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Translocation of a gut pathobiont drives autoimmunity in mice and humans

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Abstract

Despite multiple associations between the microbiota and immune diseases, their role in autoimmunity is poorly understood. We found that translocation of a gut pathobiont, *Enterococcus gallinarum*, to the liver and other systemic tissues triggers autoimmune responses in a genetic background predisposing to autoimmunity. Antibiotic treatment prevented mortality in this model, suppressed growth of *E. gallinarum* in tissues, and eliminated pathogenic autoantibodies and Tcells. Hepatocyte–*E. gallinarum* cocultures induced autoimmune-promoting factors. Pathobiont translocation in monocolonized and autoimmune-prone mice induced autoantibodies and caused mortality, which could be prevented by an intramuscular vaccine targeting the pathobiont. *E. gallinarum*-specific DNA was recovered from liver biopsies of autoimmune patients, and cocultures with human hepatocytes replicated the murine findings; hence, similar processes apparently occur in susceptible humans. These discoveries show that a gut pathobiont can translocate and promote autoimmunity in genetically predisposed hosts.

The gut microbiota are implicated in the pathogenesis of multiple gut and systemic autoimmune diseases (1). The integrity of the gut barrier is essential to prevent the microbiota of a healthy individual from triggering adaptive immune responses (2–7). When

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SUPPLEMENTARY MATERIALS

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intact commensals or pathogens escape the gut barrier, several defense mechanisms impede bacterial access to the systemic circulation. However, if these mechanisms fail, the mesenteric lymph nodes (MLNs) and liver represent further “firewalls” against commensal bacteria that escape the gut (2, 4, 7). Such mechanisms occur only during intestinal or vascular pathology, during chemotherapy, or in the absence of a functional innate immune system (2, 6, 8–11). Although recent studies show that gut commensals can reside within gastrointestinal-associated lymphoid tissues of unmanipulated, healthy hosts, it is unclear whether pathobiont translocation is involved in systemic autoimmunity (12).

In humans, systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease that is associated with marked morbidity and mortality, especially in antiphospholipid antibody–positive patients. Besides the major histocompatibility complex locus, SLE is associated with several genetic risk loci for excessive signaling of RNA-sensing Toll-like receptor 7 (TLR7) and type I interferons (IFNs) (13). In normal animals, exogenous viral infections and retroviruses contribute to these immune responses and interact with the microbiota (14–19). In the specific pathogen-free (NZW × BXSB)F₁ hybrid mouse, responses to endogenous retrovirus glycoprotein 70 (ERV gp70) have been shown to drive lupus kidney disease via TLR7 (20, 21). These mice eventually succumb to progressive autoimmune thrombi mediated by pathogenic antiphospholipid [β_2 -glycoprotein I (β_2 GPI)] antibodies. We asked whether, in animals predisposed to autoimmune responses, trans-location of gut commensals drives IFN and anti–double-stranded DNA (dsDNA), anti–ERV gp70, and anti– β_2 GPI responses. Consequently, we investigated whether pathological immune responses could be alleviated by therapeutic strategies such as antibiotic treatment or vaccination.

Mortality, lupus-related autoantibodies, and autoimmune manifestations were relieved in (NZW × BXSB)F₁ hybrid mice after oral administration of vancomycin or ampicillin, implicating involvement of Gram-positive pathobionts in disease (Fig. 1, A to C, and fig. S1). In addition to anti-dsDNA and anti-RNA autoantibodies (Fig. 1, B and C), anti– β_2 GPI immunoglobulin G (IgG), hepatic and serum ERV gp70, and anti–ERV gp70 immune complexes (ICs) were all suppressed by vancomycin treatment (fig. S2, A to L). Uptake of orally fed fluorescein isothiocyanate (FITC)–dextran into the systemic circulation of (NZW × BXSB)F₁ hybrid mice indicated grossly impaired gut barrier function relative to nonautoimmune C57BL/6 mice (Fig. 1D). At 16 weeks of age, we were able to detect marked bacterial growth in the mesenteric veins, MLNs, and liver, and 2 weeks later also in the spleen but not kidneys, which are affected by deposition of circulating immune complexes (Fig. 1, E to G, and fig. S2M). Translocation of microbiota was suppressed by vancomycin or ampicillin, both of which prevented mortality; neomycin was less effective at inhibiting translocation relative to vancomycin (Fig. 1, E to G).

