Autoantibodies to transglutaminase 2 (TG2) are hallmarks of celiac disease. To address B cell tolerance and autoantibody formation to TG2, we generated immunoglobulin knock-in (Ig KI) mice that express a prototypical celiac patient–derived anti-TG2 B cell receptor equally reactive to human and mouse TG2. We studied B cell development in the presence/absence of autoantigen by crossing the Ig KI mice to Tgm2−/− mice. Autoreactive B cells in Tgm2+/− mice were indistinguishable from their naïve counterparts in Tgm2−/− mice with no signs of clonal deletion, receptor editing, or B cell anergy. The autoreactive B cells appeared ignorant to their antigen, and they produced autoantibodies when provided T cell help. The findings lend credence to a model of celiac disease where gluten-reactive T cells provide help to autoreactive TG2-specific B cells by involvement of gluten–TG2 complexes, and they outline a general mechanism of autoimmune immunity with autoantibodies being produced by ignorant B cells on provision of T cell help.

Introduction
Celiac disease, characterized by highly disease-specific autoantibodies to transglutaminase 2 (TG2; Dieterich et al., 1997), is a common enteropathy caused by exposure to dietary gluten proteins (Sollid, 2002). Plasma cells specific for TG2 are abundant in celiac gut lesions (Di Niro et al., 2012). Subjects with the condition exclusively carry the HLA-DQ allotypes DQ2.5, DQ2.2, or DQ8, and they have gluten-specific CD4+ T cells recognizing deamidated gluten peptides bound to these disease-associated HLA-DQ molecules (Sollid, 2002). The creation of deamidated gluten epitopes is mediated by the same enzyme to which there are autoantibodies, TG2. This dual role of TG2 in celiac disease, both creating immunogenic T cell epitopes and being the target of autoantibodies, is hardly coincidental and is likely central in the disease pathogenesis.

In general, the majority of newly generated B cells have B cell receptors (BCRs) that bind self-antigens, but most of these autoreactive B cells are removed from the repertoires of mature B cells (Wardemann et al., 2003). In the bone marrow, developing autoreactive B cells can become non-self-reactive by receptor editing (Gay et al., 1993; Tiegs et al., 1993), and if receptor editing fails, they can be deleted by apoptosis (Nemazee and Burk, 1989). In the periphery, autoreactive B cells can be kept silent by anergy (Goodnow et al., 1988). What dictates the mode of tolerance is not fully understood, but avidity of the BCR appears central as multimeric or membrane-bound self-antigens typically promote B cell deletion, and monomeric soluble self-antigens typically result in B cell anergy (Goodnow, 1992). In some cases, autoreactive B cells termed “ignorant” can appear in the periphery as normal B cells, presumably because of non-exposure to self-antigen or because the BCR-binding signal is too weak to enforce tolerance induction (Adelstein et al., 1991; Akkaraju et al., 1997; Aplin et al., 2003; Hannum et al., 1996; Huang et al., 2006; Koenig-Marrony et al., 2001).

TG2 is constitutively expressed in most cell types and tissues (Lorand and Graham, 2003). The protein is transcribed on free ribosomes in the cytosol but can be externalized and bind to components of the extracellular matrix. Many tissues exhibit abundant TG2 staining in the extracellular matrix (Korponay-Szabó et al., 2004). For other extracellular matrix proteins, like basement membrane laminin, to which there are antibodies in lupus disease of mice and humans, transgenic BCR mouse models have revealed that there are strong B cell tolerance obtained by central deletion, L chain editing, or anergy (Foster et al., 2006).

To address how anti-TG2 autoantibodies are formed, we generated Ig knock-in (KI) mice based on a celiac patient–derived BCR
with equal reactivity to human and mouse TG2 (mTG2). TG2-specific plasma cells across celiac patients have a strikingly restricted usage of VH and VL genes with a particular dominance of the IGHV5-1/IGKV1-5 pair and with a low frequency of somatic mutations (Di Niro et al., 2012; Hnidia et al., 2016; Iversen et al., 2013; Roy et al., 2017). The chosen BCR is prototypic for celiac disease as it expresses IGHV5-1 in germline configuration and IGKV1-5 with two somatic mutations. By crossing the Ig KI mice to Tgm2-deficient mice, we were able to study the development and function of autoreactive B cells in the presence and in the absence of the autoantigen. We demonstrate that clonal deletion and anergy to TG2 do not develop. Instead, self-tolerance to the TG2 autoantigen appears to be regulated outside the B cell compartment and is maintained by the absence of T cell help. We also show that autoreactive anti-TG2 B cells are able to produce autoantibodies when linked to cell help is provided, suggesting that autoreactivity to TG2 in celiac disease is controlled at the T cell level.

Results

Generation of an Ig KI mouse model to study class switch–competent anti-TG2 B cells

The Ig KI mouse was based on a prototypic anti-TG2 mAb 679–14-E06 (hereafter termed 14E06) derived from a single gut plasma cell from a celiac patient (Di Niro et al., 2012). The mAb is specific for the major epitope I of TG2, and it binds with equal, nanomolar affinity to the human and mouse orthologue TG2 proteins (Table S1; Di Niro et al., 2012; Hnidia et al., 2016; Iversen et al., 2013). The H chain KI mouse was generated with the 14E06-rearranged VDJ sequence introduced into a targeting et al., 2013). The H chain KI mouse was generated with the promotor of mouse double KI mice and not the single H chain or single L chain KI crossed to obtain 14E06 double KI mice. Only B cells of the mouse double KI mice and that both the H and L chains of 14E06, as also conserved on PE-conjugated streptavidin (Fig. 1 C). These data observed by H and L chain shuffling (Chen et al., 2015), conserved on PE-conjugated streptavidin (Fig. 1 C). These data on 14E06 KI mice to Tgm2-deficient mice (De Laurenzi and Melino, 2001). We found that the frequency of TG2-binding B cells in peripheral lymphoid organs was the same for 14E06 KI mice on Tgm2+/+ and Tgm2−/− genetic backgrounds (Fig. 2 A). Moreover, no differences in spleen cellularity and the total number of B cells were found between 14E06 KI mice being Tgm2+/+ or Tgm2−/− (Fig. S1, A and B). To assess if there would be spontaneous differentiation of Ig KI B cells into antibody-secreting cells, we measured basal serum total and TG2-specific IgM, IgG, and IgA levels in Tgm2+/+ and Tgm2−/− 14E06 KI mice. Both IgM and IgG anti-TG2 antibodies, but not IgA, were detected; however, titers were not increased in the presence of the self-antigen compared with control mice on Tgm2−/− background (Fig. S1 C).

