfer of gliadin-sensitized memory T cells. Increased body weight loss over the full treatment period remained statistically insignificant because of an unexplained initial weight gain in mice challenged with oral secalin. This
confirmed that secalin-sensitized CD4+ T cells induced gluten-sensitive enteropathy in Rag1−/− mice excreted in secalin-challenged mice, consistent with more severe duodenitis/enteritis [2.32 ± 0.07 (SE) vs. 1.93 ± 0.09 g, P = 0.002, Fig. 4C]. In addition, splenocyte secretion of IFN-γ, IL-2, and IL-17 in response to secalin restimulation in vitro was increased in secalin-challenged mice [IFN-γ: cytokine secretion ratio 0.34 ± 0.11 (SE) vs. 0.03 ± 0.01, P < 0.01, Fig. 4D; IL-2: 0.137 ± 0.067 vs. 0.024 ± 0.005, P < 0.05, Fig. 4E; IL-17: 0.054 ± 0.012 vs. 0.019 ± 0.011, P < 0.05, Fig. 4F], whereas secretion of the regulatory cytokine IL-10 remained unchanged [0.26 ± 0.03 vs. 0.26 ± 0.05, not significant (NS), Fig. 4G]. These results suggested that inflammatory cytokine secretion by secalin-specific T cells was responsible for the observed exacerbation of duodenitis. Taken together, the findings confirmed that secalin-sensitized CD4+CD62L−CD44high T cells induced gluten-sensitive enteropathy in Rag1−/− mice exposed to oral secalin, similar to the effects of gliadin-sensitized memory T cells in wheat gluten-challenged recipient mice.

Effects of commensal microbes and gluten in the model. The results above demonstrated the detrimental effects of T cell recognition of oral gluten on duodenal integrity in this model. However, the causes of “baseline” duodenitis in control recipients on GFD had not previously been addressed. To investigate further 1) the role of commensal microbes in duodenitis development and 2) the role of gluten in duodenitis exacerbation, two groups of Rag1−/− recipients (n = 10) of CD4+CD62L−CD44high-memory T cells from secalin-immunized donors were treated from week 4.5 posttransfer with gut-sterilizing antibiotic cocktail, an effective treatment of intestinal inflammation in the CD4+CD45RBhigh T cell transfer model (19). Until week 4.5, both groups of mice had been fed a gluten-containing diet and had started to lose weight, indicative of intestinal inflammation (Fig. 5A). One group of mice was also changed to GFD, whereas the other group remained on gluten-containing diet. Interestingly, after an adaptation period of 1 wk, the group now on antibiotics and GFD started regaining weight and continued to do so until the end of the experiment at week 8.5. In direct comparison, the group that remained on gluten-containing diet in addition to treatment with antibiotics showed a significant delay in weight gain (P < 0.05, Fig. 5A). Furthermore, recipients on antibiotics/GFD had no or only mild duodentitis at 8.5 wk, whereas mice on gluten-containing diet suffered from significant exacerbation of duodenitis in spite of the fact that they were cotreated with antibiotics (score of 4.00 median, 2.25–6.88 interquartile range vs. 2.0, 0.0–3.13, P < 0.05; Fig. 5B). These results demonstrated both a contribution of commensal microbes to the development of duodenitis in this model and also a robust treatment effect of oral gluten (secalin), leading to severe duodenitis in recipients of secalin-primed memory T cells even in the absence of commensal microbes. The sterilizing effect of the antibiotic cocktail was confirmed by stool culture (data not shown).