Full-length 16S rDNA sequencing of single colonies from aerobic and anaerobic MLN, liver, and spleen cultures detected *Enterococcus gallinarum*, a Gram-positive gut commensal of animals and humans, in the mesenteric veins of 82% of (NZW × BXSB)F₁ hybrid mice. *E. gallinarum* was visualized in situ in MLNs and livers by fluorescence in situ hybridization (FISH) (Fig. 1H and fig. S2N). C57BL/6 control mice showed no systemic bacterial growth. Taxa identified by longitudinal fecal 16S rDNA sequencing revealed that *Enterococcus* spp. were enriched only in some fecal samples (fig. S3, A to C, and fig. S4). Species-specific

polymerase chain reaction (PCR) did not detect *E. gallinarum* DNA in stool samples from human or murine autoimmune hosts (fig. S5, A to D); however, fecal or mucosal tissue culture followed by species-specific PCR consistently revealed *E. gallinarum* in the feces and small intestine, as well as in the liver, of (NZW × BXSB)_{F1} mice (fig. S6, A to C). We also found *E. gallinarum* translocation to livers of (NZW × BXSB)_{F1} mice in two other animal facilities at Yale after transfer of newly weaned animals equilibrated to different microbiomes (fig. S6, D and E).

To test whether *E. gallinarum* induces proinflammatory pathways and alters gut barrier-related molecules in small intestinal tissue during translocation into internal organs, we performed RNA expression profiling of *E. gallinarum*-monocolonized C57BL/6 mice and compared these data with those from *Enterococcus faecalis*- and *Bacteroides thetaiotaomicron*-monocolonized mice (Fig. 2, A to J, and figs. S7 and S8). The presence of *E. gallinarum* down-regulated ileal molecules related to barrier function (e.g., occludin, claudins, Plvap, Axin2), the mucus layer (e.g., Mucin-2), and antimicrobial defense (e.g., Reg3b, Defa2) and up-regulated those related to inflammation (e.g., Cxcr2, AhR/Cyp1a1, Enpp3). Enpp3 is known to increase numbers of plasmacytoid dendritic cells (pDCs) (22), which are key cells contributing to the IFN signature in human SLE (13) ^{and} which were induced by *E. gallinarum* monocolonization (Fig. 2K).

We used confocal microscopy to visualize the gut epithelial, vascular, and lymphatic barrier molecule changes we detected by RNA. Intestinal epithelial and endothelial cells have tight junctions formed by occludin, zonula occludens-1 (ZO-1), cingulin, and junctional adhesion molecule- A (JAM-A) (23). These cells also have adherent junctions formed by vascular endothelial cadherin (VE-cadherin) and β-catenin. A loss of expression of these junctional proteins was seen in gnotobiotic C57BL/6 mice monocolonized with *E. gallinarum*, except for ZO-1 and VE-cadherin (Fig. 2L and figs. S9 to S11). Claudin-2, -3, and -5 are expressed in lymphatic endothelial tight junctions, but in *E. gallinarum*-monocolonized mice they were weakly expressed relative to germ-free controls, except for pore-forming claudin-2 (Fig. 2L and fig. S12).

Liver-resident *E. gallinarum* possibly induces hepatic overexpression of ERV gp70 that fuels anti-ERV immune complex formation and systemic autoimmunity (fig. S2). We found that (NZW × BXSB)_{F1}-derived hepatocytes cocultured with *E. gallinarum* isolated from an (NZW × BXSB) _{F1} liver induced multiple autoimmune-promoting factors, including the autoantigens ERV gp70 and β₂GPI, which were potently induced by *E. gallinarum* or *E. gallinarum* RNA (a potential TLR7/8 ligand) relative to *E. faecalis* (Fig. 3, A and B). In hepatocytes and dendritic cells, *E. gallinarum* RNA also induced expression of type I IFN and other proinflammatory cytokines (Fig. 3C and fig. S13).