Immature, mature follicular, and marginal zone B cells (as defined by CD21/CD35 and CD23 staining) in Tgm2−/− mice were present at similar proportions to those observed in Tgm2+/+ mice (Fig. 2, B and C). Peritoneal cavity lavage showed that TG2-specific B cells exclusively belonged to the B-2 cell lineage (Fig. S1 D). In the bone marrow, we detected a reduced frequency of pro/pre-B cells and a corresponding increase in immature and mature B cells compared with WT mice (Fig. 2, D and E), indicating that, as seen in other Ig transgenic mouse models (Goodnow, 1992), 14E06 KI mice had accelerated development of B cells as a result of the prearranged transgenic BCR. We next looked at the fraction of peripheral B cells expressing λ L chains as a sign of receptor editing. As the transgenic L chain is κ, these λ L chains must have been the result of expression of the endogenous λ locus. We found no increase in the percentage of λ-positive cells in TG2-expressing 14E06 KI mice (Fig. 2 F). Taken together, our data demonstrate a normal development of 14E06 B cells without evidence for receptor editing or developmental arrest at an immature stage.

Classical features of anergy are not observed in autoreactive 14E06 KI B cells

We next assessed whether the TG2-specific B cells emerging from the bone marrow could be anergic. B cell anergy is characterized by down-regulation of surface IgM levels, impaired signaling through the BCR (shown by reduced Ca2+ influx, protein phosphorylation, and induction of activation markers), and resistance to LPS-mediated activation (Cambier et al., 2007). We analyzed these features in 14E06 KI B cells from Tgm2−/− vs. Tgm2−/− mice comparing autoreactive and naive cells. We noted a very modest down-regulation of surface IgM levels on autoreactive cells (Fig. 3 A). Besides this minor difference in surface IgM, we found that autoreactive and naive cells were phenotypically indistinguishable with no increased expression of activation markers CD69, CD80, and CD86 on autoreactive KI B cells (Fig. 3 B). To determine whether autoreactive B cells were able to up-regulate activation markers in response to BCR ligation, we cultured isolated splenic B cells for 18 h in the presence of anti-IgM or TG2 antigen (Fig. 3 C). We observed that autoreactive KI B cells from Tgm2−/− mice up-regulated activation markers CD86 (Fig. 3 C) and CD80 (data not shown) to the same extent as naive KI B cells. We next compared autoreactive and naive TG2-specific KI B cells for their ability to mobilize Ca2+. Both stimulation with anti-IgM and with TG2 antigen induced a clear Ca2+ flux response in freshly isolated autoreactive B cells from Tgm2−/− KI mice, and importantly, the magnitude of this

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response was comparable to that seen for resting naive B cells isolated from both WT C57Bl/6 mice and 14E06 KI Tgm2−/− mice (Fig. 3 D). We also observed that anti-TG2 B cells isolated from the spleens of TG2-expressing 14E06 KI mice had slightly elevated basal Ca2+ levels when compared with resting naive B cells (Fig. 3 D). We next investigated the ability of autoreactive B cells to proliferate in response to antigen in the presence of T cell mimicking stimuli (anti-CD40 and IL-4), as well as the T cell–independent stimulus LPS. Both stimuli induced efficient proliferation of both autoreactive and naive TG2-specific B cells (Fig. 3 E).

Anergic B cells have been shown to compete poorly with nonanergic B cells, resulting in exclusion from B cell follicles and a shortened life span (Cyster et al., 1994). To determine if these features were applicable to autoreactive TG2-specific B cells in our KI model, we created mixed bone marrow chimeras by mixing the bone marrow of TG2-expressing 14E06 KI mice 1:1 with that of CD45.1 WT mice followed by transfer to lethally irradiated C57Bl/6 recipient mice. As a control, we used lethally irradiated Tgm2−/− mice reconstituted with mixed bone marrow from Tgm2−/− 14E06 Ig KI mice and CD45.1 Tgm2−/− mice. The composition of the peripheral B cell pool in chimeric mice was consituted with an equal ratio of donor cells was substantially skewed in favor of CD45.1+ (WT) cells in both Tgm2+/+ and Tgm2−/− chimeras, possibly due to competition with clones in the nontransgenic B cell population (Fig. S2 A). Anti-14E06+ B cells comprised 27.3 ± 8.2% of splenic B cells and were not reduced compared with those in Tgm2−/− chimeras (Fig. 4 A). As the frequency of antigen-specific B cells inversely correlates with the signal strength each B cell receives through its BCR (Cook et al., 1997), even lower frequencies of autoreactive B cells could be required in order to reach the threshold required to induce B cell tolerance in our model. We therefore also generated TG2-expressing chimeras reconstituted with 5% 14E06 Ig KI and 95% CD45.1 (WT) bone marrow, resulting in 4.6 ± 2.3% anti-14E06+ B cells in the spleen (Fig. 4 A). We did not observe differences in the proportions of immature, mature follicular, and marginal zone B cells in the spleens between low-frequency Tgm2+/+ chimeras, high-frequency Tgm2+/+ chimeras, and Tgm2−/− control chimeras (Fig. S2 B). To analyze the turnover of the autoreactive B cells among an excess of competitor B cells, we compared incorporation of BrdU.
after 8 d of continuous BrdU feeding. We found that TG2-specific B cells and WT CD45.1 B cells displayed comparable percentages of BrdU+ cells in both Tgm2+/+ and Tgm2−/− 14E06 KI mice, and this was not dependent on the frequency of autoreactive TG2-specific B cells (Fig. 4 B). In keeping with this finding, analysis of the anatomical localization of autoreactive TG2-specific B cells in mixed bone marrow chimeric mice revealed that TG2-specific B cells essentially migrated and distributed equivalently to naive control B cells in the presence of self-antigen (Fig. 4 C). We next analyzed whether TG2-specific B cells in mixed bone marrow chimeric mice were functional by investigating their ability to mobilize Ca2+. CD45.2 B cells, the majority of which were TG2-specific, demonstrated a clear Ca2+ flux upon stimulation with anti-IgM or TG2, whereas CD45.1 B cells in the same animal were, as expected, only responsive to anti-IgM stimulation (Fig. 4 D). These analyses demonstrate that 14E06 KI B cells are not anergic in the presence of the TG2 autoantigen.

