Accumulation of HLA-DR4 in Colonic Epithelial Cells Causes Severe Colitis in Homozygous HLA-DR4 Transgenic Mice

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Background: Homozygous HLA-DR4/I-E^d transgenic mice (tgm) spontaneously developed colitis similar to human ulcerative colitis. We explored whether endoplasmic reticulum stress in colonic epithelial cells due to overexpression of HLA-DR4/I-E^d was involved in the pathogenesis of colitis.

Methods: Major histocompatibility complex class II transactivator-knockout (*CIITAKO*) background tgm were established to test the involvement of HLA-DR4/I-E^d expression in the pathogenesis of colitis. Histological and cellular analyses were performed and the effect of oral administration of the molecular chaperone tauroursodeoxycholic acid (TUDCA) and antibiotics were investigated. IgA content of feces and serum and presence of IgA-coated fecal bacteria were also investigated.

Results: Aberrantly accumulated HLA-DR4/I-E^d molecules in colonic epithelial cells were observed only in the colitic homozygous tgm, which was accompanied by upregulation of the endoplasmic reticulum stress marker Binding immunoglobulin protein (BiP) and reduced mucus. Homozygous tgm with *CIITAKO*, and thus absent of HLA-DR4/I-E^d expression, did not develop colitis. Oral administration of TUDCA to homozygotes reduced HLA-DR4/I-E^d and BiP expression in colonic epithelial cells and restored the barrier function of the intestinal tract. The IgA content of feces and serum, and numbers of IgA-coated fecal bacteria were higher in the colitic tgm, and antibiotic administration suppressed the expression of HLA-DR4/I-E^d and colitis.

Conclusions: The pathogenesis of the colitis observed in the homozygous tgm was likely due to endoplasmic reticulum stress, resulting in goblet cell damage and compromised mucus production in the colonic epithelial cells in which HLA-DR4/I-E^d molecules were heavily accumulated. Commensal bacteria seemed to be involved in the accumulation of HLA-DR4/I-E^d, leading to development of the colitis.

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Key Words: HLA-DR, transgenic mice, ulcerative colitis, inflammatory bowel disease, endoplasmic reticulum stress

Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract and comprises inflammatory bowel disease (IBD) together with Crohn's disease.[1] Although both genetic and environmental factors are believed to play an important role in the development of UC, its pathogenesis is largely unknown. One possible mechanism for the onset of UC is the excessive immune response to gut commensal organisms,[2] when luminal bacteria infiltrate the lamina propria due to defects in the intestinal barrier function. In this scenario, apoptosis of the intestinal epithelial cells takes place, which leads to reduced production of mucus and antimicrobial proteins.[3] Recent experimental studies suggest that goblet cells are particularly sensitive to endoplasmic reticulum (ER) stress.[4] Once goblet cells fail to properly manage the accumulation of unfolded proteins, they will be subjected to ER stress-dependent apoptosis,[5] leading to decreased mucus secretion and reduced barrier function of the intestinal epithelium. Indeed, Heazlewood et al reported that in some UC patients, the precursor of the Muc2 protein, a major component of mucus, accumulates in goblet cells with concomitant upregulation of the ER stress marker binding immunoglobulin protein (BiP).[4]

Human leukocyte antigen (HLA)-DR is one of the class II human major histocompatibility complex (MHC) antigens. It is a glycosylated heterodimeric membrane protein comprised of almost monomorphic α and polymorphic β chains. They are

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constitutively expressed on B cells, myeloid antigen-presenting cells such as dendritic cells and macrophages, and thymic epithelial cells. In other cell types such as intestinal epithelial cells, expression of MHC class II is inducible by IFNγ under inflammatory conditions.[6] The primary role of MHC class II molecules is to present antigenic peptides to $CD4^+$ T cells. The apical α1 and β1 domains of the α and β chains of an MHC class II molecule constitute a peptide-binding groove in which the antigenic peptides are accommodated. Through T cell receptors, $CD4^+$ T cells recognize the complex of peptides and α1β1 domains of MHC class II molecules. On the other hand, the coreceptor CD4 binds to the membrane-proximal β2 domain of the MHC class II molecule.[7]

HLA-DR4 (*HLA-DRA*01:01/HLA-DRB1*04:05*) is a frequently observed HLA-DR molecule found in 26% of the Japanese population.[8] To identify the peptides potentially presented by HLA-DR4, we established C57BL/6-background HLA-DR4 transgenic mice (tgm). The tgm were designed to express HLA-DR4/I-E^d-chimeric molecules in which the α1 and β1 domains were the corresponding domains of HLA-DR4, and the remaining domains were the corresponding domains of the MHC class II I-E^d molecule, a murine homolog of HLA-DR under the control of the I-E^d promoter.[9] This chimeric construct ensured proper cell-specific expression, conferred HLA-DR4 restriction on mouse $CD4^+$ T cells,[9] and might avoid possible problems induced by the interspecies interaction between mouse CD4 and human HLA-DR4[10,11]. Unexpectedly, mice with homozygous transgenic alleles spontaneously developed severe colitis similar to human UC without any further genetic modification, adoptive cell transfer, bacterial infection or chemical treatment previously reported mouse models of experimental colitis[12,13].

The aim of this study was to clarify the mechanisms of the pathogenesis of the colitis developed in the homozygous HLA-DR4/I-E^d tgm. Here we provide evidence that aberrant accumulation of HLA-DR/I-E^d molecules inside colonic epithelial cells and abnormality of goblet cells due to ER stress resulted in the drastic reduction of mucus production in the colons of homozygous tgm showing UC-like colitis.

MATERIALS AND METHODS

Mice

Two lines of HLA-DR/I-E^d transgenic mice were established[9] and maintained under specific pathogen-free conditions in the Institute of Resource Development and Analysis at Kumamoto University and were handled in accordance with the animal care guidelines of Kumamoto University. This study was approved by the animal research committee of Kumamoto University (Permission Number: B25-115 and A27-066). MHC class II knockout mice (B6.129S2-*$H2^{dlAb1\text{-}Ea}$/J*) and MHC class II transactivator (CIITA) knockout mice (B6.129S2-*$Ciita^{tm1Ccum}$/J*) were purchased from Jackson Laboratory (Bar Harbor, ME).

An antibiotic mixture of 0.5 g/L ampicillin, 0.25 g/L vancomycin, 0.5 g/L metronidazole from Wako Chemicals Co. Ltd., and 0.5 g/L fradiomycin sulfate (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) in drinking water was provided ad libitum. Two grams of tauroursodeoxycholic acid (TUDCA; Tokyo Kasei Kogyo) suspended in water was neutralized by 0.1 N NaOH and adjusted to a volume of 1 L. The solution was provided through the prenatal and postnatal period of about 3 months.

Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were prepared by hemolysis of a few drops of blood collected from the tail vein. Colonic lamina propria T cells were prepared using the Lamina Propria Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, followed by density gradient centrifugation on Percoll and the $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec). The purity of the $CD4^+$ cells was >75% as observed by flow cytometry. Colonic epithelial cells were prepared as follows: Large intestines from heterozygous and homozygous tgm were excised and placed in 5% FCS/HBSS (-). The colons were cut open longitudinally, washed, and cut into small pieces (2–3 mm). The tissues were incubated in HBSS (-) containing 5% FCS, 1 mM DTT and 0.5 mM EDTA at 37°C for 20 minutes. After washing with the same buffer, the tissues were digested with 2.4 U/mL Dispase II (Roche Diagnostics, Mannheim, Germany) in 5% FCS in DMEM at 37°C for 100 minutes with gentle rotation. EDTA was added to a final concentration of 5 mM and the digested tissues were further incubated at 37°C for 15 minutes. The detached cells were recovered and purified by density gradient centrifugation on Percoll. The purity of the cells was >93% as measured by flow cytometry using FITC-anti-EpCAM antibody (eBioscience, San Diego, CA).