Tryptophan-derived indoles are bacterial ligands for the aryl hydrocarbon receptor (AhR), which activates the AhR-CYP1A1 pathway, a known innate antimicrobial defense mechanism and inducer of T helper 17 (Th17) cells (24–27) that we also found to be up-regulated (Fig. 3, D to F). Furthermore, whole-genome sequencing revealed that *E. gallinarum* encodes the shikimate pathway, which generates AhR ligands (fig. S14 and table S1) (26). We tested whether *E. gallinarum*-immune interactions, possibly mediated by the

AhR pathway, induced Th17 and T follicular helper (Tfh) cells in vivo that are crucial for systemic autoantibody production (28, 29). Broad-spectrum antibiotic or vancomycin treatment in (NZW × BXSB)F₁ mice reduced levels of Th17 cells, Tfh cells, and their cytokine signatures (fig. S15, A to C). Vancomycin had no direct influence on immune cells in vitro (fig. S15D). The in vivo effects of vancomycin on T cells correlated with reduced *E. gallinarum* translocation, less immunopathology, and fewer autoantibodies in these mice, as well as suppression of serum and hepatic levels of the ERV gp70 autoantigen and anti-ERV gp70 immune complexes (Fig. 1 and fig. S2). Administration of an AhR-selective antagonist abrogated the Th17- and autoantibody-inducing effects of *E. gallinarum* in (NZW × BXSB)F₁ mice in vivo (Fig. 3, G and H, and fig. S16); this finding indicates that AhR signaling is involved in *E. gallinarum*-induced autoimmunity.

Because *E. gallinarum* has the potential for translocation in (NZW × BXSB)F₁ mice but not in C57BL/6 mice under specific pathogen-free conditions, we tested translocation in germ-free C57BL/6 mice monocolonized with *E. gallinarum*. In the absence of competing microbiota, *E. gallinarum* induced barrier leakage, autoantibodies, and translocation to mesenteric veins, MLNs, and livers of nonautoimmune C57BL/6 mice (Fig. 3, I to N). Autoantibody induction in C57BL/6 mice was not seen during monocolonization with other bacteria that either remain in the gut (*B. thetaiotaomicron*) or translocate to tissues (*E. faecalis*) (Fig. 3, I to N). Monocolonization with *E. gallinarum* induced Th17 cells in the small intestinal lamina propria and MLNs of C57BL/6 mice (Fig. 3, O and P), consistent with the finding that Th17 cells are abundant in (NZW × BXSB)F₁ mice and were depleted after vancomycin treatment (fig. S15). Experiments with antibiotic-pretreated (NZW × BXSB)F₁ hybrid mice gavaged with a translocating pathogen, *Salmonella typhimurium*, did not induce autoantibodies (fig. S17). By contrast, gavage of antibiotic-pretreated (NZW × BXSB)F₁ hybrid mice with *E. gallinarum* caused systemic autoimmune pathology (fig. S17D and fig. S18, control group).

To deplete the pathobiont selectively, we developed an intramuscular vaccination strategy using heat-killed *E. gallinarum* (fig. S18A). Vaccination against *E. gallinarum*, but not against *E. faecalis* nor *B. thetaiotaomicron*, reduced levels of serum autoantibodies and prolonged survival in (NZW × BXSB)F₁ mice (fig. S18, B to F). Vaccination also prevented translocation, as no growth of *E. gallinarum* was observed in internal organs (fig. S18G). Thus, pathobiont-specific treatment can abrogate host autoimmune processes without needing to suppress the immune system, which can lead to systemic adverse events in current clinical practice.

Longitudinal stool analyses from SLE patients revealed evidence for impaired gut barrier function with increased fecal albumin and calprotectin (Fig. 4, A and B). We thus tested for *E. gallinarum* translocation to human livers in patients with SLE and autoimmune hepatitis (AIH) who display serologic features of lupus, including antinuclear antibodies and anti-dsDNA IgG (table S2) (30). Liver biopsies from three SLE patients were positive for *E. gallinarum* (Fig. 4C); of six controls obtained from healthy liver transplant donors with normal liver histology, four were positive for the presence of other *Enterococcus* species but not *E. gallinarum*. Sterilely obtained human liver tissues from the same control patients, AIH patients, and cirrhosis patients [who are known to have grossly impaired gut barriers (31)]

were subjected to 16S rDNA sequencing; the results show that *Enterococcus* spp. predominated in diseased tissues (Fig. 4D). Note that the majority of AIH liver biopsies, but not the healthy control livers, were positive for *E. gallinarum* (Fig. 4E).