**BCR occupancy of 14E06 TG2-specific B cells**

Increase in basal intracellular Ca2+ and phosphoprotein levels have been observed in models of B cell anergy (Benschop et al., 2001; Healy et al., 1997), and these findings were interpreted to reflect BCR occupancy by antigen resulting in continuous BCR signaling (Gauld et al., 2005). To assess anti-TG2 BCR occupancy in our model, we used B cells from Ig KI mice stained with a mouse TG2-reactive, non-epitope-specific (i.e., non-14E06-competing) mAb (679–14-D04; Iversen et al., 2013) to detect...
BCR-bound TG2. First, we analyzed BCR-bound TG2 on splenic TG2-specific B cells after the lysis of erythrocytes. We found that the majority of freshly isolated peripheral TG2-specific B cells in TG2-expressing 14E06 KI mice had bound TG2, whereas no BCR-bound TG2 could be detected in either WT (data not shown) or Ig KI mice on a Tgm2−/− background (Fig. 5A). As TG2 is abundantly present intracellularly in many different cell types, including erythrocytes, we wanted to control for the possible release of TG2 during erythrocyte lysis and cell death related to tissue disruption during preparation of single cell suspensions. When erythrocytes and dead cells were removed using Lympholyte M, we detected a much lower degree of receptor occupancy came from in vivo encountered TG2 or from release of TG2 during erythrocyte lysis and cell death related to the majority of freshly isolated peripheral TG2-specific B cells in TG2-expressing 14E06 KI mice. We found that also splenic B cells originating from Tgm2−/− 14E06 KI mice had BCR-bound TG2 (Fig. 5A). The same was true when lymph nodes were processed together (data not shown). Thus, TG2 thus appears to be easily released during tissue disruption and cell processing, which hinders efforts to determine whether the BCR is engaged by antigen in vivo.

Tyrosine phosphorylation is rapidly induced upon BCR ligation. We therefore next analyzed tyrosine phosphorylation in 14E06 KI B cells among Tgm2+/+, Tgm2−/−, or mixed splenocytes that were immediately fixed, followed by flow cytometric analysis. When processed separately, anti-TG2 B cells from Tgm2+/+ 14E06 KI mice demonstrated high basal tyrosine phosphorylation that was not observed in B cells from Tgm2−/− 14E06 KI mice (Fig. 5D). However, when the tissue was mixed and processed together, high tyrosine phosphorylation was also observed in TG2-specific B cells from Tgm2−/− 14E06 KI mice (Fig. 5D), indicating that ex vivo exposure to antigen rather than in vivo BCR signaling accounts for the increase in phosphoproteins. We conclude from these studies that there is minimal BCR occupancy of 14E06 KI B cells in vivo in Tgm2−/− mice.

Transcriptome analysis of autoreactive 14E06 KI B cells
To further characterize autoreactive TG2-specific B cells, we compared their transcriptome to that of their naive counterparts under resting conditions and upon BCR stimulation with TG2. We isolated total resting B cells from spleens of Tgm2-deficient or Tgm2-expressing 14E06 KI mice by negative selection using anti-CD43 magnetic beads, resulting in a B cell purity of >95%. The analysis was done on all splenic B cells, the majority of which were TG2-specific (85.6 ± 7.4%). Of note, based on the findings reported above, this procedure would likely expose B cells to TG2 antigen briefly during the ex vivo cell processing. Principal component analysis showed distinct clustering of unstimulated vs. antigen-stimulated samples, and notably the unstimulated B cells from Tgm2−/− vs. Tgm2−/− mice clustered
identify marginal zone macrophages. Right: Spleen sections were stained for CD3 (magenta) to identify the T cell zone in combination with anti-14E06 (green) and B220 (blue). Representative images of two mice (top and bottom panels) out of five mice analyzed are shown. Scale bars, 100 µm. Top panels: 10× objective; bottom panels: 20× objective. (D) Splenic B cells were isolated from Tgm2−/+ (blue), Tgm2−/+ HF (red), and Tgm2−/+ LF (green) chimeric mice, loaded with the intracellular calcium indicator Indo-1 and stained with FITC-conjugated anti-CD45.1. Baseline Ca2+ levels were acquired for 30 s before the cells were stimulated with 2 µg/ml TG2. CD45.1+ cells (WT, dotted lines) and CD45.1-negative cells (comprising mostly 14E06 KI B cells, continuous lines) were gated out and plotted for Ca2+ flux analysis. One representative plot out of three independent measures is shown.

