AUTOIMMUNITY

Clonal IgA and IgG autoantibodies from individuals at risk for rheumatoid arthritis identify an arthritogenic strain of Subdoligranulum

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The mucosal origins hypothesis of rheumatoid arthritis (RA) proposes a central role for mucosal immune responses in the initiation or perpetuation of the systemic autoimmunity that occurs with disease. However, the connection between the mucosa and systemic autoimmunity in RA remains unclear. Using dual immunoglobulin A (IgA) and IgG family plasmablast–derived monoclonal autoantibodies obtained from peripheral blood of individuals at risk for RA, we identified cross-reactivity between RA-relevant autoantigens and bacterial taxa in the closely related families Lachnospiraceae and Ruminococcaceae. After generating bacterial isolates within the Lachnospiraceae/Ruminococcaceae genus Subdoligranulum from the feces of an individual, we confirmed monoclonal antibody binding and CD4+ T cell activation in individuals with RA compared to controls in individuals. In addition, when Subdoligranulum isolate 7 but not isolate 1 colonized germ-free mice, it stimulated T helper 17 cell expansion, serum RA–relevant IgG autoantibodies, and joint swelling reminiscent of early RA, with histopathology characterized by antibody deposition and complement activation. Systemic immune responses were likely due to mucosal invasion along with the generation of colon-isolated lymphoid follicles driving increased fecal and serum IgA by isolate 7, because B and CD4+ T cell depletion not only halted intestinal immune responses but also eliminated detectable clinical disease. In aggregate, these findings demonstrate a mechanism of RA pathogenesis through which a specific intestinal strain of bacteria can drive systemic autoantibody generation and joint-centered antibody deposition and immune activation.

INTRODUCTION

The natural history of rheumatoid arthritis (RA) has been the focus of study for years, yet the causal triggers of RA remain unclear. Biomarkers that provide insights into disease mechanisms include two types of autoantibodies—anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF)—that develop years before disease onset and are predictive for future joint disease onset and severity (1–4). During this preclinical period, individuals are considered “at risk” for RA (5).

ACPA preferentially bind citrulline-containing epitopes on an array of proteins and can contribute to joint damage in a murine model of RA (6). RF targets the constant region of immunoglobulin (Ig), and persistently elevated titers correlate with clinical disease activity (7). These antibodies arise from separate B cell lineages and mechanisms (8), suggesting that they function differentially in the development of RA. ACPA+ B cells undergo up-regulation of genes that promote T cell–dependent responses, whereas RF+ B cells up-regulate translational programs that invoke innate immunological memory reactivation (8).

With regard to the initial development of RA-related autoantibodies, the mucosal origins hypothesis suggests that environmental interactions and chronic inflammation at mucosal surfaces may be important early drivers of RA pathogenesis (9). In support of this paradigm, ACPA are detected in the lungs of individuals with longstanding RA (10), as well as in the sputum of individuals at risk for RA (11), and this is associated with the presence of elevated cytokines, complement activation, and neutrophil extracellular trap formation (12). The oral mucosa is also implicated, as supported by the finding that Porphyromonas gingivalis, a bacterium causally linked to chronic periodontitis, encodes an enzyme capable of citrullinating proteins (13), and ACPA+ individuals exhibit a higher relative abundance of P. gingivalis in their oral microbiome (14). Another potential oral pathway links Aggregatibacter actinomycetemcomitans that are expanded in the periodontium of individuals with chronic RA (15) and have the capacity to induce host cell hypercitrullination (16) rather than direct enzymatic citrullination of proteins. Furthermore, an orally derived strain of Streptococcus has been found to be capable of inducing arthritis in the autoimmune arthritis-prone SKG mice (17). Additional data from both human and murine studies implicate the intestinal mucosa. Expansion of Prevotella copri in the gut of individuals with new-onset RA (18) and during the preclinical phase (19) has been described, and other groups demonstrate perturbations in Lactobacilli (20) and rare lineages of bacteria (21) in RA that can modulate experimental arthritis models. A further relationship to Prevotella is suggested by the finding that human leukocyte antigen (HLA)–DR–containing peptides, eluted from antigen-presenting cells of patients with...
chronic RA, derived from these bacteria share homology with two additional RA-specific autoantigens (22–24). Thus, certain lines of evidence support immune responses to bacteria at mucosal surfaces as modifiers of RA-relevant autoantibodies and amplifiers of underlying joint inflammation. However, despite these clinical associations, none of the proposed bacterial lineages have demonstrated an arthritogenic effect in wild-type mice in the absence of other strongly proinflammatory factors.

Plasmablasts are a potentially highly informative subset of B cells because they are circulating components of ongoing local immune responses (25–27) and could link immune responses at the mucosa with the joint. Recently, Kinslow et al. (28) described a substantial expansion of IgA+ plasmablasts in the circulation of individuals at risk for RA. After variable region sequencing, a large number of the isolated plasmablasts were found to arise in shared clonal families with both IgA+ and IgG+ members. These findings suggested a shared mucosal and systemic immune response evolution, although the specific triggers and mechanisms by which this conversion might happen remain unknown. We hypothesized that the antibodies produced by these dual IgA/IgG-containing family plasmablasts would recognize a mucosa-associated bacterium that could stimulate the development of autoimmunity. Here, we used informative monoclonal antibodies (mAbs) derived from circulating dual IgA/IgG-containing plasmablast families to identify candidate intestinal bacteria in feces from at-risk individuals. From that source, we isolated a bacterial strain that is targeted by both dual IgA/IgG family plasmablasts and CD4+ T cells from patients with RA. We found that colonizing mice with this bacterial strain stimulated RA-related autoantibodies and joint swelling with both IgG/IgA deposition and complement activation; we further demonstrated a B and T cell–dependent pathway by which these pathologic changes arise. This human bacterial species, provisionally classified as a Subdoligranulum didolesgii, provides a causal link between the mucosal immune system, RA autoimmunity, and joint-centered pathology. Studies of this strain will provide a clinically relevant model for understanding of the drivers of microbially driven RA-related autoimmunity.