Analysis of customized mouse diets. Our previous results had suggested that sourdough fermentation of germinated rye resulted in extensive hydrolysis of prolamins (17). To confirm these results, we estimated the prolamin content of customized mouse diets, prepared by mixing the gluten-free standard diet with various amounts of native or germinated rye sourdough. With the competitive R5 ELISA method, prolamins in control mouse diets, prepared by mixing the gluten-free standard diet with increasing scores. Examples represent histological scores of J) 0 (normal), 2) 3 (mild), 3) 6 (moderate), and 4) 9 (severe) duodenitis and were taken either from recipients on GFD (a–c), or gluten (d). Note increased villus diameter (hypheps), reduced villus (arrows pointing up)-to-crypt (arrows pointing down) ratios, and appearance of crypt abscesses (→) with increasing scores.
doughs were calculated at 5.6 ± 1.0 and 26.6 ± 3.6 mg/kg, respectively. This suggested >98% destruction of prolamins in germinated rye sourdoughs. Calculated diet gluten levels were very close to 20 mg/kg, i.e., the gluten-free threshold (1, 5), consistent with extensive hydrolysis of prolams during sourdough fermentation of germinated rye.

Serum immune responses to germinated rye sourdough in nude recipients of secalin-primed memory T cells. To test whether hydrolysis of prolams during sourdough fermentation of germinated rye would prevent anti-secalin antibody production in the celiac model, eight groups of nude mice (n = 5–11) were transferred with secalin-primed memory T cells and challenged over 8.5 wk with diets containing 10 or 50 g of native or germinated sourdough/kg diet. Control diets contained 100, 500, or 2,500 mg native secalin/kg, or no gluten. Interestingly, serum anti-secalin IgG (Fig. 7A) and Th1-associated IgG2c (Fig. 7B) titers at the end of the experiment were strongly reduced in mice fed with germinated vs. native rye sourdough. The decrease was significant in the high-dose treatment groups [IgG titer: 1,882 ± 578 (SE) vs. 15,849 ± 2,466, P < 0.001; IgG2c titer: 163 ± 45 vs. 1,552 ± 310, P < 0.001] but was also seen in the low-dose treatment groups (IgG titer: 725 ± 291 vs. 4,175 ± 1,014, NS; IgG2c titer: 83 ± 48 vs. 298 ± 67, NS). In addition, mean titers from mice challenged with 50 g germinated rye sourdough were below the titers from mice challenged with only 100 mg secalin/kg diet, but nonetheless were still detectable (IgG titer: 1,882 ± 578 vs. 2,466 ± 1,042, NS; IgG2c titer: 163 ± 45 vs. 182 ± 29, NS). These results indicated that in vivo B cell activation by dietary secalin was strongly reduced, but not eliminated, after germination and sourdough fermentation of rye.

Inflammatory effects of germinated rye sourdough in Rag1−/− recipients of secalin-primed memory T cells. Finally, to study the intestinal inflammatory effects of residual prolamins in germinated rye sourdough, two groups of Rag1−/− mice (n = 14–15) were transferred with secalin-primed memory T cells and challenged with diets containing 50 g germinated rye sourdough/kg vs. 50 g native rye sourdough/kg (positive control). Interestingly, after 7.5 wk of oral challenge, both groups of mice did not show significant differences in body weight (P = 0.97, Fig. 8A) or small bowel weight [1.80 ± 0.97, Fig. 8B] or T cell cytokine secretion by splenocytes in response to secalin restimulation ex vivo also remained unchanged [IFNγ: cytokine secretion ratio 0.48 ± 0.09 (SE) vs. 0.42 ± 0.08, NS, Fig. 8C; IL-2: 0.13 ± 0.02 vs. 0.26 ± 0.07, NS, Fig. 8D; IL-17: 0.09 ± 0.02 vs. 0.08 ± 0.02, NS, Fig. 8E; IL-10: 0.48 ± 0.21 vs. 0.55 ± 0.12, NS, Fig. 8F]. These results clearly demonstrated that secalin peptides in germinated rye sourdoughs retained T cell stimulatory capacity, resulting in undiminished inflammatory activ-
In summary, the findings in two variants of the CD model in nude or Rag1−/− mice did not support the hypothesis that ingestion of germinated rye sourdough, in its current composition, would be safe for celiac patients.

