Disrupting myelin-specific Th17 cell gut homing confers protection in an adoptive transfer experimental autoimmune encephalomyelitis

DUC, Donovan, et al.

Abstract

Multiple sclerosis (MS) is a common autoimmune disease of the CNS. Although an association between MS and inflammatory bowel diseases is observed, the link connecting intestinal immune responses and neuroinflammation remains unclear. Here we show that encephalitogenic Th17 cells infiltrate the colonic lamina propria before neurological symptom development in two murine MS models, active and adoptive transfer experimental autoimmune encephalomyelitis (EAE). Specifically targeting Th17 cell intestinal homing by blocking the α4β7-integrin and its ligand MAdCAM-1 pathway impairs T cell migration to the large intestine and dampens EAE severity in the Th17 cell adoptive transfer model. Mechanistically, myelin-specific Th17 cells proliferate in the colon and affect gut microbiota composition. The beneficial effect of blocking the α4β7-integrin and its ligand MAdCAM-1 pathway on EAE is interdependent with gut microbiota. Those results show that disrupting myelin-specific Th17 cell trafficking to the large intestine harnesses neuroinflammation and suggests that the gut environment and microbiota catalyze the encephalitogenic [...]
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Highlights

- TCR<sup>MOG</sup> 2D2 Th17 cells infiltrate the colonic lamina propria during EAE
- Adoptively transferred TCR<sup>MOG</sup> 2D2 Th17 cells proliferate in the colon
- Blocking 2D2 Th17 cell entry into the colon with α4β7 antibody impairs EAE
- TCR<sup>MOG</sup> 2D2 Th17 cells induce microbiota composition changes

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In Brief

Duc et al. show that Th17-polarized myelin-specific (TCR<sup>MOG</sup> 2D2) CD4<sup>+</sup> T cells migrate to the colon before the development of neuroinflammation. Encephalitogenic Th17 cells further change intestinal microbiome composition. Blocking encephalitogenic Th17 cell entry into the colon or treatment with antibiotics ameliorates EAE severity.

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Disrupting Myelin-Specific Th17 Cell Gut Homing Confers Protection in an Adoptive Transfer Experimental Autoimmune Encephalomyelitis

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SUMMARY

Multiple sclerosis (MS) is a common autoimmune disease of the CNS. Although an association between MS and inflammatory bowel diseases is observed, the link connecting intestinal immune responses and neuroinflammation remains unclear. Here we show that encephalitogenic Th17 cells infiltrate the colonic lamina propria before neurological symptom development in two murine MS models, active and adoptive transfer experimental autoimmune encephalomyelitis (EAE). Specifically targeting Th17 cell intestinal homing by blocking the α4β7-integrin and its ligand MAdCAM-1 pathway impairs T cell migration to the large intestine and dampens EAE severity in the Th17 cell adoptive transfer model. Mechanistically, myelin-specific Th17 cells proliferate in the colon and affect gut microbiota composition. The beneficial effect of blocking the α4β7-integrin and its ligand MAdCAM-1 pathway on EAE is interdependent with gut microbiota. Those results show that disrupting myelin-specific Th17 cell trafficking to the large intestine harnesses neuroinflammation and suggests that the gut environment and microbiota catalyze the encephalitogenic properties of Th17 cells.

INTRODUCTION

Multiple sclerosis (MS) is a neurological and autoimmune disorder characterized by inflammatory cell infiltrates and demyelination of the CNS. Its development is under the control of genetic and environmental factors. Although environmental factors contribute to MS development (Olsson et al., 2017), most of our knowledge about their relative contributions relies on epidemiological data, leaving the underlying pathophysiology largely unraveled. Among environmental factors, recent studies proposed a role for intestinal factors in affecting MS disease severity (van den Hoogen et al., 2017). Furthermore, changes in the intestinal microbiome were described in MS and experimental autoimmune encephalomyelitis (EAE) (Miyake and Yamamura, 2019). Although those results remain disputed, they highlight the possible interaction between the gut and the brain, called the gut-brain axis, in neuroinflammation. Interestingly, animal models indicate that increased intestinal permeability (leaky gut) plays a pathogenic role not only in gastrointestinal disorders, such as inflammatory bowel disease (IBD), but also in systemic autoimmune diseases, like type 1 diabetes and MS (Mu et al., 2017; Opazo et al., 2018). Moreover, alteration of the gut microbiome and immune cell infiltrate are described in the small intestine in EAE (Berer et al., 2011; Nouri et al., 2014) and MS (Berer et al., 2017; Cosorich et al., 2017). T helper (Th) 17 cells infiltrate the small intestine and increase intestinal permeability (Nouri et al., 2014); however, their relative contribution to neuroinflammation remains largely unknown. Furthermore, whether the large intestine, namely, the colon, promotes neuroinflammation is not established.