RESULTS
A subset of dual IgA/IgG family plasmablast–derived mAbs target RA-relevant antigens and bacteria in families
Lachnospiraceae and Ruminococcaceae

We previously identified a population of circulating plasmablasts that belong to dual IgA/IgG clonal families in individuals at risk for RA (28). Hypothesizing that these plasmablasts may inform a mucosal to systemic immune response conversion leading to targeting of RA-relevant antigens, we established mAbs derived from plasmablasts (PB-mAbs) from shared IgA/IgG clonal families. Plasmablasts were isolated from individuals at risk for the development of RA (n = 4) and individuals with early RA (<1 year from diagnosis; n = 2; tables S1 and S2), and mAbs were selected for further study due to their ability to bind RA-relevant citrullinated antigens (28). Sequences from the variable regions were cloned onto a mouse IgG2a framework and expressed. A total of 94 successfully generated PB-mAbs confirmed binding of numerous RA-relevant autoantigens by multiplex array (Fig. 1A and data files S1 and S2).

Because the PB-mAbs and many circulating autoantibodies include the mucosal IgA isotype, we queried whether the PB-mAbs could target intestinal bacteria. For broad representation of fecal bacteria, we pooled feces from healthy individuals (n = 5), individuals at risk for RA (n = 8), and individuals with early RA (n = 5; table S3 and fig. S1A). By 16S ribosomal RNA (rRNA) gene sequencing, we verified high bacterial diversity in this pool (Shannon–H index value, 2.524; fig. S1B). Using a negative control PB-mAb targeting influenza and a positive control mAb to Escherichia coli, we developed a flow cytometry assay to identify PB-mAb binding to fecal bacteria (Fig. 1B). Staining greater than 2 SDs above the mean fluorescence intensity of the negative controls was considered positive binding. Using this cutoff value, a total of 61.7% (58 of 94) of the PB-mAbs targeted intestinal bacteria (Fig. 1C), although there was no association between isotype (IgA versus IgG) and bacterial binding (fig. S2A). There was no association between autoantigen binding preference and binding of bacterial targets (fig. S2B). However, mAbs that bound bacteria used a subset of heavy chain variable region (IGHV) genes compared to the non-bacteria binding mAbs (Fig. 1D and fig. S2C). In addition, the PB-mAbs were substantially mutated from germ line, although the number of mutations in the bacteria-binding versus bacteria-nonbinding PB-mAbs did not differ, indicating that these plasmablasts are likely not from a natural antibody pool (Fig. 1E and fig. S2D).

To define the bacterial targets of the PB-mAbs, we sorted the mAb-bound bacterial fraction and sequenced bacterial 16S rRNA genes using fluorescence-activated cell sorting. Predominantly targeted taxa included the closely related families Lachnospiraceae and Ruminococcaceae (Fig. 1F and fig. S3A). Greater than 50% of the total bacteria bound by the bacteria-binding mAbs combined were from families Lachnospiraceae and Ruminococcaceae (Fig. 1G). When PB-mAbs binding Lachnospiraceae and Ruminococcaceae (mAb 4, 28, 58, and 91) were matched against individual fecal samples from humans with early RA or healthy controls, there was no difference in binding to Lachnospiraceae or Ruminococcaceae between groups (fig. S3B), suggesting as anticipated a broad range of these overall families. These data demonstrate an interesting antibody cross-reactivity between bacterial targets, specifically Lachnospiraceae and Ruminococcaceae, among a subset of mAbs that bind RA-relevant autoantigenic targets.

Ruminococcaceae Subdoligranulum strains isolated from a human sample are targeted by PB-mAbs

To investigate immune responses to specific species within Lachnospiraceae and Ruminococcaceae in RA, we first established primary bacterial isolates from an individual with greater than 40% abundance of these taxa in their feces (fig. S3C). A total of 50 isolates were established, seven identified as Lachnospiraceae/Ruminococcaceae by quantitative polymerase chain reaction (qPCR) (isolates 1 to 7), and five confirmed as pure isolates by 16S rRNA sequencing (isolates 1, 3, 4, 5, and 7). These five isolates underwent whole-genome sequencing. Sequences were assembled, scaffolded, and cleaned using Abyss (29), input into the Biobakery Workflow (30), and categorized as unidentified species within the family Ruminococcaceae and genus Subdoligranulum (Fig. 2A), which we provisionally designate as a species named Subdoligranulum didolesgii. We identified this bacterium as a distinct species by aligning the genome and inserting the full-length sequence into both the SILVA Ribosomal RNA Database and National Center for Biotechnology Information (NCBI) Blast without substantial alignment to...
other entries into these databases. We narrowed down the five isolates to two candidates (isolates 1 and 7) for further study due to their differing abilities to induce joint swelling in mono-colonized mice (fig. S4). We confirmed that a subset of PB-mAbs (numbers 4, 28, 58, and 91), selected on the basis of their binding of both Lachnospiraceae and Ruminococcaceae, bound both bacterial isolates 1 and 7, whereas mAb 7 that did not bind Lachnospiraceae or Ruminococcaceae did not bind isolates 1 or 7 (Fig. 2B).

