

Commensal microbiota influence systemic autoimmune responses

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Abstract

Antinuclear antibodies are a hallmark feature of generalized autoimmune diseases, including systemic lupus erythematosus and systemic sclerosis. However, the processes underlying the loss of tolerance against nuclear self-constituents remain largely unresolved. Using mice deficient in lymphotoxin and Hox11, we report that approximately 25% of mice lacking secondary lymphoid organs spontaneously develop specific antinuclear antibodies. Interestingly, we find this phenotype is not caused by a defect in central tolerance. Rather, cell-specific deletion and *in vivo* lymphotoxin blockade link these systemic autoimmune responses to the formation of gut-associated lymphoid tissue in the neonatal period of life. We further demonstrate antinuclear antibody production is influenced by the presence of commensal gut flora, in particular increased colonization with segmented filamentous bacteria, and IL-17 receptor signaling. Together, these data indicate that neonatal colonization of gut microbiota influences generalized autoimmunity in adult life.

Keywords antinuclear antibodies; commensal microbiota; systemic autoimmunity

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Antinuclear antibodies (ANA) are a hallmark feature of generalized autoimmune diseases (von Muhlen & Tan, 1995). These clinically heterogeneous conditions, such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), are characterized by immune-mediated tissue damage in multiple organs, caused by aberrant responses of the adaptive immune system. Immunodominant autoantigens recognized by systemic autoantibodies are often DNA- or RNA-associated protein complexes. Underlying mechanisms that mediate the breach of tolerance against these nuclear autoantigens are only partially understood. In autoimmune-prone strains of mice, antigen-producing cells have been located in secondary lymphoid tissue, and both extrafollicular and germinal center responses have been implicated in the production of such autoantibodies (William *et al*, 2002).

The lymphotoxin- β receptor (LT β R) functions as receptor for both membrane-bound lymphotoxin (LT α 1 β 2) and LIGHT (TNF superfamily member 14). Lymphotoxin- β receptor signaling controls

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the development of secondary lymphoid organs and is continuously required in adults for homeostasis and structural architecture of the thymus and secondary lymphoid organs (Futterer *et al*, 1998). LT-deficient mice thus serve as a prototypic model for studying the influence of secondary lymphoid organs in immune processes. Although antibody responses are impaired in LT-deficient animals due to the absence of follicular dendritic cell networks, germinal center formation and somatic hypermutation can still occur. Based on the presence of perivascular lymphocytic infiltrates in multiple organs as well as organ-specific autoantibodies, an autoimmune phenotype has been defined for the LT-deficient animals (Boehm *et al*, 2003). However, the role of the disturbed thymic medulla in this autoimmune phenotype is a matter of controversy (Chin *et al*, 2003; Martins *et al*, 2008). Whether systemic autoimmune responses do occur in the absence of secondary lymphoid tissues is another area of uncertainty (Boehm *et al*, 2003; Chin *et al*, 2003). Given conflicting reports on systemic autoimmune responses in *Ltbr*^{-/-} mice, we sought to investigate whether autoantibodies directed against nuclear antigens can appear in the absence of secondary lymphoid tissues utilizing *Ltbr*^{-/-} mice.

Results and Discussion

We found that ~25% of LTβR-deficient mice developed systemic autoimmune responses by three months of age (Fig 1A) using a validated immunodetection system for a broad range of nuclear antigens (Supplementary Fig S1). The immunoassay system identified diverse ANA, including anti-U1RNP, anti-Sm, anti-Scl70/topoisomerase-I, anti-centromere protein B, anti-SSA/Ro52, and anti-Jo1 (Fig 1B and C). Antibodies to these nuclear autoantigens are strongly associated with SLE, SSC, and polymyositis (von Muhlen & Tan, 1995). In contrast, no anti-dsDNA was found (Supplementary Fig S2). By six months of age, the prevalence of autoantibodies remained the same, but more mice developed multiple reactivities (Fig 1B and C). We semiquantitatively determined the titers of these mice (Fig 1B). We could not detect any autoimmune reactivity at six weeks of age despite immune maturation, suggesting a delayed stochastic penetrance of the autoimmune phenotype characteristic in most autoimmune diseases. As LT-deficient animals have a spleen, we sought to determine whether ANA can be generated in asplenic mice by intercrossing *Hox-11*^{-/-} and *Ltbr*^{-/-} mice. These double knockout mice still developed the pathological autoantibody responses at the same prevalence, demonstrating that aberrant systemic autoimmune responses can develop in the complete absence of secondary lymphoid organs (Fig 1A).

Histological examination of *Ltbr*^{-/-} mice confirmed the presence of lymphocytic infiltrates in multiple organs. Given the association of systemic autoimmune responses with generalized autoimmune disease, we specifically looked for characteristic pathological features. However, compared to wild-type mice, no difference was observed in kidney damage, and skin and esophageal sclerosis (Supplementary Fig S3). Furthermore, renal histology and proteinuria were not different between antibody-positive and antibody-negative *Ltbr*^{-/-} mice (data not shown).