Fecal Bacteria Preparation

About 0.2 grams of feces were ground between frosted microscope slides in 10 mL PBS in a 10-cm culture dish. The bacterial suspension was transferred to a 50-mL tube and centrifuged at 150 *g* for 1 minute. The supernatant was recovered and this centrifugation was repeated 4 more times to remove debris. The supernatant was then centrifuged at 3000 *g* for 5 minutes and the bacterial pellet was suspended in 10 mL PBS after removal of the supernatant. This washing step was repeated and the final bacterial pellet was resuspended in 10 mL PBS. A small aliquot was subjected to flow cytometry analysis and the remainder was autoclaved and used for the stimulation of immune responses in T cells.

Flow Cytometry

Cells were stained with phycoerythrin (PE)-conjugated or FITC-conjugated mAbs as described below and analyzed by FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software. PE-conjugated anti-HLA-DR-α-chain mAb L243 was from BD Biosciences, FITC-conjugated anti-HLA-DR-β-chain mAb TAL15.1 was from Santa Cruz Biotechnology (Dallas, TX), PE-conjugated antimouse MHC-II mAb, FITC-conjugated antimouse B220 mAb and antimouse EpCAM mAb were from eBioscience and PE-conjugated antimouse IgA was purchased from Southern Biotech (Birmingham, AL).

Mapping of Transgene Insertion Site

As reported previously, one line of the HLA-DR4/I-E^d tgm carried the transgenes on the distal region of chromosome 3 as revealed by FISH analysis.[9] To map the exact insertion site of the transgenes, paired-end whole genome sequencing, which provides sequence reads from both ends of each DNA fragment,[14] was performed by Takara Biotechnology Co. Ltd. (Shiga, Japan). The genomic DNA (5 μg) prepared from a tgm with colitis was fragmented to about ~3 kb and sequenced at about 20 to 60 Gb by the Illumina HiSeq sequencer. The genomic position close to the transgene insertion site was predicted by the reads of one end of the read-pairs of which the other end was mapped to the sequence of the transgenes. The exact insertion site was finally identified by PCR amplification using primers corresponding to 30 base-long genomic DNA sequences of both 5′- and 3′- regions close to the predicted insertion site by the paired-end sequencing analysis, and 30 base-long DNA corresponding to the $E^d\beta$ sequence in the transgene DNA (see Table 1, Supplemental Digital Content 1, http://links.lww.com/IBD/B647).

Western Blot

An equal number of colonic epithelial cells (2×10^5) was lysed with sample buffer for SDS-PAGE, and proteins were separated on a 10% polyacrylamide gel. Western blot analysis was performed as reported previously[15] using anti-BiP mAb (Cell Signaling Technology, Danvers, MA), anti-HLA-DR mAb (SPM289, Abcam, Cambridge, United Kingdom), anti-β-actin mAb (Sigma Aldrich, St. Louis, MO), and HRP-conjugated second antibody.

ELISA

Feces were suspended in PBS with Complete Protease Inhibitor cocktail (Roche), and the fecal suspension and serum were diluted with saline to 10,000-fold and 1000-fold, respectively. IgA levels in the dilutions were quantified by ELISA using a kit provided by Bethyl Laboratories (Montgomery, TX) according to the manufacturer's instructions.

Immune Responses of T Cells

Immune responses of lamina propria $CD4^+$ T cells to the fecal antigens were investigated by the enzyme-linked immunospot assay as described previously.[16] An autoclaved fecal bacterial suspension diluted as indicated and bone marrow-derived dendritic cells prepared from heterozygous tgm with *I-*$A^{b-/-}$ background were used for stimulation of T cells. Purified anti-HLA-DR mAb (L243, Biolegend, San Diego, CA) or isotype-matched control Ig (2 μg/well) were added to the culture media.

Histological Analyses

Gastrointestinal tract was fixed in 10% formalin in PBS and embedded in paraffin. The slices were stained with hematoxylin and eosin or with Alcian Blue/Periodic Acid Schiff. The blue area and total colonic area in the Alcian Blue-stained colonic sections were quantified using a microscope system of BX-X700 and BZ analyzer software (Keyence, Osaka, Japan) according to the manufacturer's instruction. To detect HLA-DR, BiP (Grp78) and EpCAM, anti-HLA-DR antibody (SPM289), anti-Grp78 BiP antibody and anti-EpCAM antibody from Abcam were used, respectively. In some experiments, frozen sections were stained with anti-HLA-DR antibody (L243) or with Alcian Blue/Periodic Acid Schiff.

In Vivo Intestinal Permeability Assay

Mice were kept without food and water for 4 hours and then FITC-dextran (MW = 4,000, Sigma-Aldrich) was administered by gavage at a concentration of 40 mg/mL in PBS at 400 μL (16 mg) per mouse (800 mg/kg).[17] Four hours later, blood was collected and allowed to clot overnight at room temperature. After centrifugation at 3000 g for 20 minutes, the serum was collected and analyzed on a plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

RESULTS

Mice with Homozygous Transgenic Alleles Spontaneously Developed Colitis

HLA-DR4/I-E^d-transgenic mice (tgm) were generated as described previously, and 2 lines of tgm from independent founder mice were established.[9] One carried the transgenes on the Y chromosome and the other on chromosome 3 as revealed by FISH analyses. Each of the offspring tgm bred with wild-type C57BL/6 mice was normal. Unexpectedly, crossbreeding of the mice with transgenes on chromosome 3 gave rise to smaller mice with body weight decreasing over time compared to their normal littermates (Fig. 1A, B). Within 3 to 4 months, many of the smaller mice developed diarrhea and rectal prolapse and most of them died within 6 months (Fig. 1B, see Fig. 4, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). The colons, but not small intestines, of the diseased mice were thick (Fig. 1C, D, see Fig. 5, Supplemental Digital Content 1, http://links.lww.com/IBD/B647) and histological analyses revealed that many mononuclear cells were infiltrating the lamina propria of the colons of diseased mice (Fig. 1E) compared to those of the healthy littermates (Fig. 1F). Crypt abscesses were observed only in the colons of the diseased mice (Fig. 1E). Collectively, the pathology observed in the diseased tgm was similar to human UC.

Mapping of the Transgene Insertion Site

We speculated that the diseased mice carried homozygous transgenic alleles. To specify the genotype of the tgm, we performed paired-end sequencing using next-generation sequencing technology and PCR analysis to identify the location of the transgenes on chromosome 3. The most probable hits obtained by paired-end sequencing were chr3: 155,683,363-155,686,542 according to GRCm38.p3 C57BL/6J and transgene beta: 3605-6899 (on 5′-junction)[9] and chr3: 155,725,576-155,728,654 and transgene beta: 3405-6672 (on 3′-junction). Therefore, we