In this report, we observed altered gut immune responses associated with an infiltration of pro-inflammatory Th17 cells in the lamina propria of the colon in two EAE models. Blocking the migration of pro-inflammatory Th17 cells in the intestinal compartment, as well as treatment with antibiotics, reduced the severity of EAE disease interdependently, pointing toward a contribution of the gut-brain axis in EAE. Although the link between gut immunity and MS remains to be clarified, a better understanding of how immune cells are regulated in the intestine during EAE could support innovative approaches to target neuroinflammation.

RESULTS

Antigen-Specific MOG Th17 Cells Infiltrate the Colonic Lamina Propria during Active EAE

We first evaluated whether immune dysregulation was observed in the colon during EAE actively induced by subcutaneous
immunization of myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 (MOG35–55). To evaluate the relative contribution of a CNS antigen-specific response versus the sole contribution of the complete Freund adjuvant (CFA), we immunized wild-type mice with the antigen MOG35–55 or PBS emulsified with CFA. Pertussis toxin was injected at days 0 and 2 for both groups. Only mice immunized with antigen MOG35–55 developed neurological symptoms (Figure 1A). The expression levels of innate immune cytokines interleukin (IL) 6, IL-1β, and tumor necrosis factor alpha (TNF-α) that play a role during IBD and experimental colitis (Papadakis and Targan, 2000) were assessed by quantitative real-time PCR in the colon when the MOG-immunized mice displayed neurological symptoms. IL-6 and IL-1β, but not TNF-α, were induced in an antigen-specific manner (Figure 1B). Because both IL-6 and IL-1β promote Th17 cell differentiation (Kom et al., 2009), we investigated by flow cytometry whether Th17 lymphocytes infiltrated the large intestine during EAE. The MOG-immunized group significantly surpassed the PBS group in upregulation of IL-17-producing CD4+ T cells (Figures 1C and 1D), but not in interferon (IFN) γ-producing CD4+ T cells (Figures 1E and 1F), in the colon. Those results suggest that Th17 cell subsets specifically respond to the CNS antigen.

We completed the analysis with the use of MOG35–55/IAb tetramers and tracked CD4+ T cells based on their myelin antigen specificity in the colon and the CNS. Notably, MOG-specific T cells are increased in the colon at the peak of EAE disease compared with non-immunized control animals, approximately reaching the levels observed in the CNS, where about 3% of CD4+ T cells stained positive for MOG tetramer, in accordance with a previous publication (Mycko et al., 2015) (Figure 1G). Collectively, these data indicate that MOG-specific Th17 cells are observed in the large intestine and suggest an activation of CD4+ T lymphocytes in the colon during EAE.

**Encephalitogenic TCRMOG 2D2 Th17 Cells Preferentially Infiltrate the Large Intestine**

To specifically study the relevance of encephalitogenic Th17 cell infiltration in the large intestine during EAE, we performed adoptive transfer of in vitro polarized Th17 cells of CD4+ T cells isolated from C57Bl6 mice with a T cell receptor (TCR) specific for the peptide MOG35–55 referred to as TCRMOG 2D2. This model has the advantage of overriding the priming phase of T cells to focus solely on the encephalitogenic potential of TCRMOG 2D2 Th17 cell subsets. Briefly, CD4+ T cells from the spleen and lymph nodes of naive 2D2 mice were differentiated in vitro into Th17 cells (Figure 2A) and transferred into C57BL/6J recipient mice as previously described (Jäger et al., 2009; Peters et al., 2015). Following TCRMOG 2D2 Th17 cell transfer, C57BL/6J recipient mice developed both typical and atypical EAE signs, characterized by an ascending paralysis and an unbalanced gait with severe axial instability, respectively (Peters et al., 2015). However, when highly activated TCRMOG 2D2 Th17 cell subsets are injected, neurological symptoms do not appear before 10 days after injection (Figure 2B), suggesting that TCRMOG 2D2 Th17 cells are activated in peripheral organs to acquire a phenotype enabling them to reach the CNS. We thus evaluated by flow cytometry analysis whether TCRMOG 2D2 Th17 cells (as defined by Vα3.2 expression) were detected in the small and large intestine before neurological symptoms appear. We detected a significant percentage of CD4+ T cells expressing Vα3.2 in the gut. The percentage of CD4+ T cells expressing TCR Vα3.2 was significantly higher in the colon (36.5% ± 2.0%) compared with the small intestine (10.4% ± 2.9%) (Figures 2C and 2D), suggesting a predominant infiltration of the large intestine. In line with previous reports using the Lewis rat model of EAE (Flügel et al., 2001; Kanayama et al., 2016; Odoardi et al., 2012), TCRMOG 2D2 Th17 cells were present in the lungs (Figure 2C). We further characterized the anatomical location of TCRMOG 2D2 cells within the colon using whole-mount immunostaining. In agreement with flow cytometry analysis, high numbers of TCRMOG 2D2 Th17 cells were found in the colonic lamina propria before EAE disease onset and were not observed in control, non-injected, wild-type animals (Figure 2E). We then asked whether Th17 cell colonic infiltration was MOG specific and took advantage of OT-II transgenic mice that display a TCR specific for ovalbumin 323–339 peptide. CD4+ T cells, obtained from OT-II and 2D2 mice on a CD45.2 background, were polarized into Th17 cells and transferred into CD45.1 recipient mice. Colonic tissue was examined by flow cytometry for the percentage of CD45.2+ on total CD45+ cells. 2D2 and OT-II cells migrated similarly to the colon, suggesting that Th17 cell migration to the colon is not MOG specific (Figure 2F). We asked whether different encephalitogenic T cell subsets similarly migrate to the colon. We generated Th17 and Th1 cells from TCRMOG 2D2 T cells in vitro as previously described (Jäger et al., 2009) and transferred them into naive syngeneic wild-type C57BL/6J recipients. The percentages of TCRMOG 2D2 Th17 cells were assessed by flow cytometry from the colon of recipient mice. A significantly higher percentage of TCRMOG 2D2 Th17 cells was detected in the colon when cells were differentiated into Th17 versus Th1 cells (Figure 2G). Those results suggest that Th17 cells migrate to the colon during EAE independent of their antigen specificity but at higher levels than Th1 cells.