We then evaluated whether structural defects in LT-deficient mice lead to ANA production. To this end, we used an LTβR-Fc fusion protein, which acts as a soluble decoy receptor blocking LTαβ

and LIGHT (Rennert *et al*, 1996). Blocking LTβR signaling at various phases of ontogeny and early postnatally results in the temporally patterned absence of secondary lymphoid organs (lymph nodes, Peyers' patches (PP), and cryptopatches (CP)) (Rennert *et al*, 1996; Bouskra *et al*, 2008). We observed that blocking LTβR signaling during late ontogeny through six weeks of age resulted in the appearance of ANA at the age of three months with a comparable spectrum and titers as LTβR-deficient mice (Fig 1D and data not shown), and as demonstrated previously, these mice lacked CP, PP, and isolated lymphoid follicles (ILF) (data not shown). In contrast, mice lacking peripheral lymph nodes and PP by blocking LTβR signaling during early ontogeny did not develop autoantibodies. In addition, blocking LTβR signaling during adulthood, which disrupts splenic architecture, also did not result in ANA formation (Fig 1D). To rule out a role of LTβR signaling in the thymus during the perinatal window, we performed thymus transplant experiments. Fetal thymi from *Ltbr*^{-/-} or wild-type mice were depleted of hematopoietic cells and then grafted under the kidney capsules of nude mice, creating *Ltbr*^{-/-}→nude mice and wild-type→nude mice. Three months after engraftment, mice were sacrificed and T-cell repopulation was verified in the liver and spleen by flow cytometry (Fig 2A and B). *Ltbr*^{-/-} and wild-type thymi contained approximately equal number of thymocytes, with similar distribution among the different T-cell subsets (Fig 2A and B). Levels of total IgG were also not different between the two groups (Fig 2C). Importantly, no ANA could be detected in the serum samples of nude mice engrafted with LTβR-deficient thymic lobes (Fig 2D). We thus concluded that systemic autoimmune responses can develop in the absence of CP and ILF.

We next wanted to resolve which membrane LT-expressing cell type in the lamina propria of the gut was involved in the maintenance of tolerance against nuclear antigens. To this end, we generated mice deficient in LTβ in T cells (*T-Ltbr*^{-/-}), B cells (*B-Ltbr*^{-/-}) or RORγ⁺ cells (*Rorγt-Ltbr*^{-/-}), and littermate controls. We could only detect autoantibodies with a similar spectrum as *Ltbr*^{-/-} in mice lacking membrane LT in RORγ⁺-positive cells (Fig 1E). As we were able to test only small sample sizes of cell-specific *Ltbr*^{-/-} mice, we also collected sera from cell-specific *Lta*^{-/-} mice. Alike cell-specific *Ltbr*^{-/-} mice, only *Rorγt-Lta*^{-/-} but neither *T-Lta*^{-/-} nor *B-Lta*^{-/-} developed ANA at the age of three months (data not shown). To resolve which LTβR-expressing cells in the lamina propria are involved in the maintenance of the tolerance, we performed reciprocal bone marrow transfer experiments between wild-type and *Ltbr*^{-/-} mice. As shown in Fig 1F, both wild-type→*Ltbr*^{-/-} and *Ltbr*^{-/-}→wild-type chimeras developed autoantibodies. We thus conclude that communication via the LT-LTβR axis between RORγ⁺ innate lymphoid cells (ILC) and both radio-resistant and bone marrow-derived cells is essential to maintain tolerance.

RORγ⁺ ILC have been shown to be essential in the defense of epithelial surfaces and play an important role in the intestinal homeostasis with symbiotic microbiota by the production of IgA, IL-17, and IL-22 production (Vivier *et al*, 2009). This regulatory control in the gut prompted us to examine the relationship between the gut microbiota and ANA production. Moreover, early postnatal blocking of LTβR signaling leads to a 10-fold expansion of the normal ileal microbiota, including bacteria belonging to the Clostridiales, Bacteroides, and Enterobacteriaceae groups (Bouskra *et al*,