Similar to the effects seen with murine hepatocytes (Fig. 3, A to F), primary human hepatocytes from healthy livers stimulated with *E. gallinarum* induced β_2 GPI, type I interferon, and AHR/CYP1A1 (Fig. 4, F to J). Consistent with enhanced adaptive immune responses to *E. gallinarum*, the majority of SLE and AIH patients also showed increased serum antibody titers against *E. gallinarum* and particularly its RNA, which can act as a potential TLR7/8 stimulus and cross-reactive trigger (Fig. 4, K to O, and fig. S19). Comparison of anti-human RNA IgG autoantibody titers in SLE and AIH patients with anti-*E. gallinarum* RNA IgG showed that both were equally elevated, by contrast to anti-*E. faecalis* and anti-*B. thetaiotaomicron* RNA antibodies (Fig. 4, K to O).

Our findings show that the Gram-positive gut pathobiont *E. gallinarum* translocates, as a result of gut barrier breakdown, into systemic organs in autoimmune-prone hosts to drive autoimmune pathogenesis (fig. S20). Translocating bacteria may not only skew T helper cell differentiation but may also directly act on colonized tissues, such as the liver, to induce autoantigens, ERV proteins, cytokines, and other autoimmune-promoting factors. If the complexity of host tissue-microbiota interactions is considered in chronic autoimmunity, it may offer new therapeutic avenues for these debilitating and potentially lethal diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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as well as via the following repositories: Whole-genome shotgun sequences have been deposited at DDBJ/ENA/GenBank under accession number PPHK00000000. The version described in this paper is version PPHK01000000. RNA-seq and 16S rRNA sequences have been deposited with the European Bioinformatics Institute under accession numbers PRJEB24586 and PRJEB24587, respectively.

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Fig. 1. Effect of antibiotics on autoimmunity and *E. gallinarum* translocation to the liver (A) Vancomycin (VANC), ampicillin (AMP), metronidazole (METR), neomycin (NEO), or control water (CTRL) were provided in the drinking water of (NZW × BXSB)_{F1} mice starting at 6 weeks of age ($n = 15$ each). Mice were followed for 30 weeks or until death from autoimmunity. (B and C) Serum anti-dsDNA (B) and anti-RNA (C) IgG at 16 weeks of age. (D) Serum levels of orally administered FITC-dextran as an indicator of gut barrier leakiness ($n = 8$ each). (E to G) Cultures of tissues from 16-week-old mice showed a selective growth of *E. gallinarum* in the mesenteric veins (E), MLN (F), and liver (G) ($n = 7$ each). (H) An *E. gallinarum*-specific FISH probe detects *E. gallinarum* in liver (scale bars, 30 μm) of *E. gallinarum*-monocolonized C57BL/6 mice 3 weeks after colonization in comparison to germ-free mice. One representative section from one mouse is shown from multiple sections with *E. gallinarum* signals within the tissues, representative of three mice in total. Data are presented as mean ± SD in (B) to (G); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; log-rank test and Gehan-Breslow-Wilcoxon test in (A), analysis of variance (ANOVA) followed by Bonferroni multiple-comparisons test in (B) to (G).

Fig. 2. RNA expression profiling and plasmacytoid dendritic cell frequencies in small intestine from germ-free mice monocolonized with *E. gallinarum*, *E. faecalis*, or *B. thetaiotaomicron* Germ-free C57BL/6 mice were monocolonized with *E. gallinarum* (EG), *E. faecalis* (EF), or *B. thetaiotaomicron* (BT) for RNA-seq and fluorescence-activated cell sorting (FACS) analyses of the small intestine. **(A)** RNA-seq was performed with ileal tissue isolated from 14-week-old monocolonized mice ($n = 3$ each). Heat map shows transcripts differentially expressed in the ileum 8 hours after commensal delivery. **(B to J)** Reverse transcription quantitative PCR (RT-qPCR) analysis of ileum RNA ($n = 6$ each) as described in (A). **(K)** Plasmacytoid dendritic cell (pDC) and conventional dendritic cell (cDC) frequencies in the small intestinal lamina propria of 12-week-old germ-free mice ($n = 4$ each) were evaluated 3

weeks after monocolonization by FACS analysis. (L) Confocal imaging of gut tissues was performed as described in the supplementary materials. Localizations of TJ proteins are shown in green for occludin, JAM-A, claudin-3, and claudin-5. Images are representative of six different mice. DAPI, 4',6-diamidino-2-phenylindole. Scale bars, 40 μ m. Data are presented as mean $\pm$ SD in (B) to (K); * P < 0.05, ** P < 0.01, **** P < 0.0001; ANOVA followed by Bonferroni multiple-comparisons test in (B) to (K).