**Encephalitogenic TCRMOG 2D2 Th17 Cells Are in Close Contact with Colonic Blood Vessels and Egress via Lymphatic Vessels**

Using 3D image reconstruction, we assessed the location of TCRMOG 2D2 Th17 cells within the colonic lamina propria. Although TCRMOG 2D2 Th17 cells were found throughout the colonic lamina propria, we observed many cells closely associated with colon blood capillaries. Quantification of TCRMOG 2D2 Th17 cells either in the immediate vicinity (on) or not (off) of colon blood capillaries showed that more than 60% of the TCRMOG 2D2 Th17 cells were contacting these vessels (Figure 3A). We further observed that a fraction of TCRMOG 2D2 Th17 cells showed an elongated morphology, suggesting a migratory phenotype (Figure 3A). Furthermore, TCRMOG 2D2 Th17 cells were detected closely associated with, and in the lumen of, dilated vessels below colonic crypts, identified as lymphatic capillaries by LYVE-1 staining (Figures 3B–3D). Because intestinal lymphatic capillaries drain into mesenteric lymph nodes (mLNs) (Bernier-Latmani and Petrova, 2017), we assessed the percentage of TCRMOG 2D2 by fluorescence-activated cell sorting (FACS) analysis in the gut-draining mLNs.
Figure 1. MOG-Specific Th17 Cells Infiltrate the Large Intestine during Active EAE

(A) Wild-type mice were immunized with either MOG35-55 or PBS in CFA. The course of EAE in these mice is shown as a clinical score (mean ± SEM; n = 8).

(B) Relative mRNA expression of IL-6, IL-1β, and TNF-α in colonic tissue as measured by real-time PCR in mice immunized with PBS and CFA or MOG and CFA when mice displayed neurological symptoms (mean ± SD; n = 4–6). Data are representative of two independent experiments.

(C–F) Flow cytometric analysis of colonic lamina propria-infiltrating CD4⁺ T cells in the PBS and CFA group versus the MOG and CFA group. Frequency of RORγ⁺/IL-17⁺ CD4⁺ T cells (C and D) and IFNγ⁺ CD4⁺ T cells (E and F) (mean ± SD; n = 5–6).

(G) Cellular suspensions from colon and CNS were prepared from non-immunized (ctl) and immunized mice at the peak of the disease (EAE). FACS analysis of the total proportion (as a percentage) of MOG-specific CD4⁺ T cells was visualized by MOG35-55/IAb tetramer staining (mean ± SD; n = 3).