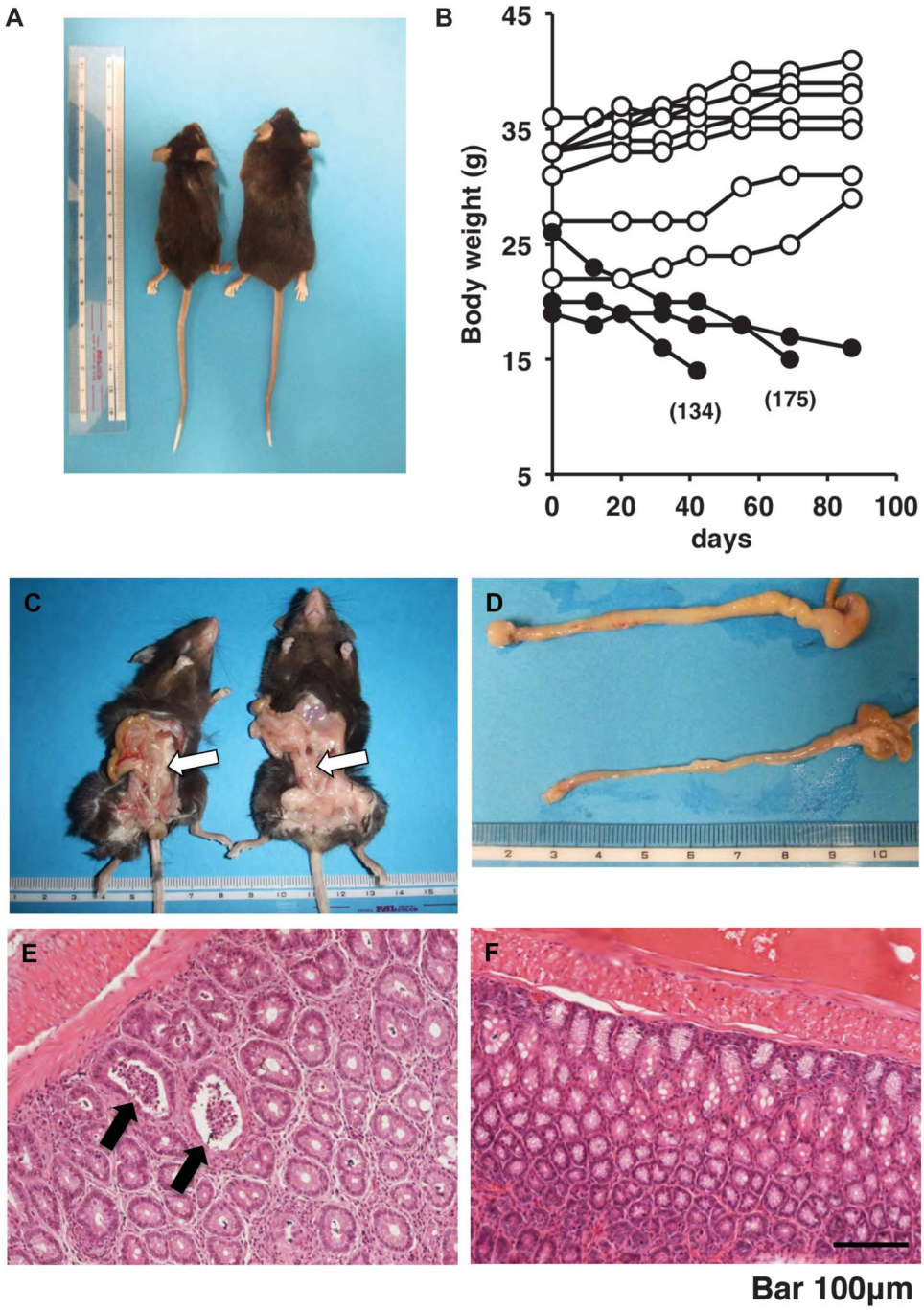

FIGURE 1. HLA-DR4/I-E^d tgm with smaller bodies developed colitis. A, Crossbreeding of tgm with transgenes on chromosome 3 gave rise to smaller mice (left) compared with their normal size littermate (right). B, Body weight change of 3- to 5-months-old tgm. Three mice (filled circles) lost body weight over time and all of them showed rectal prolapse, while others (7, open circles) gained body weight. Two out of 3 smaller mice died during the experiment (survived days shown in parenthesis). The smaller tgm had thicker colons (C, left arrow and D, upper) compared with the colon of normal littermates (C, right arrow and D, lower). A colonic section of a 4-months-old smaller tgm (E) and that of its normal littermate tgm (F). Although the crypts are tightly aligned in the normal tgm (F), the colon of the smaller tgm had many infiltrating mononuclear cells in the lamina propria (E) and crypt abscesses (arrows in E). Bar = 100 μm.

constructed 2 forward and 2 reverse primers corresponding to the transgene beta, and 2 forward and 2 reverse primers corresponding to the upstream of the 5′-junction and to the downstream of the 3′-junction, respectively (see Table 1, Supplemental Digital Content 1, http://links.lww.com/IBD/B647), and performed PCR using various primer combinations and genomic DNA from 4 diseased mice

as templates. Two combinations of the primers successfully amplified the DNA (see Fig. 1A–D, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). The insertion site of the transgenes was determined to be between bp 156,023,577 and 156,062,472 of chromosome 3 by DNA sequencing (see Fig. 1C and D, Supplemental Digital Content 1, http://links.lww.com/IBD/B647), which was in accordance with the result of our previous FISH analysis.[9] Note that the gap between the 5′- and 3′-junctions of chromosome 3 was 38,895 bp (39 kb), indicating that this region was missing possibly due to the insertion of the transgenes.

According to the junction information between the genomic and transgene sequences, we constructed 2 sets of PCR primers: one common forward primer and 2 reverse primers corresponding to the genomic DNA sequences just before and just after the 5'-junctions, respectively; and 2 forward primers corresponding to the genomic DNA sequences just before and just after the 3'-junction, and 1 common reverse primer (see Fig. 2A and B, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). Using the primer sets, we could identify the homozygous HLA-DR4/I-E^d-tgm because the 39 kb region is missing in the homozygotes, and the PCR using primers specific to the 39 kb segment did not give rise to amplified DNA (see Fig. 2C, Supplemental Digital Content 1, http://links.lww.com/IBD/B647).

At 6 months of age, 21 male and 10 female heterozygotes were all alive with an average body weight of 35.2 and 25.5 g, respectively, while 7 out of 12 male and 5 out of 8 female homozygotes were dead. The remaining male and female mice had an average body weight of 17.2 and 14.0 g, respectively (Fig. 2). Among the 12 male and 8 female homozygotes, 9 and 7, respectively, showed rectal prolapse. Thus, the homozygous HLA-DR4/I-E^d tgm had smaller bodies, less body weight and thicker colons (see Fig. 5, Supplemental Digital Content 1, http://links.lww.com/IBD/B647) due to the development of colitis irrespective of their gender.

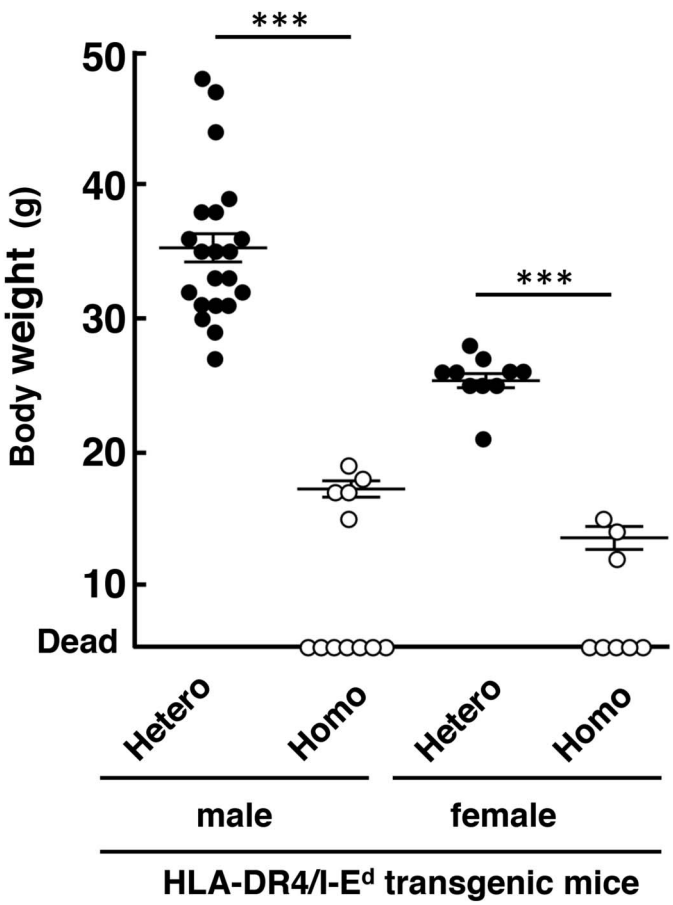

FIGURE 2. Homozygous HLA-DR4/I-E^d tgm showed decreased body weight. At 6 months of age, 21 male and 10 female heterozygotes (hetero) were all alive and had an average body weight of 35.2 and 25.5 g, respectively (filled circles). Seven out of 12 male and 5 out of 8 female homozygotes (homo) were dead, and live male and female mice had an average body weight of 17.2 and 14.0 g, respectively (open circles). ***$P < 0.001$. Indicated are mean ± SEM.