**DISCUSSION**

In this study, we employed a mouse model of gluten-sensitive enteropathy to test the safety of germinated rye sourdough, a cereal-based processed food product designed to support the GFD of celiac patients. According to current food labeling regulations, processed cereal foods with gluten contents reduced to 20 mg/kg (gluten free; see Refs. 1 and 5) may be considered safe for consumption by celiac patients. A previous study had indicated that, in germinated vs. native rye sourdoughs, prolamin levels were reduced by >99.5% (competitive R5 ELISA; see Ref. 17). This suggested that germinated rye sourdough could be used as an ingredient in gluten-free products (e.g., as a baking improver). However, the clinical safety of germinated rye sourdough as part of the GFD of celiac patients remained to be demonstrated.

We decided to first evaluate the antigenic and inflammatory effects of germinated rye sourdough in mice with gluten-sensitive enteropathy, using a modification of a memory T cell transfer model (6). For this study, CD4+CD62L−CD44high memory T cells were isolated using commercial antibody-coated columns, thus obviating a need for specialized FACS sorting equipment. Competitive R5 ELISA analysis of customized mouse diets, containing 10 g (50 g) of native or germinated rye sourdough/kg diet, suggested a reduction of prolamins by >98% as a result of rye germination, to levels of 5.6 mg (26.6 mg) secalin/kg diet (mean). These levels were very close to the threshold for gluten-free food labeling (20 mg/kg; see Refs. 1 and 5). Correspondingly, mean serum anti-secalin IgG and IgG2c titers in nude mice transferred with secalin-primed memory T cells and challenged with diets containing up to 50 g germinated rye sourdough/kg diet were below the mean serum titers for control mice challenged with only 100 mg secalin/kg diet. The result indicated that B cell activation by dietary secalins was strongly reduced, although not eliminated, in mice fed with germinated rye sourdough.

The result prompted us to compare directly the inflammatory effects of germinated vs. native rye sourdough on the intestine. Rag1−/− recipients were chosen for the analysis because glut...
Fig. 7. Comparison of serum immune responses to germinated vs. native rye sourdough in nude mice transferred with secalin-primed memory T cells. Nude recipients were challenged for 8.5 wk with 2 different doses of germinated (“malted”) vs. native rye sourdough (10 or 50 g/kg diet; means and SE). Serum anti-secalin IgG (A) and CD4\(^+\) T helper 1-associated IgG2c (B) titers were strongly reduced, but not eliminated, in mice fed germinated rye sourdough. Serum titers resulting from oral challenges with control diets (100 mg, 500 mg, or 2.5 g secalin/kg diet), or GFD, are also shown. Significance of results is depicted only for comparisons involving groups challenged with germinated rye sourdough (n = 5–11; *P < 0.05, **P < 0.01, and ***P < 0.001).

In summary, hydrolysis of secalins in germinated rye sourdoughs remained incomplete. Challenge with germinated rye sourdoughs produced secalin-specific antibodies and T cell inflammatory cytokine secretion in mice, effects likely to occur similarly in patients with CD after ingestion of equivalent dietary gluten doses. The results were consistent with the demonstration of residual fragments of secalin, likely antigenic in vivo, after sourdough fermentation of germinated rye (>15–1.4 kDa, SEC-HPLC; see Ref. 17). In conclusion, germinated rye sourdough, in its current composition, could not be considered safe for consumption by celiac patients.

Our results in vivo cautioned against the use of the current immunochemical methods (such as monoclonal antibody-based R5 or \(\omega\)-gliadin ELISAs) to predict the safety of processed food products for celiac patients. None of the current methods to determine partially hydrolyzed gluten levels have been sufficiently evaluated for this purpose (15). An explanation for the discrepancy in this study between the results for immunochemical gluten testing of germinated rye sourdough diet and its undiminished T cell stimulatory and inflammatory effects in vivo could have been an underestimation by the competitive R5 ELISA (21) of partially hydrolyzed gluten levels. Incomplete detection of prolains in peptic-(chymo)tryptic digests has been reported for this method (9).