*p < 0.05; p values were determined by Mann–Whitney test (B [IL-6]), unpaired Student’s t test (B [IL-1β and TNF-α], D, and F) and one-way ANOVA with Dunnett’s post hoc test (G).
versus the dermal inguinal lymph nodes (dLNs) of the same recipient mice 4 and 8 days after TCRMOG 2D2 transfer and in PBS-injected recipient mice (Figure S1A). Around 1% CD4+ T cells were V\(\alpha\)3.2+ in the dLNs (1.03% ± 0.1%) and mLNs (1.0% ± 0.1%) of PBS-injected recipient mice, compatible with low levels of endogenous V\(\alpha\)3.2+ expression in the lymphoid organs of C57BL/6J mice (Bettelli et al., 2003). We observed a significant 18-fold (18.7% ± 4.5%, 4 days) and 13-fold (13.3% ± 1.9%, 8 days) increase in CD4+ T cells harboring the V\(\alpha\)3.2+ marker in the mLNs after TCRMOG 2D2 Th17 cell transfer. At the same time, there was only a minor tendency toward increase in V\(\alpha\)3.2+ T cells in the dLNs of the same mice (2.6% ± 0.2%, 4 days) and (2.9% ± 0.5%, 8 days) after T cell transfer compared with the control dLNs (1.03% ± 0.1%) (Figure S1B). Altogether, these data show that TCRMOG 2D2 Th17 cells infiltrate the colonic lamina propria, migrate out of the colon via lymphatic vessels, and likely reenter blood circulation downstream of mLNs.

**Blocking \(\alpha 4\beta 7\)-Integrin and Its Ligand MadCAM-1 Interaction Inhibits TCRMOG 2D2 Th17 Cell Entry into the Large Intestine and Significantly Attenuates EAE**

To explore whether colonic TCRMOG 2D2 migration contributes to neuroinflammation, we asked whether blocking TCRMOG 2D2 entry into the gut influences EAE development. Autoimmune T cells receive signals to acquire a functional phenotype that allows them to invade their target tissues (Kassiotis and Stockinger, 2004; Salmi and Jalkanen, 2005). Intestinal targeting of T cells requires expression of the ligand \(\alpha 4\beta 7\)-integrin on immune cells and expression of its receptor mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) on intestinal venules (Gorfu et al., 2009). In our model, MadCAM-1 was
whether these cells expressed α4β7-integrin. Half (50.2% ± 2.8%) of the colonic TCRMOG 2D2 cells expressed α4β7 four days after adoptive transfer, whereas α4β7 expression was significantly reduced (14.5% ± 3.1%) at day 8, before neurological signs appear (Figure 4B). Those results suggest that α4β7 expression is involved in TCRMOG 2D2 Th17 cell migration to the gut.

In mouse models of T cell-mediated colitis, administration of α4β7 antibodies reduces inflammation and severity of colitis. Similarly, vedolizumab, a humanized specific α4β7 inhibitor, has proven effective in ulcerative colitis and Crohn’s disease (Lam and Bressler, 2014; McLean et al., 2012; Tilg and Kaser, 2010). Because we observed important TCRMOG 2D2 Th17 cell infiltration in the large intestine, we tested whether selective blocking of α4β7 could also influence EAE disease course. α4β7-blocking antibodies (DATK 32) were administered one day before adoptive transfer, followed by injections every three days until the development of EAE disease. We observed during EAE a significant reduction of TCRMOG 2D2 Th17 cell infiltration in the colon, but not in the ileum or in the lung, 4 days after T cell transfer in mice treated with anti-α4β7 compared with the isotype control group (Figures 4C and 4D). Moreover, α4β7 blockade was efficient in dampening EAE neurological disease course (Figure 4E; Table S1). In addition, a significantly reduced number of TCRMOG 2D2 Th17 cells infiltrating the CNS was recorded 10 days after T cell adoptive transfer in mice treated with anti-α4β7 compared with the isotype control group (Figure 4F). Similar results were obtained when we extended the anti-α4β7 treatment until the end of the EAE disease (Figure S2), suggesting that the contribution of colonic Th17 cells in EAE takes place early during EAE. Anti-α4β7 anti-body treatment did not influence active EAE disease course (Figures 4G and 4H). Those results suggest a contribution of Th17 cell migration to the colon when Th17 cells are adoptively transferred.

**Colonic TCRMOG 2D2 Th17 Cells Express CNS Integrin, and Their Intestinal Infiltrate Resolves upon CNS Invasion**

Because we observed TCRMOG 2D2 Th17 cell infiltration of the large intestine before neurological clinical signs, we wondered whether this infiltration persisted throughout the disease course. The frequency of TCRMOG 2D2 Th17 cells was evaluated at different time points after T cell transfer by simultaneous flow cytometry analysis in the colon and the CNS. TCRMOG 2D2 Th17 cells infiltrate the colonic lamina propria as early as 4 days after transfer, whereas no cells were detected in the CNS (Figure 5A). Eight days post-transfer, the frequency of colonic TCRMOG 2D2 Th17 cells decreased by more than two-fold compared with day 4, whereas we found a concomitant significant increase in the percentage of 2D2 cells in the CNS, from 0% at day 4 to 32.7% ± 6.4% at day 8 and then 67.5% ± 10.7% at the peak of the disease (Figure 5A). Altogether, those data demonstrate that CNS-specific Th17 cells migrate and infiltrate the large intestine during the preclinical phase of transfer EAE before reaching the CNS.