Fig. 3. Induction of hepatic AhR by *E. gallinarum*, AhR antagonism in (NZW $\times$ BXSB)F1 mice, and Th17 and autoantibody induction in *E. gallinarum* monocolonized C57BL/6 mice (A to F) *E. gallinarum*, *E. faecalis*, and *B. thetaiotaomicron* lysates or isolated RNA were cocultured with hepatocytes from 14-week-old (NZW $\times$ BXSB) F1 mice ($n = 3$ each), and expression of ERV gp70, the autoantigen β_2 GPI, IFN- α , and AhR was measured 6 hours later. (G and H) Serum anti-RNA (G) and anti-dsDNA (H) IgG of 16-week-old (NZW $\times$ BXSB)F1 mice ($n = 4$ each) gavaged with vehicle or EG and treated with AhR antagonist CH223191 or mock, as described in the supplementary materials. (I to L) C57BL/6 germ-free (GF) mice were monocolonized with *E. gallinarum*, *E. faecalis*, and *B. thetaiotaomicron* at 12 weeks of age ($n = 6$ to 10 mice) and evaluated 3 weeks later for integrity of the gut barrier with FITC-dextran (I), and for translocation to the mesenteric veins (J), MLNs (K), and liver (L). (M and N) C57BL/6 germ-free mice ($n = 4$ to 10 mice) were monocolonized as in (I), and anti-RNA (M) and anti-dsDNA (N) IgG autoantibodies were measured 8 weeks later. (O and P) Th17 cells and Th1 cell frequencies were

determined by intracellular FACS analysis of IL-17A and IFN- γ in small intestinal lamina propria (O) and MLN (P) CD45⁺ CD4⁺ CD44⁺ T cells from germ-free and monocolonized mice ($n = 5$ each). Data are presented as mean $\pm$ SD in (A) to (P); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ANOVA followed by Bonferroni multiple-comparisons test.

Fig. 4. Gut barrier function and *E. gallinarum* in liver biopsies of autoimmune patients with anti *E. gallinarum* serum reactivities

(**A** and **B**) Feces from patients with SLE ($n = 9$) were screened for increased albumin (**A**) and calprotectin (**B**) as signs of an impaired gut barrier. NHD, normal healthy donors ($n = 9$). (**C**) Multiplex PCR for eubacterial [1383 base pairs (bp)], *Enterococcus* genus (112 bp), and *E. gallinarum* (173 bp) DNA on sterily obtained and processed liver biopsies from cadaveric liver transplant donors (CTRL) or SLE patients. Lanes 10 to 12, bacterial strains as indicated; lane 13, water. (**D**) 16S rDNA sequencing of controls as in (**C**), autoimmune hepatitis (AIH) patients, and non-AIH cirrhosis (CIR) patients. (**E**) Liver biopsies from CTRL, AIH, and CIR patients ($n = 5$ to 6 each) were tested for *E. gallinarum* (EG) DNA by qPCR and normalized to any eubacterial (EUB) signal. (**F** to **J**) RT-qPCR of human primary hepatocytes ($n = 3$ each) stimulated with *E. gallinarum*, *E. faecalis*, or *B. theta* as in Fig. 3. (**K** and **L**) SLE ($n = 15$) and AIH ($n = 17$) sera were screened for anti-*E.*

gallinarum RNA IgG (K) and anti-human RNA IgG (L) by enzyme-linked immunosorbent assay (ELISA). (M) SLE and AIH serum IgG levels against human, *E. gallinarum*, *E. faecalis*, or *B. thetaiotaomicron* RNA normalized to NHD sera. (N and O) Correlation between anti-*E. gallinarum* RNA IgG and autoantibodies in SLE (N) and AIH (O) patients. Data are presented as mean $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; Student *t* test in (A) and (B), ANOVA followed by Bonferroni multiple-comparisons test in (E) to (M), and Pearson correlation in (N) and (O).