Inhibition of HLA-DR4/I-E^d Transgene Expression Prevented the Colitis

To investigate the possibility that high expression of HLA-DR4/I-E^d molecules may cause the colitis, we established MHC CIITA-deficient (*CIITA*$^{-/-}$) and sufficient (*CIITA*$^+$) homozygous HLA-DR4/I-E^d tgm and *CIITA*$^{-/-}$ heterozygous HLA-DR4/I-E^d tgm (see Fig. 3, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). The *CIITA*$^{-/-}$ homozygous tgm expressed neither HLA-DR4/I-E^d nor I-A^b (see Fig. 3A, H and I, Supplemental Digital Content 1, http://links.lww.com/IBD/B647), while *CIITA*$^+$ homozygous HLA-DR4/I-E^d tgm expressed both molecules (see Fig. 3B, Supplemental Digital Content 1, http://links.lww.com/IBD/B647), although the transgenes were detected by PCR using transgene-specific primers[9] in both *CIITA*$^{-/-}$ and *CIITA*$^+$ homozygous HLA-DR4/I-E^d tgm (see Fig. 3C, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). The *CIITA*$^{-/-}$ homozygous tgm should retain the same chromosome 3 structure as the normal background-homozygous tgm, however, they did not show any symptoms of colitis (see Fig. 3D and E, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). Histological analysis revealed that colons of *CIITA*$^{-/-}$ homozygous tgm were normal with only a few infiltrating cells in hematoxylin and eosin staining, lack of anti-HLA-DR antibody staining as expected and abundant mucus production, which were similar to those of the *CIITA*$^{-/-}$ heterozygous littermate tgm (see Fig. 3F–K, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). Histological scores[18] were 0 to 1 (no signs of inflammation - very low level of leukocytic infiltration) for 3/3 *CIITA*$^{-/-}$ homozygous and 2/2 *CIITA*$^{-/-}$ heterozygous tgm.

The endogenous MHC class II deficient (*I-A*$^{b-/-}$)-background HLA-DR4/I-E^d homozygous tgm showed rectal prolapse with similar time of manifestation, but slightly longer survival time than normal background-homozygous tgm (see Fig. 4, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). These data suggest that expression of endogenous MHC class II (I-A^b) may be involved, but does not play a critical role in the cause of the colitis. Therefore, the expression of HLA-DR4/I-

E^d molecules seems to be essential for the spontaneous development of colitis in the homozygous tgm.

High HLA-DR4/I-E^d Expression in Colitic Epithelial Cells

To examine the HLA-DR4/I-E^d expression in the colons of both homozygous and heterozygous HLA-DR4/I-E^d tgm, the colonic epithelial cells prepared from homozygous and heterozygous tgm were subjected to flow-cytometric analyses using fluorescently labeled anti-HLA-DR and anti-EpCAM antibodies. While colonic epithelial cells from heterozygotes scarcely expressed HLA-DR4/I-E^d, those from homozygotes expressed high levels of HLA-DR4/I-E^d on their surface (Fig. 3A, B). Deparaffinized 2-μm thick sections of the colonic specimens were stained with anti-HLA-DR antibody. Compared with heterozygous littermates, the epithelial cells of the colon isolated from a homozygote showed intense anti-HLA-DR staining, shrunken and fewer goblet cells, and many infiltrating mononuclear cells in the lamina propria (Figs. 3C, D, see Figs. 6 and 7, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). Flow-cytometric analysis revealed that relative population sizes of both $CD4^+$ cells and $Gr\text{-}1^+/CD11b^+$ cells were increased in the colons of homozygotes as compared with those of the heterozygotes, suggesting that $CD4^+$ T cells and neutrophils mainly accumulated in the colons of colitic mice (see Fig. 6, Supplemental Digital Content 1, http://links.lww.com/IBD/B647).

These observations suggested that the HLA-DR4/I-E^d molecules might stimulate lamina propria $CD4^+$ T cells to produce proinflammatory cytokines such as IFNγ and IL-17. Contrary to our expectations, the colonic epithelial cells did not stimulate Th1 cells and Th17 cells prepared from the lamina propria of the diseased mice at all, even in the presence of autoclaved and diluted feces as assayed by enzyme-linked immunospot assay (see Fig. 8A and B, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). These data suggest

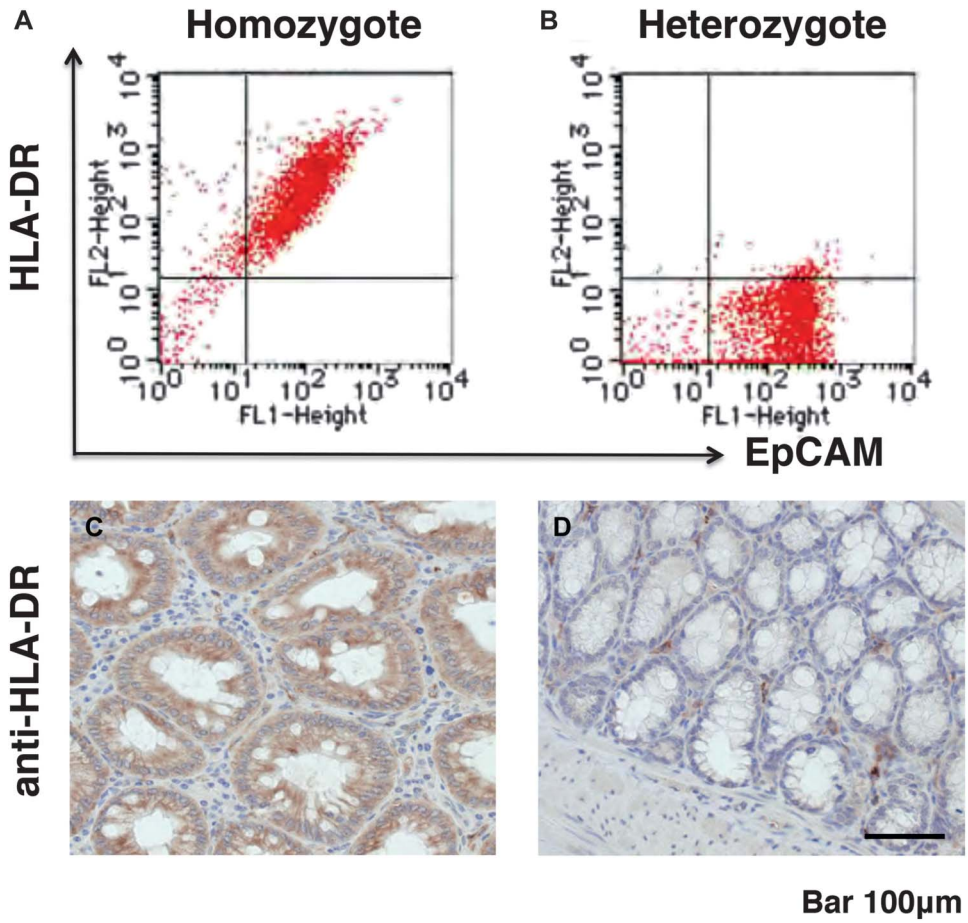

FIGURE 3. Accumulation of HLA-DR4/I-E^d in the colonic epithelial cells of colitic homozygous tgm. The colonic epithelial cells isolated from diseased homozygous tgm showed high expression of HLA-DR4/I-E^d (A), while those isolated from the heterozygous tgm hardly expressed the molecules (B). The colonic epithelial cells of homozygous tgm (C) showed intense staining of anti-HLA-DR in the cytoplasm, and distortion and a reduced number of normal goblet cells as compared with those of heterozygous tgm (D). Bar = 100 μm.