To assess whether TCRMOG 2D2 Th17 cells detected in the gut display encephalitogenic properties, we evaluated the level of...
**Figure 4. Blocking α4β7-Integrin Reduces Migration of Encephalitogenic TCRMOG 2D2 Cells to the Colon and Delays the Progression of EAE Disease**

(A) Relative mRNA expression of MAdCAM-1 in CNS and colonic tissue as measured by real-time PCR in non-injected mice (day 0) and four days after TCRMOG 2D2 Th17 cell injection. Box-and-whisker graphs show median, minimum (min.), and maximum (max.) values and 25th and 75th percentiles (n = 6).

(B) Flow cytometry analysis of the total proportion (as a percentage) of the α4β7-integrin expression on colonic TCRMOG 2D2 (Vα3.2+) CD4+ T cells at the indicated time points after TCRMOG 2D2 Th17 cell injection (mean ± SD; n = 3). Data are representative of two independent experiments.

(C and D) Representative flow cytometry analysis of TCRMOG 2D2 (Vα3.2+) expression in CD3+/CD4+ T cells obtained from the colon, ileum, and lung in isotype control versus the anti-α4β7-treated group at day 4 after TCRMOG 2D2 Th17 cell transfer (mean ± SEM; n = 3–4) (D). Data are representative of two independent experiments.

(E) Clinical scores of EAE in adoptively transferred mice treated with PBS 1X, isotype control, or anti-α4β7 antibodies. The course of EAE in these mice is shown as a clinical score (mean ± SEM; n = 6–8; p values are shown for comparison between anti-α4β7 versus isotype groups). Data are representative of three independent experiments.

(F) Absolute numbers of TCRMOG 2D2 CD4+ T cells infiltrating the CNS 10 days after adoptive transfer in mice treated with isotype control or anti-α4β7 antibodies (mean ± SD; n = 4). Data are representative of two independent experiments.

(G and H) Active EAE with mice injected either anti-α4β7 antibodies or isotype control every 3 days. The active EAE course is shown as (G) clinical scores (mean ± SEM; n = 7 per group), (H) day of disease onset (mean ± SD; n = 5–6), and maximum scores (mean ± SD; n = 7). Data are representative of one experiment.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; NS, not significant; p values were determined by unpaired Student’s t test (A, B, and F), two-way ANOVA with Sidak’s post hoc test (D), and Tukey’s post hoc test (E). See also Figure S2 and Table S1.

α4β1-integrin (CNS-specific integrin) 4 days after T cell transfer. α4β1-integrin was expressed at levels similar to those of α4β7-integrin, which is known to be gut specific (Figure 5B). However, α4β1-integrin ligand vascular cell adhesion molecule-1 (VCAM-1) was expressed solely in the CNS, not in the colon (Figure 5C). In addition, α4β1-integrin expression decreases on TCRMOG 2D2 Th17 cells of the colon over time, whereas the expression remained high in the CNS when the TCRMOG 2D2 reached the CNS (Figure 5D). Those results strengthen our hypothesis that TCRMOG 2D2 Th17 cells depict encephalitogenic properties while present in the colon.

Different Th17 cell subsets have been described in the colon, including the homeostatic Th17 cells co-expressing both Foxp3 and RORγT (Solomon and Hsieh, 2016) and the pathogenic Th17 cells that do not express Foxp3. Thus, we evaluated the phenotypic profile of the TCRMOG 2D2 Th17 cells detected in the colonic lamina propria. Four days after injection, colonic lamina propria Vα3.2+ CD4+ T cells were analyzed by flow cytometry for RORγT+, Foxp3, T-bet, and IL-17A expression. TCRMOG 2D2 Th17 cells maintained RORγT expression, whereas neither T-bet nor Foxp3 expression was detected (Figure 5E). We conducted our analysis at different time points after T cell transfer and observed a reduction of the total frequencies of RORγT+ 2D2+ cells (Figure 5F). However, the residual colonic 2D2+ CD4+ T cells kept their capacity to secrete the pro-inflammatory IL-17 cytokine in colonic lamina propria at all time points examined (Figures S3A and S3B). In addition, Foxp3 and RORγT co-expressing Th17 cells and Foxp3+ RORγT+ regulatory T cells...
were both restricted to \( V_{a3.2}^{-} CD4^{+} \) T cells (2D2\(^{-}\)), and their frequencies were not affected by time (Figure S3C). We thus demonstrated that TCR\(^{MOG}\) 2D2 Th17 cells were predominantly detected in the large intestine before reaching the CNS and maintained their pro-inflammatory phenotype after in vivo transfer.