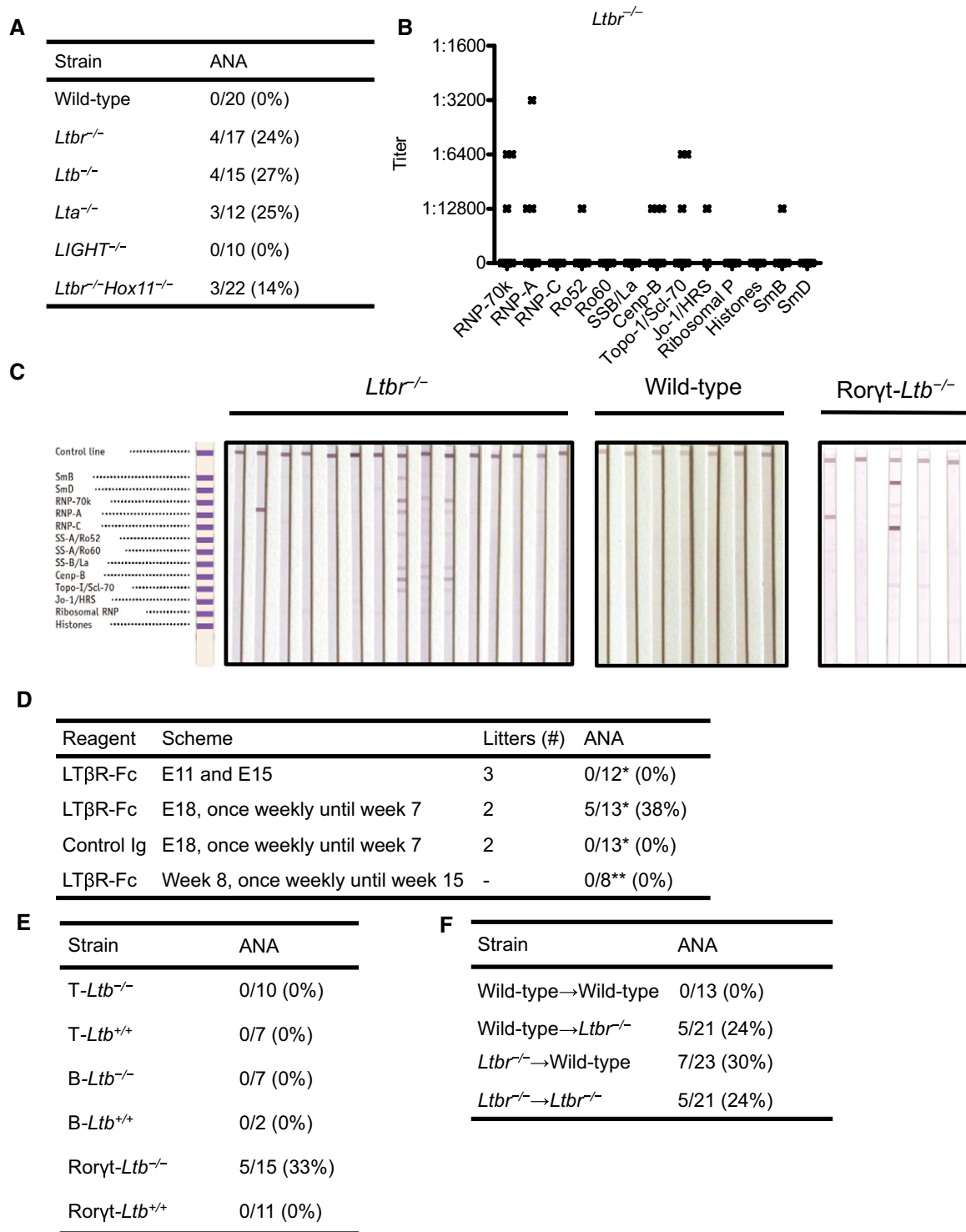


Figure 1. Systemic autoimmune responses in mice lacking secondary lymphoid organs.

A 3-month-old wild-type (C57BL/6), *Ltbr*^{-/-}, *Lta*^{-/-}, *LIGHT*^{-/-}, and *Ltbr*^{-/-}*Hox11*^{-/-} mice; percentage of mice with at least one ANA.
 B 6-month-old *Ltbr*^{-/-} mice (*n* = 15); titers of individual mice.
 C LIA of 6-month-old *Ltbr*^{-/-} mice, wild-type mice, and 3-month-old *Roryt-Ltb*^{-/-} mice.
 D LTβR-Fc or control immunoglobulin (Ig)-treated wild-type mice; percentage of mice with at least one ANA. E denotes gestational day. *ANA were determined with LIA at the age of 3 months. **ANA were determined with LIA 3 months after the start of treatment.
 E 3-month-old cell-specific LT knockout mice; percentage of mice with at least one ANA.
 F Bone marrow cells from wild-type or *Ltbr*^{-/-} mice were transferred at neonatal age into lethally irradiated wild-type or *Ltbr*^{-/-} mice; percentage of mice with at least one ANA. ANA were determined with LIA at the age of 3 months.