Next, to evaluate the immunologic relevance of the Subdoligranulum isolates, we assessed whether the bacteria were recognized by circulating T cells from individuals with RA. Peripheral blood mononuclear cells (PBMCs) from 11 individuals with RA were
Ruminococcaceae Subdoligranulum strains isolated from a human sample are targeted by PB-mAbs and stimulate CD4\(^+\) T cells from patients with RA. (A) Seven primary strains of *Ruminococcaceae Subdoligranulum* were isolated from the feces of an individual. Five isolates were selected for short-read genome sequencing based on taxonomic identification by 16S rRNA sequencing. The table represents percent genomic shared identity among the five isolates and against a reference genome found to be genetically aligned (MGYG-HGUT02424; unidentified genus in order *Clostridiales*, which includes *Lachnospiraceae* and *Ruminococcaceae*). (B) Isolates 1 and 7 were matched against four selected PB-mAbs (numbers 4, 28, 58, and 91) that bound highly to other various patterns of *Ruminococcaceae* and *Lachnospiraceae* species to verify that they targeted the strains. They were also matched against a control mAb (number 7) that was previously found to not bind bacteria. The percent of bacteria bound to mAb is displayed (y axis) against each selected mAb (x axis). Binding by isolate 7 is shown in black, and binding to isolate 1 is shown in gray. (C) Human peripheral blood mononuclear cells (PBMCs) from individuals with RA (n = 11) were stimulated with isolates 1 or 7 (50 ng/ml). Fold change of the CD4\(^+\) T cell response relative to dimethyl sulfoxide (DMSO; horizontal dotted line) is displayed against binding to isolates 1 and 7 (x axis). **P < 0.01, nonparametric Wilcoxon matched-pairs signed rank test. (D) A class II HLA-DR (clone L243) blocking antibody or an equal volume of phosphate-buffered saline was applied at 20 μg/ml for 30 min before stimulation of PBMCs from individuals with RA (n = 5) with isolate 7. Fold change of the CD4\(^+\) T cell response relative to DMSO (horizontal dotted line) is displayed against binding to isolate 7 versus isolate 7 blocked with L243 (x axis). ns, not significant by nonparametric Wilcoxon matched-pairs signed rank test. (E) Isolate 7–specific responses among CD4\(^+\) T cells was tested comparing CD4\(^+\) T cells isolated from individuals with RA (n = 11) to CD4\(^+\) T cells isolated from healthy controls (HC) (n = 12). Fold change of the CD4\(^+\) T cell response relative to DMSO (y axis) is displayed (x axis). Data were analyzed using a nonparametric Mann-Whitney test. (F) Left: Using CD45RA\(^-\)/CXCR3\(^-\)/CCR4\(^-\)/CCR6\(^-\) as a defining marker combination, we compared the relative proportion of T\(_{h}^{1}\)-like cells for isolate 7–specific (circles) or influenza-specific (inverted triangles) memory CD4\(^+\) T cell responses in CD4\(^+\) T cells from individuals with RA, observing a higher proportion of T\(_{h}^{1}\)-like influenza–specific cells (P = 0.0078, nonparametric Mann-Whitney test). Right: Using CD45RA\(^-\)/CXCR3\(^-\)/CCR4\(^+\)/CCR6\(^+\) as a defining marker combination, we compared the relative proportion of T\(_{h}^{17}\)-like cells for isolate 7–specific (circles) or influenza-specific (inverted triangles) memory CD4\(^+\) T cell responses, observing a significantly higher proportion of T\(_{h}^{17}\)-like isolate 7–specific cells (P = 0.0078, nonparametric Mann-Whitney test). Horizontal bars indicate means.
stimulated with oxygen-killed *Subdoligranulum* isolate 1 or isolate 7 (50 ng/ml) for 14 hours. Compared to isolate 1, isolate 7 significantly (*P* < 0.01) activated CD4+ T cells in the PBMCs as measured by accumulated surface CD69 and CD154 expression (Fig. 2C, fig. S5, and table S4). Blocking major histocompatibility complex class II with an anti-DR antibody did not result in significantly (*P* > 0.05) reduced CD69+ CD154+ expression (Fig. 2D). Furthermore, using previously unexamined individuals, isolate 7–specific responses were more prevalent in CD4+ T cells derived from RA cases (*N* = 11) as compared to healthy controls (*N* = 12) (Fig. 2E and table S5). Last, we examined the cell surface phenotype of isolate 7– and influenza-specific CD4+ T cells in the same RA cases (*n* = 11) using accepted definitions for T helper cell 1 (Th1)–like (CD45RA+/CXCR3+/CCR4+/CCR6+) and Th17–like (CD45RA−/CXCR3−/CCR4−/CCR6−) cells (31), observing a significantly (*P* < 0.01) higher proportion of Th1–like influenza–specific CD4+ T cells and a significantly (*P* < 0.01) higher proportion of Th17–like isolate CD4+ T–specific T cells (Fig. 2F). Our findings support the hypothesis that the *Subdoligranulum* isolate 7 elicits immunologically relevant CD4+ T cell responses and suggest that strain variations may be of importance to understand the pathogenesis of RA.

**Subdoligranulum isolates stimulate joint swelling characterized by antibody and complement deposition in mono-colonized mice**

Having associated immunity toward *Subdoligranulum* isolates with RA and deriving them from a human sample, we then investigated whether the isolates were capable of inducing RA-relevant autimmunity in mice. *Subdoligranulum* isolates 1, 3, 4, 5, and 7 were gavaged at 5 × 10^6 colony-forming units (CFU) into germ-free DBA/1 mice. Given the previously published association of *P. copri* with new-onset RA (18) and the homology between proteins in this bacterium and RA-relevant autoantigens (22–24), we also mono-colonized a group of germ-free DBA/1 mice with *P. copri* [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 18205 (32)] as a control in addition to a sterile media gavage. Four of the five *Subdoligranulum* strains, and neither control, caused joint swelling spontaneously starting about 14 days after bacterial gavage and persisted until 35 days when most mice were euthanized (Fig. 3, A and B, and fig. S4A), although when followed through 63 days, arthritis was still observed (fig. S4B). Germ-free C57BL/6j mice also developed very mild but detectable joint swelling after mono-colonization with isolate 7 (fig. S4C). We confirmed equal, stable bacterial colonization across groups (fig. S4, D to F). To confirm that our findings were not due to immunologic impairments of the germ-free state of the mice (33), we colonized specific pathogen–free (SPF) DBA/1j mice that had been pretreated with broad-spectrum antibiotics orally to deplete the microbiome. We then gavaged these mice with either isolate 7, isolate 1, or *P. copri* or left them untreated, and we were able to replicate the paw swelling phenotype under these conditions (Fig. 3C). The finding of arthritis development with introduction of a single strain alone was unexpected, because nontransgenic murine models of joint disease typically require intradermal injection of antigen with adjuvant, adjuvant alone, or intravenous transfer of pathogenic antibodies to develop disease (34, 35).

Evaluation of pathology in germ-free mono-colonized mice demonstrated a range of synovitis, osteitis, vasculitis, and soft tissue inflammation, all mild in severity (fig. S6). However, all combined, mice mono-colonized with isolate 7 had the highest incidence of pathology compared to the other treatment groups (Fig. 3D). Despite mild immune cell infiltrate, immunohistochemistry of the joints for Ig deposition and complement protein C3 demonstrated marked deposition both in the joint space and intra-dermally (Fig. 3E and fig. S7A). In addition, immunohistochemistry demonstrated IgG and IgA deposition in the joint (Fig. 3, F and G, and fig. S7, B and C). These findings suggest that an antibody-mediated process may be a key factor in driving the joint swelling in this murine phenotype.