closely together, as did the stimulated cells (Fig. 6 A). A total number of 120 genes were differentially expressed between samples from Tgm2−/− and Tgm2−/+ 14E06 KI mice (false discovery rate <0.05 and fold-change >0.5 in either direction; Fig. 6 B and Tables S2 and S3). These few genes pale in comparison to the 4,895 genes (out of a total of 8,305 genes) differentially expressed between stimulated vs. unstimulated B cells in Tgm2−/− mice. In unstimulated B cells, 73 genes were differentially expressed in Tgm2−/− vs. Tgm2−/+ mice. Of these 73 genes, 41 were also differentially expressed between stimulated B cells of Tgm2−/− vs. Tgm2−/+ mice; hence, these might include genes that have altered transcription as a direct result of Tgm2 deficiency (Fig. 6 B). Only 32 genes had uniquely altered expression among unstimulated B cells in Tgm2−/− vs. Tgm2−/+ mice. Early growth response 1 (Egr1) was on the top of the list of these 32 genes. Egr1 up-regulation in B cells is indicative of BCR ligation with antigen (McMahon and Monroe, 1996), and Egr1 is one of few genes identified as up-regulated in anergic hen egg lysozyme (HEL)–specific B cells compared with antigen-naive HEL–specific B cells, albeit we did not observe differential expression of other genes (Nab2, Nrgn) seen in the HEL model of anergy (Glynn et al., 2000; Merrell et al., 2006). While this is noteworthy, we cannot exclude the possibility that the signals of Egr1 and the other 31 genes with differential expression are the results of the short artifactual exposure to autoantigen during cell preparation in vitro. Antibody staining and flow cytometry did not show differences in expression of EGR1 protein (data not shown). Overall, the transcriptome analysis of autoreactive 14E06 KI B cells matured in the presence or absence of TG2 suggests minimal impact of this autoantigen.

**Anti-TG2 14E06 KI B cells respond to T cell help**

Having established that TG2-reactive B cells readily mature with no induction of tolerance, we next examined whether these B cells can isotype-switch and produce antibodies in vivo. First, we addressed whether autoreactive TG2–specific B cells would respond to endogenous T cell help. We produced in Escherichia coli a recombinant fusion protein consisting of TG2 and the 2W1S peptide (TG2-2W1S), which is highly immunogenic in C57Bl/6 mice as it stimulates alloreactive CD4+ T cells (Moon et al., 2007). We adoptively transferred autoreactive TG2–specific B cells to WT C57Bl/6 recipients that were...
unprimed or previously primed with the 2W1S peptide, and that were subsequently immunized with TG2 or TG2-2W1S. While mice immunized with TG2 did not develop an IgG anti-TG2 antibody response, immunization with TG2-2W1S elicited activation of TG2-specific B cells and the production of class-switched anti-TG2 IgG antibodies (Fig. 7, A and B). The anti-TG2 IgG titer was higher in mice that were primed with 2W1S peptide compared with mice that were not previously primed (Fig. 7, A and B).

For celiac disease, a model was outlined where TG2-specific B cells with involvement of hapten-carrier like TG2-gluten complexes can receive help from gluten-specific T cells (Mäki, 1992; Sollid et al., 1997). To address whether 14E06 KI B cells could cooperate with gluten-specific T cells in vivo, we generated a gluten-specific TCR transgenic mouse strain that recognizes the DQ2.5-glia-α2 epitope and introduced human HLA-DQ2.5 into the mice by breeding to comply with MHC restriction. Proper TCR expression (Fig. S3 A) and proliferative response to cognate gluten peptide antigen were confirmed in these TCR transgenic mice (Fig. S3 B). We next isolated naive CD4+ T cells from HLA-DQ2.5 × TCR-glia-α2 double transgenic mice and naive B cells from HLA-DQ2.5 transgenic 14E06 KI mice and adoptively transferred the T and B cells into nonirradiated HLA-DQ2.5 transgenic recipient mice. The recipient mice were administered a recombinant fusion protein of TG2 with the deamidated immunodominant gluten 33mer peptide without adjuvant on the day after the adoptive cell transfer. Importantly, the anti-TG2 antibodies were formed with a clear effect of gluten-specific T cells (Fig. 7, C and D). Using a similar fusion protein produced in insect cells, the antibody production after adoptive cell transfers was weak with a very modest effect of gluten-specific T cells (Fig. S3 C). This might possibly indicate that endotoxin contamination in the E. coli-produced protein induced the expression of costimulatory molecules on TG2-specific B cells, making them better equipped to interact with naïve gluten-specific T cells. Future studies will need to address this issue. Taken together, these experiments reveal that 14E06 KI B cells can get activated when provided with T cell help.

Figure 5. Receptor occupancy of 14E06 anti-TG2 B cells. (A–C) Splenocytes from Tgm2+/+ 14E06 KI and HLA-DQ2.5 Tgm2−/− 14E06 KI mice were prepared and stained for anti-B220, anti-HLA-DQ2.5, and anti-TG2 (14D04). Red blood cells were removed either by ACK lysis or using Lympholyte media. (A) Representative plots gated on B220+ splenocytes showing the percentage of B cells with surface-bound TG2. (B) Pooled data of at least 14 Tgm2+/+ 14E06 KI mice per group showing the percentage of B cells with surface-bound TG2 after red blood cell lysis (ACK lysis) or removal of red blood cells using Lympholyte media. *, P < 0.0001, as determined by Student’s t test. (C) Analysis of mean fluorescent intensity (MFI) of surface anti-TG2 staining on splenic B220+ B cells isolated from Tgm2+/+ 14E06 KI mice. Paired analysis showing representative data from n = 3 mice in one experiment. Data show mean ± SD. *, P < 0.01, as determined by Student’s t test. (D) Flow cytometric detection of phosphorylated proteins in immediately fixed splenocytes isolated from Tgm2+/+ 14E06 KI (red) and HLA-DQ2.5 Tgm2−/− 14E06 KI mice (blue) that were processed separately or together. Data are representative of two (D) or three (A and C) independent experiments.
Discussion

Exploring an Ig KI mouse model system, we report that there is absence of B cell tolerance for TG2 autoreactive B cells, and that such autoreactive B cells can produce autoantibodies when provided with T cell help. The observations support a model of celiac disease autoimmunity where gluten-specific T cells by involvement of TG2–gluten complexes provide help to TG2-specific B cells. This finding of B cell clonal ignorance to TG2 antigen is surprising as transgenic B cell tolerance models of other extracellular matrix antigens have generally reported lack of anergy.