that the overexpressed HLA-DR4/I-E^d molecules on the colonic epithelial cells did not play a major role in direct stimulation of the lamina propria CD4$^+$ T cells and raised the possibility of playing another pathogenic role.

Reduced Mucus Production and Upregulation of BiP in Colitic Epithelial Cells

It was reported that mutated Muc2 precursor protein accumulates in the goblet cells of the *Winnie* and *Eeyore* mice, resulting in reduced numbers and mucus production of these cells due to ER stress.[4] By analogy, the HLA-DR4/I-E^d molecules would accumulate in the colonic epithelial cells of the homozygotes since the anti-HLA-DR staining was not restricted to the plasma membrane of the colonic epithelial cells, but was also observed inside of the cells (Fig. 3C), even though the HLA-DR4/I-E^d molecules were supposed to be present only on the cell surface. This observation raised the possibility that overexpressed and accumulated HLA-DR4/I-E^d molecules cause ER stress in colonic epithelial cells.

To examine mucus production in the colonic epithelial cells of homozygous HLA-DR4/I-E^d tgm, the serial sections used for anti-HLA-DR staining were subjected to Alcian Blue staining (Fig. 4A, B). As shown in Figure 4A, mucus production in the colonic epithelial cells of the HLA-DR4/I-E^d homozygous tgm was drastically reduced as compared with that of heterozygous tgm (Fig. 4B). These observations suggested that the expression of HLA-DR4/I-E^d molecules in the homozygous tgm might induce ER stress in the colonic epithelial cells, resulting in the dysfunction of these cells and reduced mucus production.

The histological analysis using an antibody to the ER stress marker BiP (Grp78) showed apparent BiP staining in the colonic epithelial cells of homozygotes as compared with that of the heterozygotes (Fig. 4C, D and see Fig. 7, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). The colonic epithelial cells prepared from heterozygous and homozygous HLA-DR4/I-E^d tgm were subjected to western blot analysis. As shown in Figure 4E, only colonic epithelial cells isolated from diseased homozygous tgm showed a drastic amount of anti-HLA-DR positive protein and upregulation of BiP.

Administration of the Molecular Chaperone TUDCA Ameliorated the Colitis

TUDCA is a small molecule that functions as a chemical chaperone, facilitating protein folding and reducing ER stress both in vitro and in vivo by stabilizing protein-folding intermediates and preventing protein aggregation.[19] We examined whether the defects observed in the colonic epithelial cells of the homozygous tgm could be ameliorated by administration of TUDCA solution (2 g/L) ad libitum or not. As shown in Figure 5A, accumulation of HLA-DR4/I-E^d and BiP was reduced in the colonic epithelial cells from homozygous tgm that received TUDCA compared to control mice. The mucus production was also restored in the colonic epithelial cells of the homozygotes that received TUDCA, as demonstrated by the restored blue area of the colonic sections stained by Alcian Blue (Fig. 5B).

The FITC-dextran-administrated homozygous tgm that had received only water showed significantly higher serum fluorescence compared with heterozygous tgm (Fig. 5C). On the other hand, the FITC-dextran-administrated homozygous tgm that had received TUDCA solution showed reduced serum fluorescence as compared with homozygous tgm that had received water, suggesting that TUDCA restored the compromised barrier function of the colonic epithelial cells caused by ER stress. Taken together, in the colitic homozygous tgm, the accumulation of HLA-DR4/I-E^d molecules might trigger ER stress in the colonic epithelial cells, resulting in the dysfunction of those cells and reduced mucus production.

Higher Secretory IgA Production in Colitic HLA-DR/I-E^d Homozygous tgm

The decrease of mucus production by the epithelial cells of homozygous tgm might result in reduced barrier function of the epithelium and cause bacterial translocation into the lamina propria, which could cause inflammation leading to the observed spontaneous colitis. To examine the antimicrobial responses of the colitic HLA-DR4/I-E^d homozygous tgm, secretory IgA content of feces and serum were measured (Fig. 6A, 6B). The concentration of IgA in the feces of homozygous tgm was higher than that of heterozygous tgm (Fig. 6A). Similarly, the serum IgA concentration in homozygous tgm was significantly higher in comparison with heterozygous tgm (Fig. 6B).

The bacteria prepared from feces of homozygous tgm were coated with IgA much more than those from heterozygous tgm (Fig. 6C), suggesting that the homozygous tgm exhibited an antimicrobial immune response much stronger than that of the noncolitic mice. Lamina propria CD4$^+$ T cells from homozygous tgm responded to fecal bacterial antigens which were partially inhibited by treatment with an anti-HLA-DR antibody (Fig. 6D). Notably, the CD4$^+$ T cells responded to the bacterial antigens isolated from both homozygotes and heterozygotes, suggesting that the bacterial antigens were not specific to the diseased mice.

Antibiotics Administration Prevented Development of the Colitis

If invading bacteria were critically involved in the development of colitis, administration of antibiotics may show some effect on the onset of colitis. About 2–months-old homozygous tgm (before manifestation of colitis) were provided with an antibiotic mixture in the drinking water. After 74 days from the start of antibiotics administration, the homozygous tgm given with the antibiotic mixture had significantly higher body weights than the homozygous tgm not given with antibiotics (Fig. 7A). At the age of 8 months, the homozygous tgm given antibiotics had no signs of colitis and had no obvious differences in body size as compared with heterozygous littermate tgm (Fig. 7B). Histological analysis showed that the homozygous tgm provided with antibiotics had