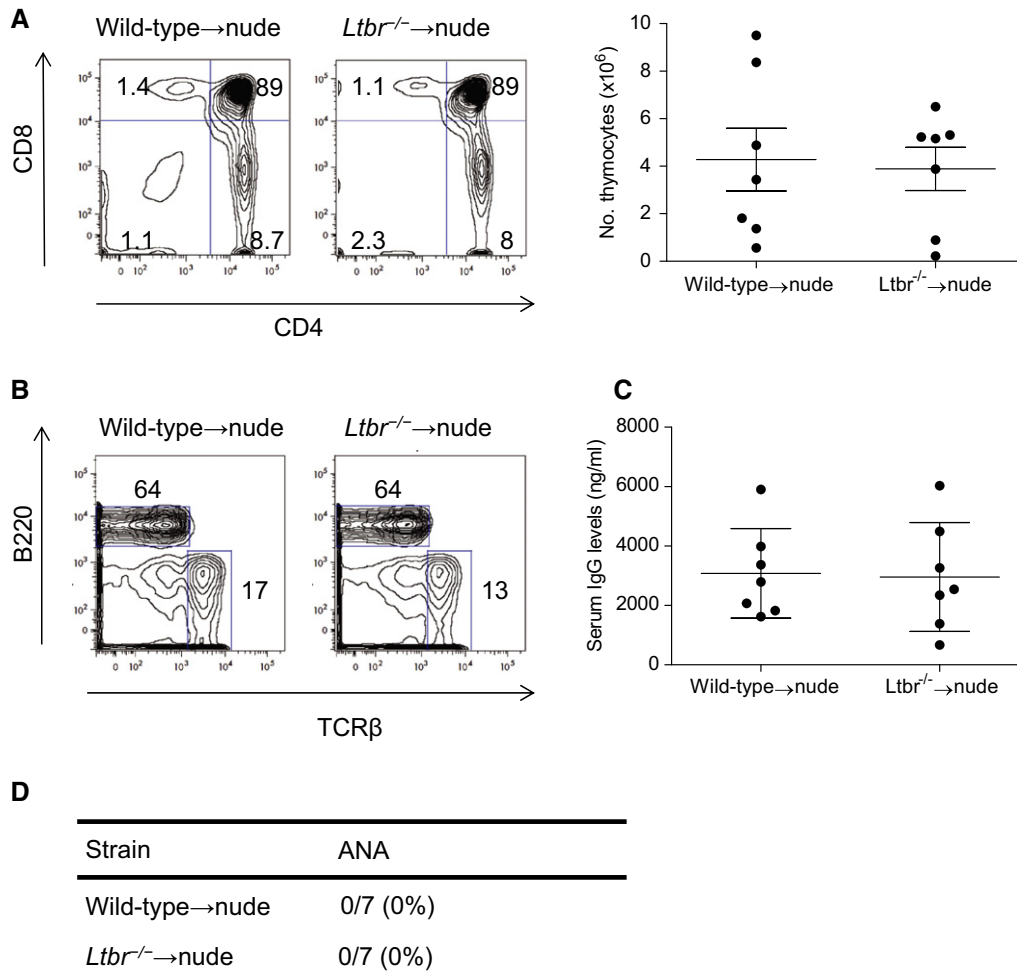


Figure 2. Central tolerance against nuclear antigens is not impaired in lymphotoxin-deficient mice.

- A Thymic reconstitution was analyzed by counting total cell number and flow cytometry for cell percentages. Numbers represent the percentages within the indicated regions (left panel). No significant differences were found. Data represent means \pm s.e.m.
- B T-cell reconstitution of spleen was assessed by flow cytometry. Numbers represent the percentages within the indicated regions.
- C Total IgG from nude mice engrafted with *Ltbr*^{-/-} or wild-type thymi was determined by ELISA. No significant differences were found. Data represent means \pm s.e.m.
- D Sera were collected 3 months after transplantation and tested for ANA; percentage of mice with at least one ANA reactivity.

2008). We first assessed whether elimination of the gut microbiota influenced ANA production. Pregnant mice and their offspring were treated with broad-spectrum antibiotics until the age of 3 months. As previously observed in germfree mice, this caused a significant enlargement of the cecum (data not shown). *Ltbr*^{-/-} mice receiving antibiotics had a reduced prevalence of ANA compared to a large series of 369 untreated consecutively born three-month-old animals (Fig 3A, upper panel). In this series, we observed some variation in the ANA positivity between litters, but no significant differences in prevalence between cages weaned from the same breeding cage (data not shown). This points to a maternal but not a cage effect on the phenotype. Therefore, we opted to analyze offspring of at least 2 litters in further experiments. To further substantiate a role for the gut microbiota, we treated germfree C57BL/6 mice with the LTβR-Fc fusion protein from gestational day 18 until six weeks after birth as described above. These experiments were performed in the INRA Anaxem germfree animal facilities. Similarly, germfree animals had a reduced prevalence of ANA (Fig 3A, lower panel).

Furthermore, we observed a higher frequency of ANA-positive animals compared to the experiments with LTβR-Fc fusion protein performed in the Ghent University vivarium.

We next assessed whether gut microbiota composition differed between wild-type, antibody-positive, and antibody-negative LTβR-deficient animals existed. In a first screening, we performed a community profiling on the luminal, mucosal, and fecal microbiome by DGGE (data not shown) and 16S rRNA sequencing, both of which showed the same results. As shown on Fig 3B, we observed genotype and antibody-specific clustering of the three animal groups. The most striking differences between groups revealed a species belonging to the segmented filamentous bacteria (SFB), characterizing animals with multiple reactivities as a separate group compared to controls that are in a cluster dominated by *Methylobacterium* (Fig 3B). These data were confirmed by quantitative PCR with primers specific for SFB genes (Fig 3C). The presence of the *Methylobacterium* genus has been associated with contamination in samples with low biomass (Barton *et al*, 2006; Salter *et al*, 2014).