The enzyme is ubiquitously expressed in many organs (Lorand and Graham, 2003), including the thymus (Szondy et al., 1997). The transamidating/deamidating activity of TG2 is Ca²⁺-dependent, and hence TG2 is likely to serve an extracellular function. Staining for TG2 in frozen tissue sections gives a characteristic distribution pattern that reflects the binding of TG2 to fibronectin (Upchurch et al., 1987) and an as yet unidentiﬁed extracellular matrix component, TG2 interaction partner (Stamnaes et al., 2016). The ﬁndings of the current study call into question the asserted abundance of extracellular TG2 under normal physiological conditions, particularly in the bone marrow. Importantly, the abundant extracellular distribution of TG2 observed in frozen sections may upon tissue sectioning result from release of intracellular TG2 binding to adjacent extracellular matrix components, and as such represent an in vitro artifact (Stamnaes et al., 2016).

Tolerance induction in the T cell compartment is known to be much more efﬁcient than tolerance induction in the B cell compartment (Adelstein et al., 1991; Chiller et al., 1971), and it is thus widely assumed that a lack of cognate T cell help prevents B cell autoimmunity. In line with this notion, there is no substantial evidence for autoreactive TG2-speciﬁc T cell responses (Jabri and Sollid, 2017) resonating with the abundant thymic expression of TG2. However, although unlikely a target for T cell autoimmunity, TG2 does efﬁciently target gluten peptides and generate covalent TG2–gluten complexes in vitro (Fleckenstein et al., 2004). These TG2–gluten complexes can conceivably act as hapten carrier–like complexes to allow TG2-specific B cells to receive help from gluten-reactive T cells (Sollid et al., 1997). This model neatly explains the clinical observations that TG2 autoantibodies are only seen in subjects who consume gluten (Sulkanen et al., 1998) and who express HLA-DQ2/DQ8 (Björck et al., 2010). Using a TG2–gluten fusion protein, we demonstrate that gluten-speciﬁc T cells can indeed provide help to ignorant TG2-speciﬁc B cells in vivo. Whether and under what conditions TG2–gluten complexes are formed in situ remain to be resolved.
Lack of B cell tolerance and presence of ignorant B cells that will become producers of autoantibodies once T cell help is provided are mechanisms of autoimmunity not only applying to celiac disease but also relevant to other autoimmune diseases whose autoantigens are shielded from B cells or present at very low concentrations. Our findings resemble the observations obtained for the nuclear protein La, where no evidence for deletion or anergy for high-affinity transgenic La-specific B cells was obtained (Aplin et al., 2003). In this model, ignorant La-specific B cells were stimulated to produce autoantibodies when cognate T cell help was provided (Keech et al., 2001).

The conceptual model of generating autoantibodies by means of coupling a B cell autoantigen with foreign T cell antigens is not unique to celiac disease. It has been shown that B cells producing anti-nuclear antibodies can receive help from T cells recognizing epitopes of a viral antigen that complexes with the self-nuclear antigen (Dong et al., 1994), and moreover, that BCR recognizing a self-membrane protein can capture this from membranes of virus-infected cells and simultaneously cocapture viral proteins that become recognized by virus-specific CD4+ T cells (Sanderson et al., 2017). Fundamentally, the conceptual model is reliant on weak or absent B cell tolerance, which we indeed demonstrate for TG2. Thus, in essence the results presented here support the concept where anti-TG2 autoimmunity develops when nontolerized TG2-specific B cells receive help from foreign antigen-specific T cells.

**Materials and methods**

**Generation of 14E06 VH VL KI and glia-α2 TCR transgenic mice**

The 14E06 antibody genes were originally amplified from a single TG2-reactive plasma cell from a celiac lesion (Di Niro et al., 2012). 14E06 Ig H and Ig L KI mice were prepared by Ozgene following an established protocol (Taki et al., 1993). To make the Ig H chain targeting construct, the B1-8 promotor with leader sequence (kindly provided by K. Rajewsky, Max Delbrück Center for Molecular Medicine, Berlin, Germany) and rearranged 14E06 VDJH were cloned downstream of a flippase recognition target (FRT)–flanked phosphoglycerate kinase I neomycin resistance cassette (Fig. 1 A). For the generation of the TCR-glia-α2 transgenic mice, cDNA sequences representing V(D)J regions of rearranged TCRα and TCRβ chains of the T cell clone 364.1.0.14 (Qiao et al., 2011), specific for the DQ2.5-glia-α2 epitope, were synthesized (GenScript) and cloned into a pair of cassette expression vectors...
containing the murine TCR constant sequences as well as the natural mouse TCR promoter/enhancer elements as described (kindly provided by D. Mathis, Harvard Medical School, Boston, MA; Kouskoff et al., 1995). Transgenic mice were generated at the Norwegian Transgenic Center by microinjecting the constructs into one of the pronuclei of one-cell-stage embryos of C57Bl/6 mice.