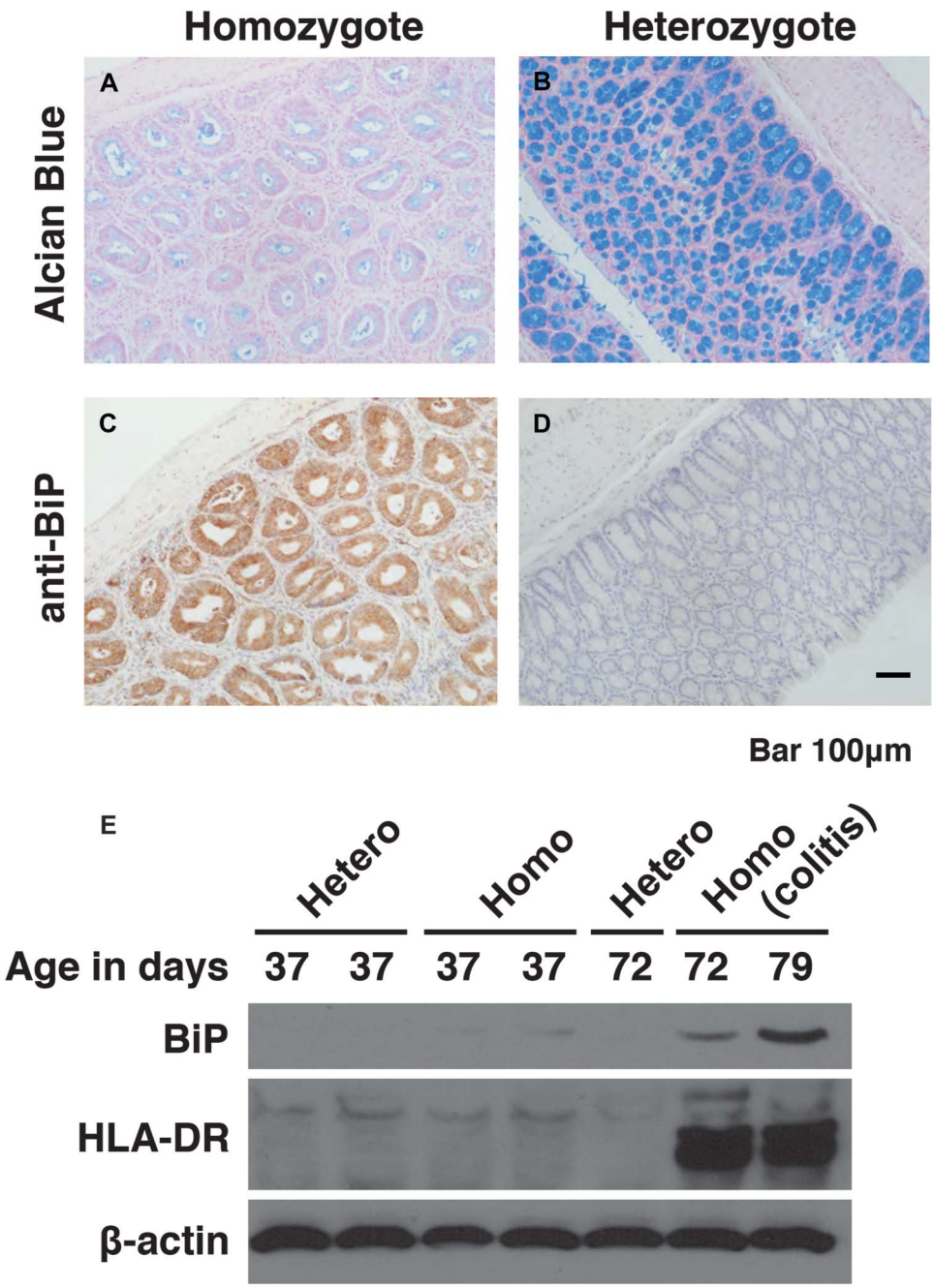

FIGURE 4. Mucus production is drastically reduced in the colons of homozygous HLA-DR4/I-E^d tgm with accumulation of BiP. Alcian Blue staining (A and B) and anti-BiP antibody staining (C and D) of the colonic sections of 4–months-old homozygous (A and C) and heterozygous (B and D) tgm. Bar = 100 μm. E, Western blot analysis of the colonic epithelial cells from 37-day old heterozygotes (Hetero) and homozygotes (Homo) before manifestation of colitis and 72- to 79-day-old heterozygote and homozygotes after development of colitis using anti-BiP and anti-HLA-DR antibodies. Anti-β-actin antibody was used as loading control.

normal mucus production (Fig. 7C, D, see Fig. 9C, Supplemental Digital Content 1, http://links.lww.com/IBD/B647) in the colonic epithelial cells, which was similar to that of the heterozygous littermate tgm (Fig. 4B, see Fig. 9D, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). It should be noted that there were a few infiltrating cells in the colonic lamina propria of the antibiotics-treated homozygous tgm (Fig. 7C) in contrast to many infiltrating cells observed in that of the colitic mice (Fig. 1E). Similarly, the aberrant accumulation of anti-HLA-DR antibody-reactive proteins in the colonic epithelial cells was not observed in the antibiotics-treated homozygous tgm, and there was no apparent difference compared with the heterozygous

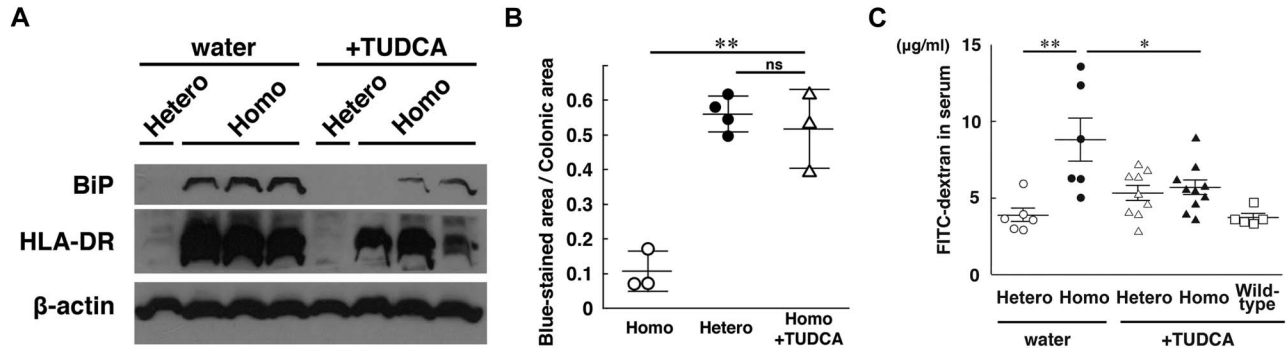

FIGURE 5. Administration of TUDCA ameliorates the colitis. A, Western blot analysis of the colonic epithelial cells from 72- to 79-days-old heterozygote (Hetero) and colitic homozygotes (Homo) orally administrated with TUDCA or water. B, Relative Alcian Blue-stained area/total colonic area of water-administrated homozygotes and heterozygotes, and TUDCA-administrated homozygotes are plotted. Nine to 13 different fields per homozygote, and 6 to 11 different fields per heterozygote were investigated. $^{**}P < 0.01$, ns: not significant. Indicated are mean ± SEM. C, Serum fluorescence of FITC-dextran-administrated tgm and wild-type mice. The mean and SE of serum concentration of FITC-dextran are shown. Data are representative of 2 independent experiments with similar results. ($^{**}P < 0.01$, $^{*}P < 0.05$).

littermate tgm (see Fig. 9A and B, Supplemental Digital Content 1, http://links.lww.com/IBD/B647). These data suggest that commensal bacteria in the intestinal tract stimulate and induce the expression and accumulation of HLA-DR4/I-E^d molecules, leading to ER stress in the colonic epithelial cells and reduced mucus production.

DISCUSSION

The transgene insertion site of the HLA-DR4/I-E^d tgm was identified by paired-end whole genome sequencing[14] to be in the distal region of chromosome 3, accompanied by a deletion of about 39 kb genomic segment. Less expensive methods for the identification of transgene insertion sites, for example improved genome walking,[20] were not successful probably due to the multiple copy numbers of the transgenes that might prevent the PCR elongation reaction to walk from the known transgene sequence to the unknown genomic region. The homozygous tgm were fertile, however, the litter size was smaller (4.8, n = 9) compared with the average (7.67 in C57BL/6J, Jackson Laboratory), and individuals showed less parenting capability. Therefore, the homozygous tgm were maintained by mating heterozygous male and female mice. The null mice were easily identified by the absence of anti-HLA-DR positive cells in the PBMC by flow cytometry, however, the discrimination of heterozygotes and homozygotes was difficult since the expression levels of HLA-DR4/I-E^d on the PBMCs of the tgm were similar. The identification of the transgene insertion site allowed us to accurately discriminate the homozygous and heterozygous tgm by performing PCR using a minimum amount of genomic DNA.