It remained possible, however, that the sensitivity to gluten of mice in the CD model might have been higher than the sensitivity of celiac patients. Experimental mice consumed gluten-containing diets as the only source of nutrition, resulting in relatively high daily gluten intake. Based on these considerations, levels of prolains in germinated rye sourdough might already have been tolerable to celiac patients, particularly if used as one of several ingredients to a final product.

In this study, gliadin- or secalin-primed CD4\(^+\)CD62L\(^-\)CD44high-memory T cells induced a gluten-sensitive enteropathy in Rag1\(^{-/-}\) mice, similar to results reported previously for gliadin-primed CD4\(^+\)CD45RB\(^{-}\)lowCD25\(^{-}\) memory T cells (6). These two CD4\(^+\) memory T cell populations showed only a partial overlap in CD44 and CD45RB marker expression by FACS (results not shown). Because the majority of regulatory T cells in mouse lymphoid organs coexpressed the markers CD25 and CD62L (14, 25), both memory T cell isolation protocols likely resulted in quantitative depletions of regulatory T cells. This was further suggested by the development of intestinal inflammation, a result of effector T cell dysregulation, in recipients of both different CD4\(^+\) memory T cell populations. Preliminary data indicate that the comparatively high baseline of duodenitis in control recipients on GFD in this study, now shown to be caused mainly by T cell recognition of commensal microbes, may be reduced by selecting lower numbers of T cells for adoptive transfers (e.g., \(3 \times 10^5\) CD4\(^+\)CD62L\(^-\)CD44high T cells/mouse), without compromising the performance of the model.
Feeding germinated rye sourdough to memory T cell recipient mice most likely caused a polyclonal response of secalin-specific T cells. This would appear similar to T cell responses in human CD. A study in children estimated that up to 50 different gluten epitopes can be recognized by T cells from celiac patients (29). Therefore, for predicting tolerability to celiac patients of processed foods, in vivo models based on multiple clonal specificities may be considered superior to in vitro assays based on one single or a small number of monoclonal antibodies. However, both approaches should be evaluated in clinical trials before recommendations regarding food choices for celiac patients can be made.

More recent results have shown that prolamin levels in germinated rye hydrolysates may be further decreased by the addition of prolyl endoprotease from Aspergillus niger (18). The combined use of germinated cereals (17) and protease(s) from Aspergillus species (2, 18) in sourdough fermentation therefore appears particularly promising for gluten-free food applications.

In summary, we have developed a CD animal model that can be applied in food safety testing for celiac patients. The model may also prove useful to determine the in vivo efficacies of treatments aiming at the degradation, neutralization, or exclusion of dietary gluten in CD and other gluten-related disorders. Drug candidates include oral proteases (8, 28), polymeric binders (16), hyperimmune bovine IgG concentrate (12), and tight junction regulators (13).

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Fig. 8. Comparison of inflammatory effects of germinated vs. native rye sourdough in Rag1–/– mice transferred with secalin-primed memory T cells. Two groups of Rag1–/– recipients were challenged with 50 g/kg of germinated vs. native rye sourdough. After 7.5 wk, there were no significant differences for body weight (means and SE; ns) (A), histological duodenitis (medians; ns) (B), or secretion of T cell cytokines IFNγ (C), IL-2 (D), IL-17 (E), or IL-10 (F) by secalin-restimulated splenocytes ex vivo (means and SE, ns). Significant differences within the groups between ovalbumin control and secalin-restimulated samples are shown (*P < 0.05 and **P < 0.01). The results demonstrated that germinated rye sourdough could not be considered safe for consumption by celiac patients.
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DISCLOSURES
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AUTHOR CONTRIBUTIONS

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