**Mice**

Hemizygous 14E06 Ig KI mice on a C57Bl/6 background were generated by crossing VDJH14E06 and VJγ14E06 single KI mice. VDJH14E06 and VJγ14E06 single KI mice were crossed to Tgm2+/− mice (De Laurenzi and Melino, 2001) and crossed to generate hemizygous Tgm2+/− 14E06 Ig KI mice. HLA-DQ2.5 transgenic mice (d’u Pre et al., 2011) were backcrossed into C57Bl/6 for at least 10 generations and thereafter crossed to both Tgm2+/− and Tgm2−/− 14E06 Ig KI mice. TCR-glia−1γ1 transgenic mice (d’u Pre et al., 2011) specific for the DQ2.5-glia−1γ epitope were backcrossed into C57Bl/6 for at least 10 generations. Mice were bred at the Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet (Oslo, Norway) under specific pathogen-free conditions. CD45.1 mice (B6.SJL-Ptprc Pep-c/BoyCrl) were purchased from Charles River Laboratories, crossed to Tgm2−/− mice, and bred in house. C57Bl/6 mice were purchased from Janvier Laboratories. All experiments were approved by the Norwegian Food Safety Authority.

**Antigen production**

Recombinant TG2 protein was expressed in *E. coli* or in SF2-express insect cells. For insect cell expression, mouse Tgm2 or human Tgm2 was cloned into the pACAB vector, and protein was expressed for 3 d followed by cell lysis and purification by nickel-nitritolactric acid affinity chromatography as previously described (Stamnaes et al., 2016). For *E. coli* expression, mouse Tgm2 was cloned into the pET28a (+) vector (Novagen) and expressed as previously described (Iversen et al., 2013). Protein was purified by nickel-nitritolactric acid affinity chromatography and if required also by gel filtration chromatography using a Superdex 200 10/300 column (GE Healthcare). For TG2 T cell epitope fusion protein production in *E. coli*, the 2W1S peptide (EAWGALANAVDSDA) or the deamidated 33mer (LQLQPFQPELPQPELPQPELPQPFQPF) was cloned into the pET28a vector to the C-terminal end of the mouse Tgm2 gene spaced by a three glycine residue linker. For insect cell production, deamidated 33mer was cloned to the C-terminal end of the mouse Tgm2 gene in pACAB3. Protein purity was assessed by SDS-PAGE and Coomassie Blue staining. Protein antigen used for in vivo experiments was buffer exchanged by spin filter centrifugation to sterile PBS before use. In the 2W1S experiment, *E. coli* produced TG2 and TG2-2W1S were further purified by size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) using PBS as running buffer. To generate biotinylated antigen, TG2 was biotinylated using BirA ligase (Avidity) according to the manufacturer’s instructions. For surface plasmon resonance analysis, insect cell–produced mouse and human TG2 with a C-terminal biotin-BirA site were used.

**Surface plasmon resonance analysis**

Fab fragment production of 14E06 in HEK293 cells has been described elsewhere (Hnida et al., 2016). Binding kinetics of Fab 679-14-E06 for human and mouse TG2 was assessed by surface plasmon resonance analysis (BIAcore T200 instrument; GE Healthcare) using antigen with a controlled open or closed conformation. To immobilize TG2 in an open conformation, biotinylated, insect cell–produced TG2 was incubated with the irreversible active site inhibitor DP3-3 (Ac-P[DON]-LPF-NH2; Zedira) at room temperature for 15 min before addition of 5 mM CaCl2 followed by incubation on ice (TG2-DP3-3). To generate TG2 in a closed conformation (TG2-DGP), biotinylated, insect cell–produced TG2 was preincubated with GDP to a final concentration of 1 mM. NeutrAvidin-coated sensor chips were generated by coupling 10 µg/ml NeutrAvidin (Thermo Fisher Scientific) in 10 mM sodium acetate, pH 4.5, to CMS sensor chips to reach 500 response units. Unreactive moieties were blocked with 1 M ethanolamine. Biotinylated TG2-DGP or TG2-DP3-3 was injected at 16°C at a flow rate of 10 µl/min to reach ~200–800 resonance units. Kinetic measurements were done by injection of serial dilutions of 14E06 Fab fragment at a flow rate of 30 µl/min for 60 s at 16°C. Binding data were automatically zero-adjusted and reference cell binding subtracted by the BIAcores T200 software. Kinetic rate constants were estimated using a Langmuir 1:1 binding model provided by the software.

**Flow cytometry**

Single-cell suspensions from spleens and lymph nodes were prepared by carefully grinding and filtering tissues through a 70 µm diameter nylon mesh (BD Biosciences). Bone marrow was flushed from the femur/tibia. Blood was collected using heparin. For removal of erythrocytes, cell suspensions were treated with ammonium-chloride-potassium (ACK) lysis buffer or subjected to Lympholyte M gradient (Cedarlane). Peritoneal cells were harvested by lavage with 5 ml PBS. Cells were resuspended in PBS containing 2% FCS (vol/vol) and incubated with optimal amounts of antibodies against the following molecules: B220 (RA3-6B2; BioLegend), CD19 (6D5; BioLegend), CD21/CD35 (7G6; BD), CD23 (R3B4; eBioscience), CD3 (145-2C11; BioLegend), CD45.1 (A20; BioLegend), CD45.2 (104; BioLegend), CD5 (53–7.3; BioLegend), CD69 (H1.2F3; BioLegend), CD80 (16-10A1; BioLegend), CD86 (B7-2; eBioscience), Igλ1/λ2/λ3 (R26–46; BD), IgD (11-26c.2a; BD), IgM (II/41; eBioscience), and Vι6.7 (OT145; En- dogen). Unconjugated hIgG1 anti-14E06 and anti-TG2 (679-14-D4) were produced in our own laboratory as previously described (Di Niro et al., 2012; Haydahl et al., 2016) and detected with phycoerythrin-conjugated anti-hIgG1 (4E3; Acris Antibodies). TG2 tetramers were generated by incubating 0.4 µM of biotinylated mTG2 with 0.1 µM of phycoerythrin- or allophycocyanin-conjugated streptavidin (Thermo Fisher Scientific). Detection of BrdU-labeled cells was done using the BrdU Flow Kit (BD Biosciences). EGRI staining was performed on fixed and permeabilized cells using the Fixation/Permeabilization kit (BD Biosciences), using rabbit monoclonal antibody T.126.1 (Thermo Fisher Scientific) followed by goat-anti-rabbit IgG-alexa647 (Invitrogen). For detection of phosphoryrosine, total splenocytes were immediately fixed by incubating in RPMI.
containing 2% paraformaldehyde for 15 min at room temperature. Cells were briefly incubated with H₂O and then permeabilized with ice-cold methanol and incubated for 30 min on ice. Cells were washed twice with PBS and incubated with anti-phosphotyrosine (PY20; BioLegend) for 30 min, combined with surface antibodies against B220 and HLA-DOQ.5 (2.12.E11; Viken et al., 1995). Cells were analyzed on a FACS Calibur, Fortessa (BD Biosciences), or Attune NxT Flow Cytometer (Thermo Fisher Scientific), and data analysis was done using FlowJo software (Tree Star).