**Serum IgA, RA-related autoantibodies, and splenic Th17 cells expand in *Subdoligranulum* isolate 7–mono-colonized mice**

To understand the systemic immune response after gavage with isolate 1, isolate 7, *P. copri*, or sterile media, serum from mice was collected on days 14 and 35 after gavage. The total serum IgA was significantly (*P* < 0.0001 compared to isolate 1, *P* < 0.05 compared to *P. copri*, and *P* < 0.05 compared to sterile media) increased in isolate 7–mono-colonized mice at day 14 as compared to the other groups (Fig. 4A), but this normalized by day 35 (fig. S8A) and was not observed for IgG (fig. S8B). In SPF mice mono-colonized with isolate 7, both IgA and IgG were increased in the serum at days 14 and persisted at day 35 (fig. S9).

We next evaluated serum autoantibodies using a planar array containing about 350 RA-relevant autoantigens. Isolate 7–gavaged mice developed and maintained serum autoantibodies against RA-relevant autoantigens at higher proportions 14 and 35 days after gavage (*P* < 0.05) than mice in the other groups (Fig. 4B). Furthermore, isolate 7–mono-colonized mice developed several autoantibodies targeting RA-relevant antigens, such as fibromodulin, at greater titers in comparison to the other groups (Fig. 4C). There was reactivity to both citrullinated and native peptides, analogous to the reactivity profiles of the human PB-mAbs (Fig. 1A). These data indicate that mono-colonization with isolate 7 allows for the establishment of persistent RA-relevant autoantibodies in circulation.

DBA/1 mice are prone to generate anti–murine type II collagen (anti-CII) antibodies in the collagen-induced arthritis (CIA) model. Thus, we specifically assayed these by enzyme-linked immunosorbent assay (ELISA) in day 14 and 35 serum from our mono-colonized mice. Mice mono-colonized with isolate 7 developed anti-CII autoantibodies comparable to mice with CIA (Fig. 4D).

Given CD4+ T cell reactivity to isolate 7 in patients with RA, we evaluated splenic CD4+ T cell populations at days 14 and 35 in mice mono-colonized with isolate 1, isolate 7, or *P. copri* or gavaged with sterile media (fig. S10A). We focused on Th17 and activated regulatory T cell (*Treg*) populations due to the demonstrated role of intestinal microbiota in their development (36, 37), and on T follicular helper cells (Tfh) because of our observed changes in autoantibodies. At day 14, splenic Th17 cells were significantly increased in percentage (*P* < 0.05 compared to *P. copri* and *P* < 0.01 compared to sterile media), absolute number (*P* < 0.05 compared to *P. copri* and sterile media), and Th17/*Treg* ratio (*P* < 0.05 compared to *P. copri* and *P* < 0.01 compared to sterile media) in isolate 7–mono-colonized mice (Fig. 4E). However, we did not observe...
Fig. 3. A specific *Subdoligranulum* strain stimulates joint swelling and inflammation in mono-colonized mice that is characterized by IgG, IgA, and complement C3 deposition in joints. (A and B) *Subdoligranulum* isolates 1 and 7 as well as *Prevotella copri* or sterile media were gavaged separately into germ-free DBA/1 mice (*n* = 6 isolate 1, *n* = 6 isolate 7, *n* = 5 *P. copri*, and *n* = 6 sterile media). Mice were observed weekly for 35 days for the development of joint swelling and assessed a score based on the number of joints affected. (A) The means ± SEM score is shown (y axis) over time after bacterial gavage (x axis). **** *P* < 0.0001, repeated measures ANOVA. (B) Representative photographs of paws from the treatment groups are shown to demonstrate the swelling observed in mice mono-colonized with isolate 7. (C) SPF DBA/1j mice were treated with oral broad-spectrum antibiotics (neomycin, ampicillin, metronidazole, and vancomycin) for 5 days to deplete the microbiome. After antibiotic treatment, mice were gavaged with either *Subdoligranulum* isolates 1 and 7 or *P. copri* (*n* = 6 isolate 7, *n* = 6 isolate 1, *n* = 6 *P. copri*, *n* = 5 antibiotics only). Mice were observed weekly for 35 days for the development of joint swelling and assessed a score based on the number of joints affected. The means ± SEM score is shown (y axis) over time after bacterial gavage (x axis). **** *P* < 0.0001, repeated measures ANOVA. (D) Paw histology was assessed by a pathologist in a blinded fashion. Displayed is the incidence of pathology (y axis) separated by treatment group (x axis). Data were analyzed using Fisher’s exact test. (E) Immunohistochemistry (IHC) for the C3 component of complement was performed on decalcified paw sections. C3 staining intensity scores are displayed (y axis) separated by treatment group (x axis). Symbols represent individual mice, and bars indicate the means ± SEM. ** *P* ≤ 0.01, one-way ANOVA with Tukey’s posttest. A representative section for isolate 7 is displayed, with deposition indicated by arrows. Scale bar, 100 μm. (F) IgG deposition in decalcified paw sections was assessed by IHC. Intensity scores with symbols as individual mice and bars as means ± SEM are displayed (y axis), separated by treatment group (x axis). * *P* ≤ 0.05 and ** *P* < 0.01, one-way ANOVA with Tukey’s posttest. A representative section is displayed, with deposition indicated by arrows. Scale bar, 100 μm. (G) IgA deposition in decalcified paw sections was assessed by IHC, and intensity scores are displayed (y axis), separated by treatment group (x axis). Symbols represent individual mice, and bars indicate the means ± SEM. ** *P* ≤ 0.01, one-way ANOVA with Tukey’s posttest. A representative section is displayed, with deposition indicated by arrows. Scale bar, 100 μm. Data are from *n* = 12 isolate 1–gavaged, *n* = 11 isolate 7–gavaged, *n* = 11 *P. copri*–gavaged, and *n* = 11 sterile media–gavaged animals across two experiments.
Fig. 4. *Subdoligranulum* isolate 7 causes development of increased serum IgA, systemic RA-related autoantibodies, and expanded splenic Th17 populations. (A to C) Serum from mice mono-colonized with either isolate 1 (n = 12), isolate 7 (n = 12), or *P. copri* (n = 10) or given sterile media (n = 10) was collected at days 14 and 35 after gavage. (A) The total serum IgA at 14 days after gavage was determined by ELISA; serum IgA is displayed (y axis) against treatment group (x axis). Symbols represent individual mice, and bars indicate the means ± SEM. *P < 0.05 and ****P < 0.0001, Kruskal-Wallis test with Dunn’s posttest. (B) Serum was analyzed on a planar array containing about 350 citrullinated and native peptides for auto-antigens relevant in RA. A cutoff for positivity was established at the 80th percentile of autoantibody reactivity for individual samples on this assay (1.5 relative units) and the proportion of murine samples meeting or exceeding this threshold (11 antigens as displayed) at each time point is shown (y axis). Each treatment group is shown from left to right. (C) Means ± SD values for serum reactivity from each treatment group with specific RA-relevant autoantigens shown in (B) are shown in the table, comparing the four groups. P values were determined by Kruskal-Wallis test with Dunn’s posttest. *n = 6 CIA mice, n = 6 isolate 7–gauged mice, n = 6 isolate 1–gauged mice, n = 6 *P. copri*–gauged mice, n = 6 sterile media–gauged mice. (D) Serum was analyzed at days 14 and 35 after bacterial gavage for the presence of murine collagen type II (CII) autoantibodies by ELISA. Means ± SEM relative values are shown (y axis) comparing groups (x axis), including mice with collagen-induced arthritis (CIA). *P < 0.05, **P < 0.01 by Kruskal-Wallis test with Dunn’s posttest. (E to G) Spleens were collected from each mouse at 35 days after gavage, and CD4+ T cell populations were analyzed by flow cytometry (n = 7 isolate 7, n = 7 isolate 1, n = 6 *P. copri*, n = 8 sterile media). The percentage (left) and the absolute number (middle) of Rorty+ Th17 cells and the Th17 to Th0 ratio (right) are displayed. Symbols represent individual mice, and bars indicate the means ± SEM. *P < 0.05 and **P < 0.01 using Kruskal-Wallis test with Dunn’s posttest. (F and G) Splenocytes were collected from mice gavaged with *Subdoligranulum* isolate 1 or isolate 7 at 35 days after gavage, and CD4+ T cells were isolated. CD4+ T cells were cocultured with bone marrow dendritic cells loaded with either isolate 1, isolate 7, or no bacterial antigen for 14 hours. (F) The percentage of CD154+ CD69+ CD4+ T cells is shown (y axis) compared by treatment group (x axis). The CD4+ T cell stimulation assay was performed in technical triplicate for each mouse and condition in vitro.
differences in activated T_{reg} or Tfh subsets in the isolate 7 group (fig. S10, B and C). By day 35, the expansion of T_{H17} cells remained in isolate 7–mono-colonized mice compared to sterile media–gavaged mice (fig. S11). At day 35, a similar expansion of T_{H17} cells was observed in SPF DBA/1j mice that were gavaged with isolate 7 (fig. S12, A and B) and is accompanied by a significant decrease in absolute number (P < 0.05 compared to isolate 1 and P. copri, P < 0.01 compared to antibiotics only) and percentage (P < 0.05 compared to isolate 1, P < 0.01 compared to P. copri) of splenic activated T_{reg} populations (fig. S12C). However, similar to germ-free mono-colonized mice, there was not an observable difference in Tfh frequencies between groups (fig. S12D).