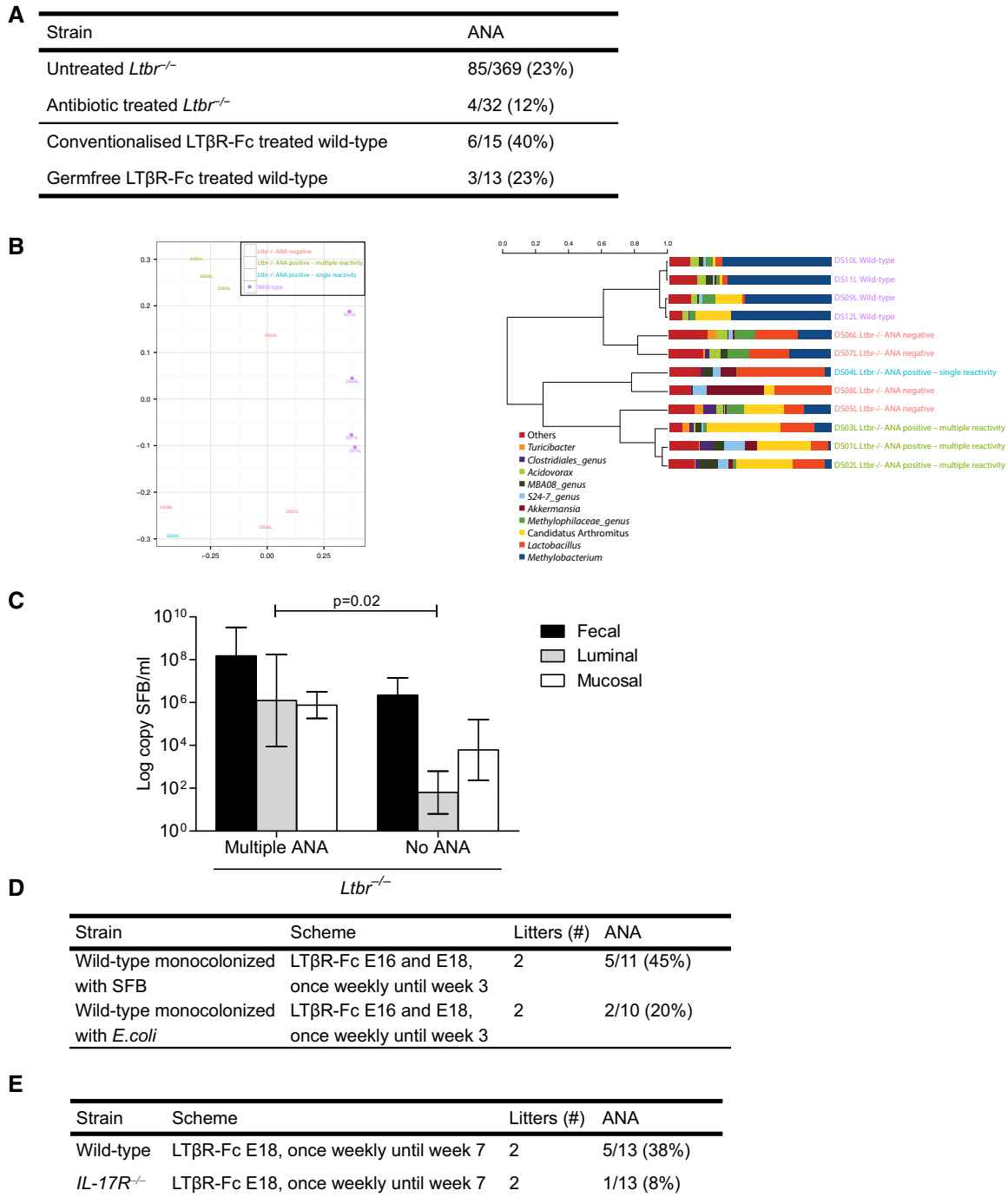


Figure 3. Development of antinuclear antibodies in lymphotoxin-deficient mice is influenced by gut microbiota.

A *Ltbr*^{-/-} mice were treated or not with antibiotics from birth (upper panel), conventionalized or germfree wild-type mice were treated with the LTβR-Fc fusion protein from gestational day 18 until 6 weeks after birth (lower panel); percentage of mice with at least one ANA. ANA were tested with LIA at the age of 3 months.

B 16S rRNA gene analysis of luminal samples of wild-type, ANA-positive, and ANA-negative *Ltbr*^{-/-} mice. Left panel: Beta diversity plot using multidimensional scaling on Bray–Curtis dissimilarities distances. Right panel: Hierarchical cluster analysis using Pearson’s correlation. Bacteria community compositions differ in the different groups. Please note that *Candidatus arthromitus*, according to Thompson *et al* (2012), should be renamed *Candidatus savagella*.

C Real-time PCR for SFB in luminal, mucosal, and fecal samples of multiple ANA-positive and ANA-negative *Ltbr*^{-/-} mice. Analysis with ANOVA, *P* = 0.02 for differences between groups, *n* = 4 mice per group. Data represent means ± s.e.m.