**Encephalitogenic TCR\(^{MOG}\) 2D2 Th17 Cells Proliferate in the Large Intestine**

Having established that TCR\(^{MOG}\) 2D2 cells are detected in the colonic lamina propria, we explored their fate within the large intestine and evaluated their proliferative status with Ki67 staining. FACS analysis demonstrated that almost 100% of TCRMOG 2D2 cells proliferated in the colonic lamina propria 4 days after T cell transfer. This proliferation rate significantly decreased to less than 20% 8 days post-transfer (Figure 6A). In comparison, \( V_{a3.2}^{-} CD4^{+} \) T cells (host cells) showed a constant proliferation rate of 25% with no significant changes between day 4 and day 8 (Figure 6A).

We assessed the location of proliferating TCRMOG 2D2 cells in the colon by whole-mount immunostaining (Figure 6B). At day 4, 30% of TCRMOG 2D2 detected above the lumen-adjacent blood vessel (lumen, yellow line in Figure 6B) were \( \text{Ki67}^{+} \), whereas only 15% of TCRMOG 2D2 cells were located below the lumen-adjacent blood vessel (stroma cells). In agreement with FACS analysis, less than 5% of total TCRMOG 2D2 cells were \( \text{Ki67}^{+} \) at day 8 after injection, regardless of location (Figure 6C). However, among \( \text{Ki67}^{+} \) TCRMOG 2D2 cells at both day 4 and day 8, approximately two-thirds of \( \text{Ki67}^{+} \) cells were found near the colon lumen (Figure 6D). These results show that TCR\(^{MOG}\) 2D2 cells proliferated at the maximum rate 4 days after T cell transfer and are found near colonic lumen.
Encephalitogenic TCR\textsuperscript{MOG} 2D2 Th17 Cells Induce an Intestinal Dysbiosis, and Antibiotic Treatments Decrease the Encephalitogenic Properties of Th17 Cells

We hypothesized that factors present in the colonic lumen, more specifically gut microbiota, could affect TCR\textsuperscript{MOG} 2D2 T cells. We thus evaluated whether TCR\textsuperscript{MOG} 2D2 were influenced by and/or could affect intestinal microbial composition, because changes in gut microbiota composition were reported in other EAE models (Berer et al., 2011; Lavasanii et al., 2010; Lee et al., 2011; Ochoa-Repa´raz et al., 2009, 2010). We performed metatransomic analysis by sequencing of 16S ribosomal RNA gene amplicons from fecal samples of the same recipient mice before and 9 days after, when mice were still clinically asymptomatic, TCR\textsuperscript{MOG} 2D2 Th17 cell transfer. Analysis of the Shannon index revealed that TCR\textsuperscript{MOG} 2D2 Th17 cell transfer resulted in markedly different (permutational multivariate analysis of variance [PERMANOVA], p = 0.0009) gut microbial communities (Figure 7A). Analysis of the variance among microbial communities showed that TCR\textsuperscript{MOG} 2D2 T cell transfer significantly increased the diversity of the gut microbiota (Figure 7A). Analysis of the variance among microbial communities showed that TCR\textsuperscript{MOG} 2D2 T cell transfer resulted in a significant (permutational multivariate analysis of variance [PERMANOVA], p = 0.0009) gut microbial community shift (Figure 7B). Analysis of the microbiota at various taxonomic levels showed a shift in the gut microbiota composition after Th17 cell transfer, including an increase in phyla with gram-negative cell wall structure (Proteobacteria and Bacteroidetes) and a decrease of the gram-negative bacteria belonging to the class Bacilli (Firmicutes). In the Bacteroidetes phylum, genus Alistipes and several operational taxonomic units (OTUs) of the Bacteroidales order increased in relative abundance after TCR\textsuperscript{MOG} 2D2 Th17 cell transfer. In the Firmicutes phylum, the proportion of genus Lactobacillus and three OTUs assigned to it decreased significantly following adoptive transfer (Figure 7C; Table S2).

Concomitantly, several members of the Clostridiales order, including the family Ruminococcaceae, an OTU from the family Lachnospiraceae, and the genera Eisenbergiella, KE159538, Eubacterium, Oscillibacter, and Pseudoflavonifractor, increased in relative abundance.