The insertion site of the transgene (from bp 156,023,577–156,062,472 of chromosome 3 of GRCm38) was relatively close to the *cytokine deficiency colitis-susceptible* (*Cdcs*) *1* locus (corresponding to bp 127,041,233–127,041,356 of chromosome 3 of GRCm38). Swapping from colitis-susceptible C3Bir-*Il10*$^{-/-}$ mice to resistant B6-*Il10*$^{-/-}$ mice altered the resistant phenotype of B6-*Il10*$^{-/-}$ mice to susceptible[21–23] however, the insertion site harbored no known genetic elements according to the Mouse Genome Informatics database (http://www.informatics.jax.org) except for one predicted pseudogene *Gm6510*.

The homozygous tgm with *CIITA*$^{-/-}$ background showed a normal phenotype with no body-weight loss, no diarrhea, and no colonic ulceration. Those mice had very few, if any, HLA-DR4/I-E^d-positive B cells in the PBMCs and colonic epithelial cells, while sharing the same genomic structure of chromosome 3 as wild-type background homozygous tgm. This indicates that it is not the genomic disruption by the insertion of the transgenes but the HLA-DR4/I-E^d-expression that was essential for the development of the colitis. In accordance with this, colonic epithelial cells of the wild-type background homozygous tgm with colitis showed drastic anti-HLA-DR staining compared with those cells of the heterozygous littermate tgm. Of note, the anti-HLA-DR staining was more evident in the cytoplasm rather than on the cell surface, suggesting that the HLA-DR4/I-E^d-molecules accumulated inside the epithelial cells. Moreover, there were a few normal-shape goblet cells in the crypts of the wild-type background homozygous tgm and the size of the crypts was on average larger than that of the heterozygous tgm. The reduced number of the goblet cells coincided with the drastic decrease of mucus production in the colonic epithelial cells of wild-type background homozygous tgm. All these observations suggested that the epithelial cells were under ER stress possibly due to the accumulation of HLA-DR4/I-E^d-molecules and lost mucus secretion, leading to a reduced barrier function of the epithelial cells, which could be the primary cause of the observed colitis. In accordance with this hypothesis, administration of the molecular chaperone TUDCA, which ameliorates ER stress both in vitro and in vivo, reduced accumulation of HLA-DR4/I-E^d and BiP, and restored mucus

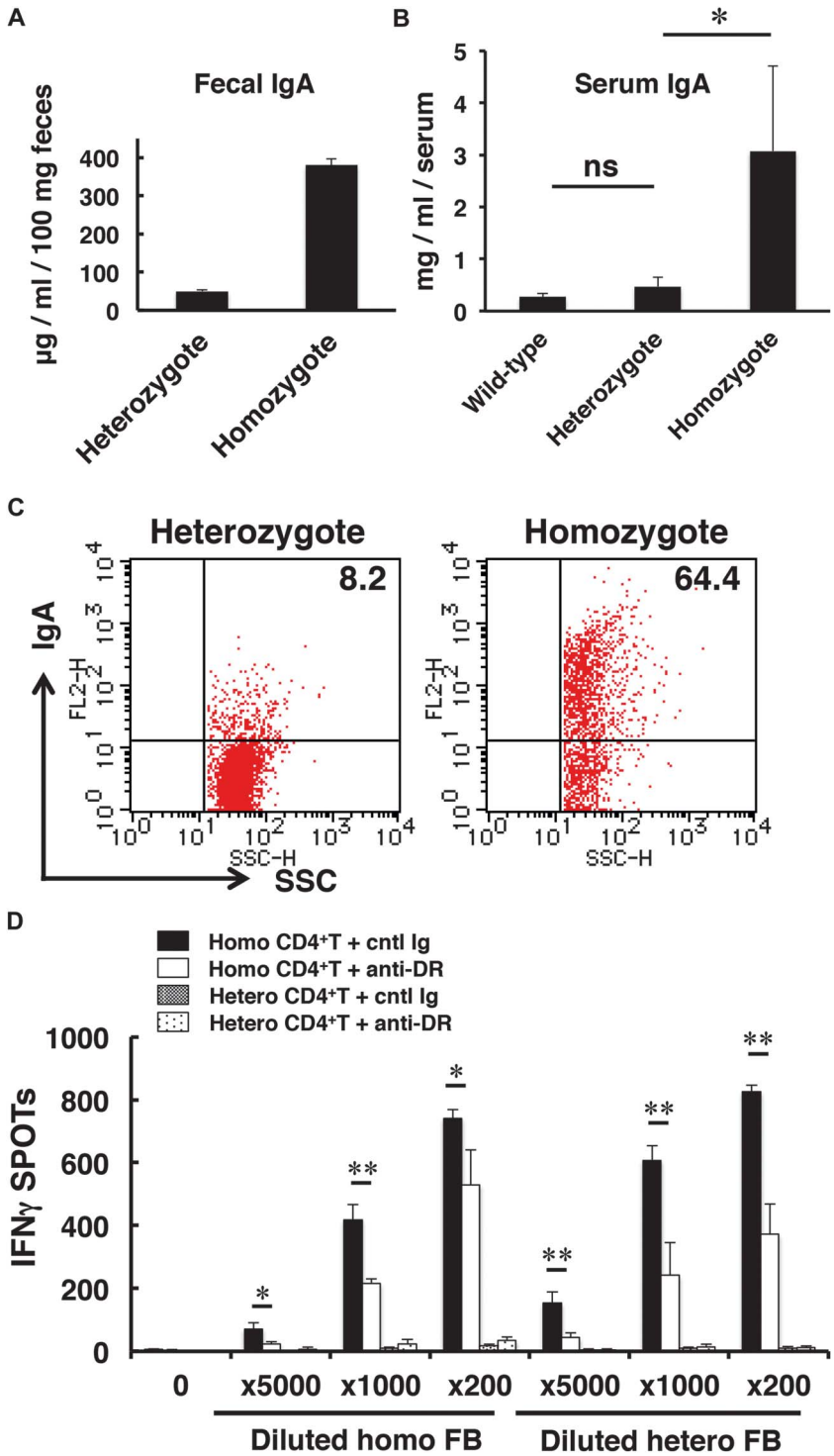

FIGURE 6. Immune responses of the tgm to commensal bacteria. IgA content in feces (A) or serum (B) of heterozygous and homozygous tgm and wild-type mice. *$P = 0.009$, ns = not significant. Mean + SD are shown. C, The bacteria in feces of the homozygous tgm were heavily coated with IgA as compared with those of the heterozygous tgm. The percentages of IgA-positive bacteria are shown. (D) Lamina propria $CD4^+$ T cells isolated from homozygotes (black and white bars) but not from heterozygotes (shaded and dotted bars), responded to autoclaved and diluted fecal bacteria (FB) prepared from both homozygous and heterozygous tgm in an FB concentration-dependent manner. The T-cell responses were significantly lower under the presence of anti-HLA-DR-antibody (white and dotted bars) than under the presence of irrelevant control Ig (black and shaded bars), showing that the responses were HLA-DR-restricted. Mean + SD are shown. **$P < 0.01$, *$P < 0.05$. Data are representative of 2 independent experiments with similar results.