D C3H/HeN germfree pregnant mice were treated with the LTβR-Fc fusion protein on day 16 and 18 of gestation to prevent the development of PP in their offspring. Pups were also treated in the neonatal period to prevent the development of ILF. Mice were colonized at 8 weeks of age with SFB or *E. coli* and were sacrificed on d20 or on d60 postcolonization; percentage of mice with at least one ANA. E denotes gestational day.

E LTβR-Fc-treated wild-type or *IL17R*^{-/-} mice; percentage of mice with at least one ANA. E denotes gestational day. ANA were determined with LIA at the age of 3 months

However, we feel that this is not the case here—the fecal material used in this study has large biomass that should dilute the contaminant rather than allowing for its dominance in the profile. Furthermore, a neighbor-joining tree shows that the observed *Methylobacterium* forms a separate cluster than the one produced by the reported contaminants (Supplementary Fig S5). Histological analyses of different gut parts comparing ANA-positive and ANA-negative LT β R-deficient animals, however, revealed no inflammatory differences (Supplementary Fig S6). We also directly compared the impact of monocolonization of LT β R-Fc-treated mice with SFB versus the nonadherent and nonvirulent strain of *E. coli* MG1655 on autoantibody induction in the INRA facilities. We found an increased prevalence of ANA in SFB as opposed to *E. coli* monocolonized mice (Fig 3D). In SFB monocolonized mice, ANA frequency was markedly higher in mice lacking all gut-associated lymphoid tissue compared to mice lacking PP only (Supplementary Fig S4B).

ILF and PP contain the stromal microenvironment for IgA production, a critical antibody that helps maintain gut homeostasis (Eberl, 2007). Furthermore, it was shown that SFB can also induce IgA responses via *de novo* induction of tertiary lymphoid tissue in contrast to *E. coli* in mice lacking PP and cryptopatch-derived ILF (Lecuyer et al, 2014). Because an aberrant expansion of SFB was reported in IgA-deficient mice, it could be anticipated that IgA was protective against ANA induction. Yet ANA are present at comparable levels in mice with gut tertiary follicles, which have substantial concentrations of IgA in feces, as in mice lacking all gut lymphoid tissues, which have hardly any IgA detectable in the feces (data not shown). This argues against a critical role for IgA in LT-dependent ANA induction.

SFB are important for inducing a robust T-helper cell type 17 population in the small intestinal lamina propria of the mouse gut (Wu et al, 2010) and has the ability to induce such responses inside and outside gut organized lymphoid tissue (Lecuyer et al, 2014). To assess the potential role of IL-17 or IL-25 in the model as an effector cytokine, we treated IL-17R-deficient mice with the LT β R-Fc fusion protein from gestational day 18 until six weeks after birth as described above. In contrast to wild-type mice, this treatment did not induce significant systemic autoimmune responses (Fig 3E). In BXD2, a mouse model for lupus, IL-17 was also identified as an important effector cytokine for systemic autoimmune responses (Hsu et al, 2008).

It was reported that intestinal IgA and IgG production plasma cells, while mostly antigen specific, include a relatively high frequency of cells secreting autoreactive and polyspecific antibodies (Benckert et al, 2011; Scheid et al, 2011). This is in contrast to the bone marrow IgG plasma cells where autoreactivity is relatively rare. Because we were unable to find immune-mediated pathology despite the presence of multiple ANA reactivity, this could point to production to polyreactive intestinal B cells due to impaired architecture rather than active regulation of autoantibody responses. In such a scenario, SFB could alter the threshold for induction of ANA given its marked ability to generate potent Th17 responses.

In summary, we report that in the absence of LT IL-17R-dependent systemic autoimmune responses are associated with increased SFB colonization. Overall, our findings enforce a new paradigm that neonatal colonization of the gut impacts systemic autoimmune responses against nuclear antigens in adulthood.

Materials and Methods

Mice

C57BL/6, *Rag2*^{-/-} (C57BL/6 background) and nude mice (C57BL/6 background) were originally purchased from The Jackson Laboratory. Mice deficient in LT α (De Togni et al, 1994) (backcrossed 8 times on C57BL/6), LT β R (Futterer et al, 1998) (backcrossed 6 times), LIGHT (Scheu et al, 2002) (backcrossed 6 times), HOX11 (Dear et al, 1995) (backcrossed 10 times) and IL-17R (Ye et al, 2001) (backcrossed more than 10 times) have been described previously. *Ltb*^{-/-}, *T-Ltb*^{-/-}, *B-Ltb*^{-/-}, and *Ror γ t-Ltb*^{-/-} were generated by crossing LT β -floxed mice (Tumanov et al, 2002) with K5-cre (Ramirez et al, 2004), CD4-cre (Lee et al, 2001), mb1-cre (Hobeika et al, 2006), and ROR γ t-cre (Lochner et al, 2008), respectively. *MRL/lpr*^{-/-} and (NZB/NZW)F1 mice were purchased from Harlan Europe. Sera from NOD mice were kindly provided by Prof. C. Mathieu (University Hospital Leuven, Belgium). Pristine-induced lupus in C57BL/6 was performed as described previously (Lee et al, 2008). All these mice were housed and bred in the Ghent University vivarium in a specific pathogen-free conditions. Germfree C57BL/6 and C3H/HeN were bred in the INRA Anaxem germfree animal facilities (Jouy-en-Josas, France) and housed in isolators. For colonization, 8- to 9-week-old female germfree mice were gavaged twice at a 24-h interval with 0.5 ml of fresh anaerobic cultures of *E. coli* MG1655 or of fecal homogenate from SFB monoassociated mice (Gaboriau-Routhiau et al, 2009). Colonization by *E. coli* was monitored by bacterial counts after culture on nonselective brain heart infusion agar plates (Difco). For SFB monitoring, DNA was extracted from frozen ileal biopsies and 16S rRNA was amplified by qRT-PCR by using specific primer pairs for SFB or total bacteria on an ABI Prism 7300 Sequence Detection System (Life Technologies) as described (Gaboriau-Routhiau et al, 2009). Gnotobiotic mice were sacrificed on day 20 and 60 postcolonization. All animal procedures were approved by the Institutional Animal Care and Ethics Committee of Ghent University.