To evaluate whether the TCR\textsuperscript{MOG} 2D2 Th17 cells required the presence of microbiota to induce EAE, we injected TCR\textsuperscript{MOG} 2D2 Th17 cells into antibiotic-treated mice. The antibiotic treatment, used to eliminate a broad bacteria community (Zaiss et al., 2015), significantly reduced EAE severity (Figure 7D) without impairing the ability of TCR\textsuperscript{MOG} 2D2 T cells to infiltrate the colon (Figure S4). We then wondered whether blocking Th17 cell colonic migration and antibiotic treatment could have additive effects. We treated mice with antibiotics and administered anti-\textalpha4\textbeta7 or control isotype antibody (Figure 7E). Antibiotic treatment reduced EAE severity; however, blocking the \textalpha4\textbeta7-integrin and its ligand MadCAM-1 pathway did not enhance the protective effect of antibiotic treatment, thus orienting our interpretation toward an interdependent role for the intestinal microbiome and anti-\textalpha4\textbeta7 treatment.

In summary, in TCR\textsuperscript{MOG} 2D2 Th17 cell adoptive-transfer EAE, Th17 cells migrate and proliferate at the highest level in the large intestine at the preclinical stage of EAE disease. Myelin-specific Th17 cells change gut microbiota composition. Finally, blocking myelin-specific Th17 cell entry into the colon or disrupting gut microbiota with antibiotic treatments dampens EAE development.
DISCUSSION

The implication of the gut-brain axis has received growing attention in the field of neuroimmunology, with recent works showing Th17 cell accumulation in the small intestine, as well as intestinal dysbiosis, in MS patients (Cosorich et al., 2017). However, whether those findings are a consequence of neuroinflammation or instead they affect the development of neuroinflammation remains disputed. We report here that CNS-specific Th17 cells migrate to the intestine during EAE. Furthermore, blocking the $\alpha_4\beta_7$-integrin and its ligand MAdCAM-1 pathway not only limits intestinal Th17 cell infiltration, to a large extent in the large intestine, but also significantly damps EAE severity in an adoptive-transfer model. Mechanistically, myelin-specific Th17 cells proliferate in the gut and alter gut microbiota composition. Antibiotic treatment dampens EAE development in the Th17 cell adoptive-transfer EAE model. However, blocking the $\alpha_4\beta_7$-integrin and its ligand MAdCAM-1 pathway does not synergize with the beneficial effect of antibiotic treatments. These data strongly suggest that during CNS autoimmunity, encephalitogenic Th17 cells enter the large intestine, where their pro-inflammatory functions are strengthened at least partially by gut microbiota changes.

An association between MS and IBD has been described in humans (Gupta et al., 2005), as has an increased risk of MS and IBD comorbidity (Kosmidou et al., 2017). Animal models have demonstrated a crucial role for gut-homing effector T cells in IBD, and mice lacking $\beta_7$-integrin have fewer intestinal T cells as a consequence of a reduced rate of migration to the gut (Steeber et al., 1998; Wagner et al., 1996). Moreover, $\alpha_4\beta_7$-integrin-blocking antibodies attenuate mouse models of intestinal inflammation and are clinically effective in the treatment of human ulcerative colitis and Crohn's disease (Feagan et al., 2005; Picarella et al., 1997). Combined anti-$\alpha_4$-integrin (natalizumab), blocking both $\alpha_4\beta_1$/VCAM-1 and $\alpha_4\beta_7$-integrin and its ligand MAdCAM-1 interactions, is effective, in addition to...
intestinal inflammatory diseases, in targeting both MS and EAE (Brandstadt and Katz Sand, 2017; Steinman, 2012; Yednock et al., 1992). For MS, the effect is attributed specifically to the α4β7 integrin directly in the CNS. Indeed, the impact of α4β7-integrin and its ligand MAdCAM-1 interactions in neuroinflammation remains controversial, and studies specifically addressing their role in EAE pathogenesis have led to inconsistent results. Using the SJL/N mouse strain, Engelhardt and colleagues found no effect of anti-α4β7- or anti-β7-neutralizing antibodies on the development of EAE (Engelhardt et al., 1998). Similarly, vedolizumab does not prevent CNS inflammation or demyelination in a rhesus monkey EAE model (Haanstra et al., 2013). In apparent contrast, β7-integrin-deficient C57BL/6 mice are partially resistant to adoptive EAE transfer and to neutralizing antibodies against MAdCAM-1 (Kanwar et al., 2000a, 2000b). Furthermore, the development of active EAE is attenuated in MAdCAM-1-knockout (KO) mice, whereas MAdCAM-1 blockade 5 days after immunization does not attenuate EAE (Kuhbandner et al., 2019). Indeed, the timing of intestinal T cell blocking is relevant. We observe that anti-α4β7 treatment is efficient when administered only until the appearance of the first neurological signs and that the extension of the treatment during the entire EAE disease course did not enhance its protective effect. Those results are in accordance with our observation of significant TCR\(^{M}2D2\) Th17 cell colonic proliferation at 4 days, but not at 8 days, after T cell transfer. Furthermore, the observed differences in the previous publications can be attributed to the use of different mouse strains and to methodological differences in inducing EAE that may or may not favor Th17 cell polarization. We show here that blocking α4β7-integrin inhibits Th17 cell entry into the colonic lamina propria during adoptive EAE transfer and contributes to a reduction of neurological symptoms. We did not observe an impact of anti-α4β7 treatment in the active EAE model, suggesting that in the absence of a MOG-CFA depot, Th17 cells depend more on gut for their activation. Alternatively, the MOG-CFA model depends on both Th1 and Th17 cells, whereas in the adoptive transfer, we used purified 2D2 Th17 cells. However, the differences obtained between the two EAE models remain to be investigated, as does the initial localization of Th17 cell activation/reactivation during autoimmunity in humans. We propose that the colon might be a niche for reactivation and proliferation of immune cells during EAE, in particular for Th17 cells. The effect on myelin-specific Th17 cells observed in the colon during EAE could be related to their production of the IL-17 cytokine family more than on their antigen specificity. This would be in accordance with the intrinsic properties of IL-17A as modulators of microbiota composition (Douzande-Mobarrez and Karimnik, 2019). Because IL-17 cytokines can be produced not only by adaptive immune cells but also by other cell types (ILC3 and γδ T cells), it remains to be elucidated whether IL-17 family cytokines, such as IL-17A, could per se affect neuroinflammation independent of their cellular sources.