To further understand this splenic T_{H17} skewing in vitro, splenic CD4^+ T cells were isolated from germ-free DBA/1 mice mono-colonized with either isolate 1 or isolate 7 and cocultured with bone marrow–derived dendritic cells loaded with either isolate 1 or isolate 7. Markers of T cell activation CD69 and CD154 were increased in CD4^+ T cells from isolate 7–mono-colonized mice and stimulated with isolate 7 after 14 hours of coculture as compared to CD4^+ T cells stimulated with isolate 1 (Fig. 4F and fig. S13), as well as markers of T_{H17} proliferation and function (Fig. 4G). Our data are consistent with others' findings of T_{H17} expansion aiding in the development of autoantibody-mediated arthritis in mice (38) and in the evolution of human RA (39, 40). These findings in aggregate suggest a possible mucosal-to-systemic immune system response driven by IgA, resulting in the targeting of autoantigens by the IgA that reach the systemic circulation after being educated in the mucosa.

**Subdoligranulum isolate 7 generates intestinal isolated lymphoid follicles and increased IgA and T_{reg} skewing**

We next investigated the effects of *Subdoligranulum* isolate 7 on intestinal immunity. First, to determine whether isolate 7 relative to isolate 1, *P. copri*, or sterile media affected intestinal permeability, fluorescein isothiocyanate (FITC)–dextran was administered orally to mice 4 hours before euthanasia, at which time serum was collected and the concentrations of FITC–dextran was measured. All three mono-colonizations resulted in improved barrier compared to sterile media (Fig. 5A), indicating that the two *Subdoligranulum* isolates and *P. copri* were capable of at least partially restoring the barrier defect of germ-free mice (41). Intestinal histology revealed significantly increased numbers of isolated lymphoid follicles (ILFs) in isolate 7–mono-colonized mice as compared to *P. copri*– and isolate 1–mono-colonized mice (P < 0.05 compared to *P. copri* and sterile media); no difference in mean ILF size was observed (Fig. 5, B and C). We did not observe changes in colonic crypt depth or small intestinal villus morphology among isolate 7–gavaged mice (fig. S14). There was an increase in villus width and crypt depth among isolate 1–gavaged mice, which could suggest a mild injurious intestinal effect among mice gavaged with this strain (fig. S14). The ILFs in isolate 7–mono-colonized mice more closely resembled classical mature ILF morphology (Fig. 5C and fig. S15) (42), suggesting increased mucosal IgA generation (43). Luminal IgA secretion was significantly increased in isolate 7–mono-colonized mice (P < 0.05 compared to sterile media, P < 0.0001 compared to isolate 1) (Fig. 5D), although, similar to the serum, this difference resolved by day 35 (fig. S16A). There was no difference (P > 0.05) in fecal IgM among groups (fig. S16B). An increase in fecal IgA was similarly seen in SPF mice that were gavaged with isolate 7 compared to mice gavaged with isolate 1 and *P. copri* at day 14 and compared to *P. copri* and antibiotics at day 35 (fig. S17). This indicates the possibility that T-dependent IgA is being produced and is coating the bacteria in the lumen, thereby reducing the total free IgA in the lumen. This hypothesis is supported by the presence of IgA^+ B cells and IgA^- B220^- plasma cells being generated in the mature ILFs seen in isolate 7–colonized mice (Fig. 5E and fig. S18). In associated mucosal lymphoid tissues mesenteric lymph nodes and Peyer’s patches, the ratio of T_{H17}/T_{reg} T cell subsets increased significantly (P < 0.05) in the isolate 7–mono-colonized mice compared to sterile media–gavaged mice at day 14 after gavage (Fig. 5F), although, the percentages and absolute numbers within the T_{H17}, T_{reg}, and Tfh populations individually were not different across groups (fig. S19, A to C). There is evidence of increased host access by isolate 7–mono-colonized mice compared to isolate 1– and *P. copri*–gavaged mice as demonstrated by the bacterial presence in the host epithelium (Fig. 5, G and H), and mucus area in square millimeters was reduced in isolate 7–mono-colonized groups compared to the *P. copri*–mono-colonized groups, but greater than germ-free mice (fig. S20), suggesting that isolate 7 potentially affects mucus thickness. In aggregate, our observations suggest that *Subdoligranulum* isolate 7 can directly access the host to stimulate a robust intestinal immune response characterized by the formation of ILFs functioning to secrete IgA.