Antibodies, fusion protein, and antibiotics

Pregnant mice were injected simultaneously i.v. and i.p. with 50 μ g of LT β R-Fc on day 11 and day 15 of gestation, or only on day 18 of gestation. When the mother was injected only at day 18, progeny received weekly i.p. injections with 25 μ g of LT β R-Fc (continuous) starting at day 7 after birth until the age of 6 weeks. Controls consisted of C57BL/6 mice treated with control IgG. In addition, adult C57BL/6 mice (8 weeks) were injected i.p. weekly with 100 μ g of LT β R-Fc or control immunoglobulin for 7 weeks. Prevention of development of gut lymphoid tissues was performed with a slightly different scheme in experiments with monocolonized mice. Pregnant germfree mice were i.v. injected with 150 μ g of LT β R-Fc on day 16 and 18 of gestation. Some germfree pups born from LT β R-Fc-treated mothers were further i.p. injected with 10 μ g LT β R-Fc once a week during the first 3 weeks of life. Ampicillin, streptomycin, and colistin (Sigma-Aldrich) were supplied to mice in the drinking water at a concentration of 1, 5, and 1 g/l, respectively.

Thymus transplants

Thymi were isolated from newborn *Ltbr*^{-/-} or wild-type mice and cultured in 1.35 mM 2-deoxyguanosine (Sigma-Aldrich) for 5 days to deplete bone marrow-derived cells. Two thymic lobes were then transplanted under the kidney capsule of adult nude mice (Martins *et al*, 2008). Mice were bled 6 weeks after the procedure to verify the presence of circulating T cells by flow cytometry. After 3 months, mice were bled and sacrificed. Thymic lobes, liver, and spleen cells were analyzed by flow cytometry.

Bone marrow transfers

Bone marrow was isolated from the femur and tibia of *Ltbr*^{-/-} or wild-type mice. Three million bone marrow cells were injected in the liver of sublethally irradiated (400 rad) 7-day-old C57BL/6 and LTβR-deficient recipient mice.

Antinuclear antibodies

Specific ANA except for anti-dsDNA were detected by line immunoassay (INNO-LIA ANA Update, Innogenetics NV). The nylon strips were incubated with serum at a 1:200 dilution. Following washing, a 1:2,500 dilution of an alkaline phosphatase-conjugated anti-mouse IgG was added (Chemicon). After washing, the reaction was revealed with the chromogen 5-bromo-4-chloro-3-indolyl phosphate, producing a dark brown color in proportion to the amount of specific autoantibody in the test sample. Sulfuric acid was added to stop the color development. The cutoff of the reactivities was determined by testing 20 serum samples from 6-month-old C57BL/6 mice. None produced any background staining higher than a 1:12,800 dilution of a strong anti-RNP-A reactivity of a *MRL/lpr*^{-/-} mouse. Thus, we considered a higher intensity as a positive test result. To determine the intensity of the color as quantification for the amount of antibody in the test sample, further dilutions of the reference anti-RNP-A reactivity were used (at least as intense as 1:12,800 was considered 1+, 1:6,400 2+, 1:3,200 3+, 1:1,600 4+, 1:800 5+, and 1:200 6+). The assay contains the following recombinant and natural antigens: SmB, SmD, RNP-A, RNP-C, RNP-70k, Ro52/SSA, Ro60/SSA, La/SSB, CenpB, Topo-I/Scl70, Jo-1, ribosomal P, and histones. Anti-dsDNA IgG antibodies were detected with ELISA according to the manufacturer's instructions (Alpha Diagnostic International).