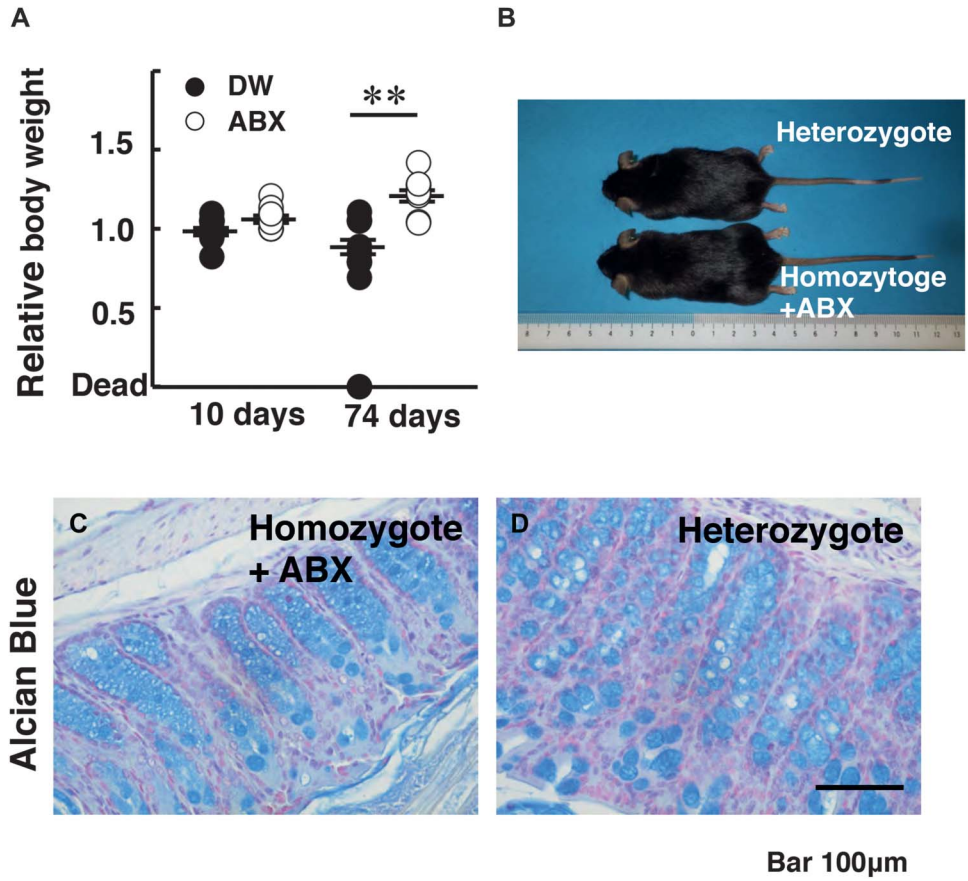

FIGURE 7. Administration of antibiotics mixture prevents the development of colitis. About 2-months-old homozygous tgm (before manifestation of colitis) were orally provided with an antibiotics mixture (ABX; 0.5 g/L ampicillin, 0.25 g/L vancomycin, 0.5 g/L metronidazole and 0.5 g/L fradiomycin) in the drinking water. A, Relative body weight of 9 homozygous tgm provided with ABX (open circles) or 10 not provided with ABX (closed circles). By day 74, 2 homozygous tgm without antibiotics died. $^{**}P = 0.0002$. Indicated are mean ± SEM. B, Body size of the heterozygous littermate (top) and the homozygous tgm provided with antibiotics mixture for 74 days (bottom). C and D, Alcian Blue staining of colonic sections of tgm. C, Four-months-old-homozygous tgm provided with antibiotics mixture and (D) its heterozygous littermate without antibiotics treatment. Bar = 100 μm.

production and barrier function in colonic epithelial cells of homozygotes.

The unfolded protein response responds to and manages ER stress conditions in mammalian cells.[24] The unfolded protein response is mediated mainly through 3 unfolded protein sensors in the ER membrane, activating transcription factor 6α (ATF6α), inositol-requiring kinase 1 (IRE1), and pancreatic ER kinase (PERK)[24,25] Mice with targeted genes encoding for those molecules[19,26] or XBP1, a downstream molecule in the signaling pathways from IRE1,[27,28] show spontaneous enteritis or enhanced susceptibility to DSS-induced colitis. Thus, ER stress in intestinal epithelial cells has recently been implicated as a novel mechanism that leads to IBD,[25] through which the mucus producing cells are damaged and the barrier function of the epithelium may be decreased.[12,29]

Apart from the unfolded protein response-related gene targeting models, animal models of misfolded protein accumulation have been established and investigated.[4,30] Muc2 is the major macromolecular component of intestinal mucus and mice with missense mutations in the *Muc2* gene show an accumulation of Muc2 precursors in the intestinal epithelial cells.[4] This leads to ER stress in those cells and reduced mucus secretion, and the mice show a phenotype similar to UC. Several lines of HLA-B27 (*HLA*B27:05*)/human β2m transgenic rats show inflammation of multiorgans, including the gastrointestinal tract,[30] in a transgene copy number-dependent manner.[31,32] Interestingly, one of the transgenic rat lines called 21 to 3 was normal in heterozygotes, but developed diseases in homozygotes, an observation similar to that of our HLA-DR4/I-E^d tgm. Therefore, the copy numbers of the transgenes for α and β chains in the tgm should be determined. However, the most important difference seems to be that the intense staining of the anti-HLA-B antibody in the colons of diseased HLA-B27 transgenic rats was observed on hematopoietic cells infiltrating the lamina propria, while no detectable staining was observed in the crypts.[31] Another difference is the higher tendency of HLA-B27 to be misfolded due to the unpaired Cys67 at the bottom of the B pocket of the peptide-binding groove,[33] or even due to the other

Cys residues at position 101 and 164[34] that potentially form disulfide-linked complexes. In the HLA-DR4/I-E^d tgm, the numbers of Cys residues in both α and β chains are not different from those of the I-E^d molecule, and the Cys residues in the α1 and β1 domains are conserved between HLA-DR4 and I-E^d. Therefore, the intermolecule and intramolecule disulfide-linked complex formation did not seem to be the primary cause of the accumulation of the HLA-DR4/I-E^d molecules in the colonic epithelial cells.

The barrier function of the intestinal epithelial cells physically prevents penetration of intact bacteria, and its reduction allows the invasion of the lamina propria by commensal microorganisms, leading to enhanced host immune responses and inflammation.[12,29] The IgA content of feces and serum of colitic homozygous tgm was much higher than that of the heterozygous tgm. Moreover, the fecal bacteria isolated from colitic homozygous tgm were heavily coated with IgA compared with those from heterozygous tgm, indicating that the host immune system was responding to the invading bacteria. Notably, the CD4$^+$ T cells from homozygotes responded to the fecal bacterial antigens derived from both heterozygous and homozygous tgm at least in part in an HLA-DR-restricted manner, indicating that the immune response was not specific to intestinal bacteria in homozygous tgm, but to commensal bacteria commonly present in both homozygous and heterozygous tgm.

It was reported that in IBD patients, the luminal bacteria were more frequently coated with IgA than those from healthy controls, suggesting that there may be a breakdown of the mucosal barrier to the commensal gut flora in IBD,[35] which is in accordance with our hypothesis that ER stress in the colonic epithelial cells in the tgm leads to the breakdown of the mucosal barrier. Since administration of the antibiotics mixture to homozygous tgm completely suppressed the development of colitis, the inflammatory conditions induced by the antimicrobial host immune responses are likely to be the primary mechanism of colitis pathogenesis.

In conclusion, the mechanism of pathogenesis of the spontaneous colitis observed in homozygous HLA-DR4/I-E^d tgm might be ER stress in the colonic epithelial cells accompanied by aberrant accumulation of HLA-DR4/I-E^d molecules. Commensal bacteria were shown to be involved in the onset of the disease, however, what kind of bacteria and why and how they stimulated only homozygous tgm remains to be elucidated. If the mechanisms for initiation of the colitis were clarified, the tgm would be a good model for the analysis of ER stress-induced colitis and useful for the development of therapy for UC patients.

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