**Joint swelling in *Subdoligranulum* isolate 7–colonized mice is dependent on T and B cells but not granulocytes**

To determine whether the observed paw swelling was truly mediated by adaptive immunity, we selectively depleted B cells, CD4^+ T cells, or granulocytes using mAbs (fig. S21) 2 days before mono-colonizing mice with isolate 7. The depletions were efficacious at depleting the B cells by 2-fold, the CD4^+ T cells by 10-fold, and granulocytes by 5-fold on average. Mice depleted of CD4^+ T or B cells did not develop paw swelling, whereas control antibody–treated mice did (Fig. 6A), indicating that adaptive immunity is required for our phenotype. Although mice depleted of granulocytes developed paw swelling equal to treatment with control antibody, the onset of swelling was delayed by about 1 week (Fig. 6A), suggesting that, although granulocytes aid in the phenotype, they are not essential. ILFs were not affected in the CD4^+ T cell–, B cell–, or granulocyte-depleted mice (Fig. 6B). Circulating and fecal IgA was significantly decreased in the B- and CD4^+ T cell–depleted mice at days 14 (P < 0.01) and 35 (P < 0.05 for B cell and P < 0.01 for CD4^+ T cell depletion) after bacterial gavage, and circulating and fecal IgG was decreased at day 35 after bacterial gavage (Fig. 6, C to F). Circulating IgA was significantly reduced in the granulocyte-depleted mice at day 14 (P < 0.05) and day 35 (P = 0.05), and circulating IgG was reduced in granulocyte-depleted mice at day 35 (P < 0.05). These findings suggest that granulocyte-dependent IgA and IgG synthesis at the mucosal surface that spreads systemically may be important in this model.

As the isolate 7–colonized mice develop serum autoantibodies to RA-relevant antigens, we queried whether they were pathogenic. Serum was collected from mice 35 days after colonization with isolate 1, isolate 7, or *P. copri* and pooled by colonization group. Intraperitoneal injection of serum from isolate 7–colonized mice into healthy germ-free DBA/1 mice (Fig. 6G and fig. S22) and SPF DBA/1j mice (Fig. 6H) resulted in paw swelling observed within days and
Subdoligranulum isolate 7 is detectable in the feces of individuals in the at-risk period and early stages of RA

To determine the potential biological relevance of Subdoligranulum isolate 7 in humans at risk for and early RA (less than 1 year from diagnosis), we used regions of the genome unique to isolate 7 and not present in isolate 1, to design a specific qPCR assay testing for the presence of isolate 7. The qPCR assay demonstrates specificity toward isolate 7 and sensitivity down to 10,000 bacteria per 100 mg
Fig. 6. Joint swelling is dependent on T and B cells but not granulocytes. (A) Mice were selectively depleted of their B cells (n = 4), CD4+ T cells (n = 5), or granulocytes (n = 5) through administration of depleting mAbs (n = 5 isotype control). Joint swelling was assessed as previously, and the means ± SEM score (y axis) is shown over time (x axis). ****P < 0.0001 by repeated measures ANOVA. (B) ILF area in square micrometers (left) and numbers (right) were assessed in colon histology from isotype control and cell-depleted mice at day 35 after bacterial gavage. Symbols represent individual mice, and bars indicate the means ± SEM. P values were determined by one-way ANOVA with Tukey’s posttest. (C to F) Total IgA and IgG in serum (C and D) and feces (E and F) at days 14 and 35 after bacterial gavage were determined by ELISA. Symbols represent individual mice, and bars indicate the means ± SEM. *P < 0.05; **P < 0.01; and ns, not significant as determined by Kruskal-Wallis test with Dunn’s posttest. (G and H) Pooled day 35 serum from mice mono-colonized with isolate 1, isolate 7, and P. copri was injected into healthy germ-free DBA/1 mice or SPF DBA/1j mice, and these mice were monitored for the development of joint swelling. (G) The mean clinical score ± SEM (n = 6 isolate 7 serum transfer, n = 6 isolate 1 serum transfer, n = 7 P. copri serum transfer) is shown relative to time after-serum transfer (x axis) for germ-free mice. *P < 0.05; **P < 0.01, Kruskal-Wallis test with Dunn’s posttest. (H) The mean clinical score ± SEM (n = 6 isolate 7 serum transfer, n = 6 isolate 1 serum transfer, n = 6 P. copri serum transfer) is shown relative to time after-serum transfer (x axis) for SPF mice. *P < 0.05; **P < 0.01; ***P < 0.001, Kruskal-Wallis test with Dunn’s posttest.
of fecal bacteria (Fig. 7A). We then screened the feces of healthy controls (n = 12), individuals at risk for RA (n = 12), and with early RA (n = 12; table S6). The prevalence of isolate 7 above the limit of detection was 16.7% in the at-risk and early RA groups but 0% in healthy controls (P < 0.001; Fig. 7B). The abundance of isolate 7 varied among the positive samples, with an average of 1.637 × 10^7 ± 3.059 × 10^7 CFUs per positive sample (Fig. 7C). Previous studies have estimated that 100 mg of fecal weight contains roughly 4.1 × 10^{12} bacteria (46), indicating that our positive samples have an average of 0.04 ± 0.07% abundance of isolate 7 in their microbiomes (table S7). We similarly tested 12 unmanipulated mice from our SPF colony for the presence of isolate 7. Isolate 7 was undetectable in the feces of all of the mice (Fig. 7B). Thus, the presence of isolate 7 appears to be restricted to a subgroup of individuals at risk for and with RA.