Isolation of T and B cells, and cell culture
Splenect B cells were purified using the Dynabeads Mouse CD43 kit (Invitrogen). The purity of B cells, measured by the percentage of B220+ cells, was typically >96%. For measurement of activation markers, cells were cultured overnight with LPS (from E. coli O111:B4; Sigma-Aldrich), mTG2 or F(ab′)2 goat-anti-mouse IgM (Jackson ImmunoResearch) at a density of 1.25 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% (vol/vol) FCS, penicillin, streptomycin, and 10 µM β-mercaptoethanol at 37°C. For cell proliferation assays, cells were labeled with CFSE (Molecular Probes) and cultured in RPMI as above with additional supplements 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 mM Hepes (all from Sigma-Aldrich) for 72 h with the stimuli indicated above as well as anti-CD40 (5 µg/ml; BD) and rmIL-4 (50 ng/ml; R&D Systems). For 3T4-thymidine incorporation assay, 5 × 10⁶ splenocytes from HLA-DOQ.5 × TCR-glia-γ1 transgenic mice and HLA-DOQ.5 × TCR-glia-α2 transgenic mice were cultured in the presence of 5 µg/ml plate-bound anti-CD3 and 2 µg/ml anti-CD28 (both eBioscience) or with 10 µM synthetic gluten peptides (γ-gliadin, YQQLPQPEQPQQSFPEQERPF; α-gliadin, LQLQFPFPQPELPYPQEPYLDPQEPYQPQPF; GenScript) for 72 h. Proliferation was assessed by incorporation of 3H-thymidine. CD4+ T cells were isolated from pooled lymph nodes and spleens from gliadin TCR transgenic mice by negative selection using the EasySep Mouse CD4+ T cell isolation kit (StemCell Technologies). The purity of CD4+ T cells was typically >85%.

Intracellular Ca²⁺ mobilization
B cells were loaded for 60 min at 37°C with Indo-1 acetoxyethyl (final concentration, 1 µM; Molecular Probes) in RPMI containing 10% FCS. Baseline measurements were acquired for 30 s, and then cells were stimulated with 10 µg/ml F(ab′)2 goat-anti-mouse IgM (Jackson ImmunoResearch) or 2 µg/ml mTG2 for 2.5 min. Mean relative [Ca²⁺]i was monitored over time using an LSR II flow cytometer (BD) with analysis using FlowJo software (Tree Star).

Adoptive transfer and immunization
For the 2W1S experiment, C57Bl/6 recipients were immunized i.p. with 50 µg 2W1S peptide (EAWGALANWAVDSA) in CFA (Sigma-Aldrich) 6 d before adoptive transfer of 10⁶ TG2-binding B cells from Tgm2+/+ 14E06 KI mice in 200 µl PBS by intravenous injection. 24 h after transfer, recipient mice were i.p. injected with 100 µg mTG2 or 100 µg mTG2-2W1S in IFA (Sigma-Aldrich). For the gluten-specific T cell experiment, 10⁶ TG2-binding B cells from Tgm2+/+ HLA-DOQ2 transgenic 14E06 KI mice and 10⁶ CD4+ T cells from HLA-DOQ2 × gliadin-α2 TCR transgenic mice were adoptively transferred to HLA-DOQ2 tg recipient mice. The next day, recipient mice received 100 µg mTG2 or 100 µg mTG2-33merEEE in PBS by i.p. injection.

Mixed bone marrow chimera
C57Bl/6 and Tgm2−/− recipient mice were lethally irradiated (1000 Rad) and reconstituted by i.v. injection with equal mixtures of 5 × 10⁶ WT CD45.1 bone marrow cells and 5 × 10⁶ Tgm2−/− 14E06 IgK1 bone marrow cells, or 5 × 10⁶ CD45.1 Tgm2−/− and 5 × 10⁶ Tgm2−/− 14E06 IgK1 bone marrow cells. Bone marrow chimeric mice with low frequencies of autoreactive B cells were generated by mixing 0.5 × 10⁶ Tgm2−/− 14E06 IgK1 bone marrow cells with 9.5 × 10⁶ WT CD45.1 bone marrow cells. Mice received 2 g/liter neomycin sulfate (Thermo Fisher Scientific) and 1 g/liter glucose in drinking water until day 14 after irradiation. 6 wk after bone marrow transfer, mice were i.p. injected with 1 mg BrdU (BD PharMingen) and given 1 mg/ml BrdU (Sigma-Aldrich) in their drinking water. 1 g/liter glucose was added to overcome taste aversion.