Histopathology

Tissues for histological examination were fixed in 4% buffered formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin, periodic-acid Schiff, silver staining, and Masson's trichrome using the standard technique. De-identification and coding of slides was done so that a blinded evaluation could be performed. Slides were scored by trained pathologists (LA and CAC for the gut sections and AD for the renal sections). Renal slides were scored as described previously (Keeton *et al*, 1995). Briefly, for glomerular damage, 10 random glomeruli of each animal were scored using a semiquantitative grading system. In this grading system, a grade 0 corresponds with specimens with no glomerular lesions, grade I lesions show minimal mesangial thickening, grade II

lesions contain increases in both mesangium and glomerular cellularity, and grade III lesions contain the preceding features plus superimposed inflammation and/or capsular adhesions. Grade IV lesions show obliteration of glomerular architecture involving more than 70% of the glomeruli. Furthermore, glomerular crescents, interstitial inflammation, and vascular inflammation were described. Dermal sclerosis and esophageal sclerosis was determined using ImageJ to measure the thickness of dermis and submucosa. Inflammatory scores of different part of the gut were performed according to an adapted scoring scheme (Van der Sluis *et al*, 2006; Supplementary Fig S5B).

Flow cytometry

Liver mononuclear cells were isolated using an adjusted 33% Percoll gradient (GE Healthcare) (Franki *et al*, 2006). Cell suspensions from thymus and spleen were prepared by conventional methods. Mononuclear cells were isolated from peripheral blood using a Ficoll-Paque Plus gradient (GE Healthcare). Cell stainings were performed as described previously (Franki *et al*, 2006). Cells were acquired on a FACSCanto II (Becton Dickinson) flow cytometer and analyzed using FlowJo software (Tree Star). Primary antibodies used were from eBioscience: anti-TCRβ (H57-597), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-FOXP3 (FJK-16s), and B220-APC-Cy7.

Analysis of the gut flora

The most prominent shifts within the microbiota were monitored via denaturing gradient gel electrophoresis (DGGE). After DNA extraction and PCR, gels were run using an Ingeny PhorU apparatus (Ingeny International). DNA-fragment bands on the DGGE gel that distinguished autoantibody-positive from autoantibody-negative samples on the DGGE gel were identified through sequence analysis. Specific bands were cut from the gel, after which the 180-bp sized fragment was sequenced (AGOWA). Normalization and further analysis of the gels was carried out using BioNumerics software v5.10. To identify the band of interest in more phylogenetic detail, a clone library was made from the samples of interest. PCR was performed on the sample with primers F27/R1492. PCR products were cut from the gel, purified (QIAquick Gel Extraction Kit, QIAGEN), and cloned with a TOPO TA Cloning Kit with PCR2.1-TOPO Vector (Invitrogen). Clone DNA was amplified with primers M13F/M13R, after which nested PCR was followed with DGGE primers GC-338F/518R. Clones from which the DNA band on DGGE matched with the DNA band of interest (distinctive between autoantibody-positive and autoantibody-negative) were sent for sequencing (AGOWA). Sequences (480 bp) were manually inspected and compared with databases at the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Following the identification of segmented filamentous bacteria (SFB) as distinctive DNA band between autoantibody-positive and autoantibody-negative mice, we next performed a quantitative analysis of SFB with qPCR using primers SFB736F and SFB844R as reported previously (Barman *et al*, 2008). The 16S rRNA gene was characterized by sequencing the V4 variable region. Samples were prepared using a modification of a previously described protocol (Caporaso *et al*, 2011) using the same primers as the Earth Microbiome Project (515F/806R). Amplification reactions were prepared in triplicate. Agilent Bioanalyzer was used

for quantification of the amplicons, and the Illumina MiSeq platform was used for sequencing. Paired-end reads were processed using LotuS (<http://psbweb05.psb.ugent.be/lotus/downloads.html>), and further analysis was performed using Phyloseq (McMurdie & Holmes, 2012) and R scripts. From the greengenes database (v. 2013), we identify *Candidatus arthromitus* (ID: 376862) that, according to Thompson *et al* (2012), should be *Candidatus savagella* (Thompson *et al*, 2012). Greengenes reference sequence for *Candidatus arthromitus* (ID 376862) is 100% identical to EU791150.1 (isolated from mouse distal small intestine and reported as uncultured bacteria) and 99% to several *Candidatus arthromitus* sp. SFB-mouse sequences (NR_074545.1, NR_074460.1, AP012209.1, AP012202.1, D86305.1, LK932435.1, CP008713.1, EU791182.1). All these sequence entries are annotated as *Candidatus arthromitus* in the NCBI record. Also, the closest sequence coming from full genomes is that from a SFB-Mouse-NL, a commensal bacterium associated with maturation of gut immune function published recently and reported as *Candidatus arthromitus* (Bolotin *et al*, 2014).

Statistical analysis

For categorical data analyses, Fisher's exact tests were used, whereas continuous data were analyzed by Student's *t*-tests, Mann-Whitney *U*-tests, or ANOVA with Bonferroni *post hoc* tests, using PASW 18.0 (IBM). Pearson's correlation and UPGMA (unweighted pair group method using arithmetic mean) clustering were used to calculate dendrograms, using BioNumerics v5.10 (Applied Maths).

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Author contributions

JTVP, ED, and DE conceived of and designed the study. JTVP, ED, IV, MBD, FW, AD, LA, CAC, FvdL, TVdW, PSN, SAN, SR, AAK, RT, JR, VG, NC, and CFW performed the experiments. JTVP, ED, TVdW, GE, VG, NC, and ED analyzed the data. JTVP and DE wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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