**DISCUSSION**

The mucosal origins hypothesis for the development of RA is based on compelling human immunologic and phenotypic data: IgA plasmablasts and dual IgA/IgG clonal families are expanded in the circulation during the at-risk period preceding clinically apparent RA (28); ACPA have been detected at several mucosal surfaces throughout the human body (9–11, 47) and are often of an IgA isotype (28); periodontitis, *P. gingivalis*, and *A. actinomyctetemcomitans* in the oral mucosa link to ACPA generation (13, 14, 16); and cross-reactivity between proteins in *P. copri* and RA-relevant autoantigens has been suggested (22–24). Nevertheless, no specific microbe-derived organism has to date been shown to be both recognized by patients with RA and able to singularly cause joint disease in experimental models. Other studies have demonstrated that strains can modulate existing arthritis (17, 48, 49), but the demonstration of the ability of a strain to incite pathology independently in genetically unmanipulated and nonmutant mice is unique. Using dual IgA/IgG plasmablast–derived mAbs from human participants at risk for RA as a tool to probe the mucosal origins hypothesis, our findings establish a line of data linking a specific intestinal bacteria in the genus *Subdoligranulum* with local intestinal ILF formation, T cell– and RA-related autoantibody development, and, ultimately, paw swelling associated with IgG, IgA, and C3 deposition in mice.

Not only do the presence of IgA plasmablasts and shared clonality with IgG plasmablasts during the at-risk period in RA suggest a mucosal trigger for ACPA generation and disease (28), but they also provide an important tool to probe for potential antigens driving their clonal expansion. Although the hallmark of ACPA is the citrulline specificity, recent studies of PB-mAbs from the peripheral blood of patients with RA challenge this concept, often finding cross-reactivity with antigens containing and lacking posttranslational modifications (50, 51). Finding that poly-autoreactive PB-mAbs from at-risk individuals similarly reacted with *Lachnospiraceae* and *Ruminococcaceae* further supports that bacteria could potentially drive a polyreactive response with RA-related antigens, as suggested for ACPA. We are aware of the intricacies of the terms polyreactive and cross-reactive and have chosen to term these PB-mAbs as cross-reactive, because we hypothesize that there may be a discrete antigen(s) on the *Subdoligranulum* isolates that is a binding target for these antibodies. However, further studies are necessary to confirm the identity of this putative antigen(s) and determine whether molecular mimicry is relevant to the interaction.

Our initial cohort of PB-mAbs was derived from a relatively small sample size of individuals at risk for and with RA, potentially limiting the generalizability of the results. We used this initial small cohort as a probe to identify potential intestinal bacteria that could stimulate systemic autoantibody responses. Future directions are aimed at elucidating the antigenic properties of *Subdoligranulum* isolate 7 to which a larger population of individuals at risk for and with RA can be screened for serum antibody reactivity by ELISA. Given that antibodies derived from each of the initial cohort of six individuals were reactive with families *Lachnospiraceae* and *Ruminococcaceae*, we hypothesize that many individuals at risk for and with RA will have serum reactivity but acknowledge that there are multiple other likely relevant bacterial candidates within the intestine and at other mucosal sites.

The bacterium of interest in this study, *Subdoligranulum* isolate 7, is a member of a highly heterogeneous group of bacteria belonging to the family *Ruminococcaceae* that is phylogenetically interlinked with the family *Lachnospiraceae* (52, 53), both within order
Clostridiales. We propose to name this bacterial isolate Subdoligranulum didolesgii, because Didolesgii is the Cherokee word for arthritis or rheumatism, and the first author is an enrolled member of the Cherokee Nation of Oklahoma (54). As we have not yet determined whether this bacterium is a commensal organism or a pathobiont, we have avoided using these terms here. Ruminococcaceae and Lachnospiraceae are two of the most abundant families in both the human and murine gut microbiome (55, 56), raising the question of why certain strains could lead to the development of arthritis without RA being a ubiquitous disease in the population. Several potential mechanisms could explain this observation. Both Lachnospiraceae and Ruminococcaceae live deep in the mucosa, occupy a niche that allows them close access to their host organism and subsequently greater immunomodulatory potential compared to bacteria localized in the lumen (57). Furthermore, heterogeneity in families Lachnospiraceae/Ruminococcaceae may be due in part to their engagement in lateral gene transfer or, alternatively, infection by bacteriophages, leading to the expression of distinct proteins and gain of functions that could lead to host immunomodulation. At-risk individuals who are serum ACPA⁺ harbor increased abundances of Lachnospiraceae and Ruminococcaceae phages in their feces compared to ACPA⁻ at-risk individuals and controls (58). Molecular mimicry between bacterial and host proteins may lead to the development of autoimmunity, or bacterial products may alter mucosal immune system dynamics. In support of molecular mimicry is the recent discovery of T and B cell–targeted autoantigens in RA with shared homology between synovial and P. copri proteins (22). Alternatively, murine models of autoimmune arthritis, as evidenced by the K/BxN, CIA, SKG, and HLA-B27/β2m models, demonstrate that microbial stimulation of the T₃₁₁十七 pathway is required for the development of arthritis (59–63). Last, community dynamics within microbial populations under different environmental pressures could lead to altered metabolite generation, which affect immune responses within the host. For example, bacterial metabolism generates short-chain fatty acids like butyrate and propionate that promote Treg development and function, protecting from CIA and HLA-B27/β2m-mediated arthritis (64, 65). Thus, Subdoligranulum didolesgii may be able to promote RA-relevant autoimmunity through multiple pathways.

Mice gavaged with Subdoligranulum isolate 7 developed paw swelling in a manner that is highly reproducible through blinded scoring. Although a profound immune infiltrate into the joints was absent, there was an increase in IgG, IgA, and C3 deposition. These findings could resemble the stage of RA development in humans known as tenosynovitis, which is tendon sheath inflammation that can be associated with microscopic synovitis in the hands and feet during the at-risk and early RA periods (66, 67). We see evidence of mild synovial inflammation in the joints of mice colonized with Subdoligranulum isolate 7. If we are indeed capturing a stage of joint swelling similar to tenosynovitis in our mice, then this model may be more aligned with the typical pattern of human RA development than previous murine arthritis models. In addition, this model may be more aligned with the current two-hit hypothesis for the development of RA, the first hit being an environmental stimulus that catalyzes the development of circulating RA-relevant autoantibodies and tenosynovitis, and the second hit being an additional factor, whether genetic or environmental, that triggers the overt synovitis in classifiable RA (9, 68).