Immunohistochemistry
Spleens from bone marrow chimera mice were embedded in optimal cutting temperature compound and snap frozen in liquid nitrogen. 4-µm cryosections were briefly fixed in 4% neutral buffered formalin and stained with anti-14E06, anti-CD169/SIGLEC (clone 3D6.112; BioRad), and anti-IgM (donkey-anti-mouse-IgM-A488; Jackson ImmunoResearch) followed by donkey-anti-rat-IgG-Cy3, donkey-anti-human-IgG-biotin (both Jackson ImmunoResearch), and streptavidin-APC (ProZyme), or anti-14E06, anti-B220-biotin (clone RA3-6B2; BioLegend), and anti-CD3-PE (clone 145-2C11; BioLegend) followed by donkey-anti-human-IgG-A488 and streptavidin-aminomethylcoumarin acetate (both Jackson ImmunoResearch). Slides were mounted with ProLong Diamond Antifade (Thermo Fisher Scientific). Images were acquired on an inverted fluorescence microscope (Nikon Eclipse Ti-S; Nikon) with a Nikon 9 10/0.3 or 9 20/0.45 Plan Fluor lens (Nikon) and NIS ELEMENTS BR3.2 software (Nikon). Single channel images were merged in FIJI/ImageJ (Schiindelin et al., 2012; Schneider et al., 2012). Linear adjustment of brightness and contrast was done equally across images. Anti-14E06 staining acquired in the blue channel was pseudocolored as green and anti-IgM staining acquired in the green channel was pseudocolored as blue for visualization purposes.

RNA sequencing (RNA-seq) analysis
Total RNA was isolated from B cells with an RNeasy minikit (Qiagen). RNA integrity was analyzed on an Agilent Bioanalyzer using an Agilent RNA 6000 Pico kit. RNA integrity was 9.1 ± 0.7. RNA-seq libraries were generated from purified ribosomal RNA–depleted RNA samples using the strand-specific TruSeq stranded mRNA library prep kit (Illumina), and sequenced at the Norwegian Sequencing Centre using the Illumina HiSeq 3/4000 with a read length of 150 bp and paired end reads. Salmon version 0.7.2 (Patro et al., 2017) was used for the quantification of transcript counts. In quasi-mapping mode, a variational
Bayesian expectation-maximization algorithm was used for optimizing abundance estimates. With the default k-mer length of 31, the quasi-mapping index was built on the transcriptome of Ensembl genome build GRCh38 patch release 5 that includes alternative loci. Transcript-level counts were aggregated to gene level (including those on alternative loci). Models that correct sequence-specific and fragment-level biases were applied while quantifying the transcript counts.

To identify and retain the absolutely expressed genes for downstream analyses, finite normal mixture models implemented in the Mclust package (Scrucca et al., 2016) were used as suggested (Hebenstreit et al., 2011). Briefly, a model-based clustering of the log-transformed transcripts per million values of all protein-coding genes in each sample was performed to categorize each gene as either “expressed” or “not expressed.” This exercise has been performed independently for each group of mouse samples. Further, to be categorized as “expressed” in a sub-group, we imposed another criterion that the gene has to be detected in all the samples within that group. DESeq2 version 1.20 (Love et al., 2014) was used for differential expression analysis. All the samples were processed in a single batch, ruling out any batch-induced variation. Genes were categorized as differentially expressed at a false discovery rate of 10%.

**ELISA**

Serum was collected from recipient mice serially bled through the saphenous vein by incubating blood for 20 min at room temperature followed by centrifugation for 15 min at 2,000 g, 4°C. Supernatant was collected and stored at −20°C until assayed. Total IgM, IgG, and IgA serum antibodies were measured using the ELISA Ready-SET-Go! kits (eBioscience) according to the manufacturer’s instructions. Sera were diluted 1:10,000. For detection of TG2-specific antibodies, wells were coated with 5 µg/ml mTG2 in PBS overnight at 4°C. After washing and blocking, serial dilutions of serum were incubated for 2.5 h, washed, and detected with alkaline-phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich), or biotinylated mouse anti-mouse IgM (BD) or goat anti-mouse IgA (Southern Biotech) followed by alkaline-phosphatase-conjugated streptavidin (Southern Biotech). OD was determined at 405 nm. Monoclonal mouse IgM, IgG2c, and IgA 14E06 antibodies were purified from 14E06 B cell hybridomas (see below) using HiTrap protein L columns (BD) and used as standard curves.

**Generation of B cell hybridomas**

Single-cell suspensions were prepared from spleens, lymph nodes, Peyer’s patches, and bone marrow of Tgm2+/+ and Tgm2−/− 14E06 KI mice and fused with murine plasmacytoma cells (OURI cells, an α2t transgenic mouse; L.M. Sollid conceptualized and project 275053), the South-Eastern Norway Regional Health Authority (projects 2010048, 2011050, and 2016113), and Stiftelsen KG Jebsen (project SKGJ-MED-017).

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Author contributions: M.F. du Pré designed and performed experiments, interpreted data, and wrote the manuscript; J. Blazevski and A.E. Dewan designed and performed experiments and interpreted data; J. Stamnaes designed the recombinant TG2 fusion proteins, performed immunohistochemistry, and edited the manuscript; C. Kanduri and G.K. Sandve analyzed RNA-seq data; M.K. Johannesen prepared critical reagents and knowledge and Ig-gene plasmids during the process of generating the Ig KI mice, Diane Mathis for sharing TCR-expression plasmids, Klaus Rajewsky for sharing Ig-gene plasmid, Rasmus Iversen for advice, and Mark Shlomchik for helpful discussions.

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Author contributions: M.F. du Pré designed and performed experiments, interpreted data, and wrote the manuscript; J. Blazevski and A.E. Dewan designed and performed experiments and interpreted data; J. Stamnaes designed the recombinant TG2 fusion proteins, performed immunohistochemistry, and edited the manuscript; C. Kanduri and G.K. Sandve analyzed RNA-seq data; M.K. Johannesen prepared critical reagents and expression vectors; C.B. Lindstad made 14E06 B cell hybridomas; K. Hnida performed surface plasmon resonance analysis; J. Stamnaes designed TCR-glia-a2 transgenic mice; S.W. Qiao designed TCR-glia-a2 transgenic mice; L.M. Solli conceptualized and...
supervised the study, interpreted data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

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