We were also able to appreciate substantial local histopathologic and immune changes in the gut of the mice gavaged with Subdoligranulum isolate 7. One key finding was the generation of mature ILFs in the colon, which are known to develop dynamically in response to bacteria in the intestine (69). They are composed mostly of B cells with T cells and CD11c⁺ dendritic cells interspersed (70). Mature ILFs can form germinal centers, and the B cell repertoire inside of them has been shown to resemble systemic B cell populations (71). Clinically, ILF hyperplasia is linked with IgA dysfunction (72) and has been seen in children with circulating IgA and IgG against milk proteins (73), signaling the potential for mature ILFs to stimulate systemic immune responses. Subdoligranulum isolate 7 seems to stimulate mature ILFs, likely after direct interactions with immune cells after penetrating the epithelial barrier. This finding is corroborated with our observation that Subdoligranulum isolate 7 gains increased access to the host epithelium as compared to isolate 1. In aggregate, these findings could suggest a mechanism by which isolate 7 produces an immune response that triggers a mucosal to systemic immune response conversion. Although we observed indications of inflammation at the gut, spleen, and accompanying arthritis, other sites of potential inflammation have not been studied and could provide more mechanistic clues for this observed phenotype. We propose that similar patterns of mucosal and systemic immune responses may occur in humans, as evidenced by the targeting of isolate 7 by plasmablast-associated antibodies, CD4⁺ T cell responses against isolate 7, and our ability to detect isolate 7 in the feces of individuals at risk for and with early RA.

Our study has limitations. First, as discussed above, our initial plasmablast cohort was developed from six individuals at risk for or with early RA, and our conclusions would be strengthened by additional PB-mAbs from a larger group of individuals and from individuals without RA or risk thereof. Next, our data do not identify differences in PB-mAb binding to Lachnospiraceae and Ruminococcaceae in the feces of healthy controls versus individuals with RA. This does not necessarily indicate that there are no intrinsic differences in the microbial composition between groups and could indicate a degree of nonspecificity in the binding to the bacteria of interest. PB-mAbs could be binding both pathogenic and non-pathogenic Ruminococcaceae and may do so at varying degrees of affinity. Future studies aimed at examining the “pathogenic” properties of isolate 7, and identifying target antigens may elucidate this limitation. Last, although our qPCR data can detect the presence of Ruminococcaceae Subdoligranulum isolate 7 in the feces of individuals at risk for and with early RA, but not in healthy controls, the tested sample size was very limited; stronger conclusions regarding the prevalence of this isolate 7 in humans at risk for and with RA will require a larger and longitudinal population study.

Together, our data suggest one pathway by which the intestinal microbiome and mucosal immune responses can lead to systemic autoreactivity and joint pathology that is potentially a pathway in human RA. These data are strengthened by our ability to detect Subdoligranulum isolate 7 in the feces of individuals at risk for and with early RA. Numerous outstanding uncertainties remain: the prevalence of our specific Subdoligranulum strain in the general population versus those with RA and strain variations that could explain our findings; specific interactions between Subdoligranulum and the host and the mechanism for T cell and B cell responses toward it; the necessity of epithelial invasion for the observed phenotypes; the relationship between Subdoligranulum itself and the
rest of the microbial community; and lastly, the role of host genetics, especially shared epitope, during the immune responses to *Subdoligranulum*. Nevertheless, our results highlight the utility of using host immune responses, here the IgA/IgG response in plasmablasts, to identify relevant microbiota at mucosal surfaces that contribute to RA.

**MATERIALS AND METHODS**

**Study design**

The objective of this study is to understand the bacterial targets of human autoantibodies in the context of RA, to isolate these bacteria, and to understand their role as drivers of murine joint disease. For all experiments, the number of replicates, statistical test used, and *P* values are reported in the figure legends. The reported replicates refer to biological replicates, either murine or human. For human studies, individuals were recruited at either the University of Colorado or Benaroya Research Institute under institutional review board approval. Full details of the human cohorts used can be found in the Supplementary Materials and Methods. For murine studies, mice were derived germ-free and housed at the University of Colorado gnotobiotic facility under institutional animal care and use committee approval. Cages of mice were randomly assigned to different treatment groups, and the assessor of joint swelling was blinded to treatment group. To ensure animal welfare, weight loss and signs of pain and distress were used as rationale for the premature end point of any study. For all histopathological analysis, the scorer was blinded to treatment group. Full experimental details can be found in the Supplementary Materials and Methods.

**Statistical analysis**

All raw, individual-level data for experiments where *n* < 20 are found in data file S3. Unless noted elsewhere, normally distributed data as determined by D’Agostino test were evaluated by analysis of variance (ANOVA) and a post hoc *t* test with Tukey’s correction. Nonparametric data were analyzed using Mann-Whitney or Kruskal-Wallis with Dunn’s correction. Qualitative data were compared using chi-square and Fisher’s exact tests where noted. Data were analyzed using GraphPad Prism 9 Software.

**Supplementary Materials**

This PDF file includes:

- Materials and Methods
- Figs. S1 to S22
- Tables S1 to S11
- References (74–95)

Other Supplementary Material for this manuscript includes the following:

- Data files S1 to S3
- MDAR reproducibility checklist

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**


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Clonal IgA and IgG autoantibodies from individuals at risk for rheumatoid arthritis identify an arthritogenic strain of *Subdoligranulum*


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**A bacterial driver of arthritis**
Autoantibodies can be detected in individuals at risk for developing rheumatoid arthritis (RA) before development of clinical disease. The source of these autoantibodies, however, remains unclear. Here, Chriswell *et al.* identified that IgG and IgA autoantibodies from individuals who are at risk for RA cross-react against gut bacteria in the *Lachnospiraceae* and *Ruminococcaceae* families. Further analysis identified a bacterial strain from the *Subdoligranulum* genus that was associated with autoantibody development. Mice colonized with this *Subdoligranulum* isolate developed arthritis with pathology similar to human RA. These findings suggest that this *Subdoligranulum* strain may be a major contributor to RA autoantibody development.

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