Whether MAdCAM-1, an intestinal protein, is also expressed in the CNS and thus contributes to our observation has been debated (Allavena et al., 2010; O’Neill et al., 1991; Steffen et al., 1994, 1996; Vercellino et al., 2008). We detected low levels of MAdCAM-1 mRNA in the CNS at lower rates compared with the colon. MAdCAM-1, if expressed in the CNS, is expressed at a low level and has been shown not to contribute to neuroinflammation (Döring et al., 2011). In addition, even ectopic expression of MAdCAM-1 on the blood-brain barrier does not influence EAE in the C57BL/6J model (Döring et al., 2011). This corroborates with the publication from Korn and colleagues that proposed, in an adoptive-transfer EAE model, that Th17 lymphocytes reach the CNS independent of α4-integrin expression during EAE (Rothhammer et al., 2011). Here we observed a beneficial effect on neuroinflammation when blocking α4β7-integrin and its ligand MAdCAM-1 interactions. However, the beneficial effect was transient. This could be a consequence of the insufficient blocking of Th17 cell trafficking in the small intestine and the colon. Lymphocyte migration to the gut is controlled by additional chemoattractant receptors, such as the C-C motif chemokine receptor 9 (CCR9) for the small intestine or the orphan G-protein-coupled receptor 15 (GPR15) for the colon (Habtezion et al., 2016). Concomitant blocking of CCR9/ GPR15/chemokine interaction could contribute to evaluation of the role of the intestine in driving EAE.

Gut mucosa harbors the highest concentration of immune cells in the body, and studies in humans and mice suggested that the small intestine is a possible location for the generation, activation, and expansion of effector T cells that cause autoimmune responses (Nouri et al., 2014). However, it is unclear how mucosal immune responses elicited in the gut modulate CNS inflammation and whether the large intestine is further involved in EAE pathogenesis. Our observations that myelin-specific Th17 cells preferentially infiltrate the large intestine further demonstrate the occurrence of compartmentalization and suggest the existence of distinctive mechanisms of autoimmune cell recruitment that may allow functional specialization of immune responses in different segments of the intestine.

Th17 cell adoptive-transfer EAE had a notable effect on gut microbiota composition, with a decreased representation of the Firmicutes phylum, in particular of the proportion of the Lactobacillus species that have been associated with beneficial effects, including inflammatory immune response reduction during EAE (Umbrello and Esposito, 2016). We observed a significant reduction in L. johnsonii, which are known to have immunomodulatory properties (Owaga et al., 2015). Interestingly, L. johnsonii are induced by intermittent fasting, which dampens EAE severity (Cignarella et al., 2018). Intestinal microbiota have been proposed to contribute to the accumulation or activation of Th17 cells in the intestine (Goto et al., 2014; Yang et al., 2014b). In the context of neuroinflammation, commensal species residing in the small intestine affect the development of the disease in humans and in their mouse models. Encephalitogenic Th17 cells can be generated in the gut with segmented filamentous bacteria monoclonization (Lee et al., 2011), whereas Bacteroides species or colonic clostridia, respectively, suppress IL-17 production or support the development of regulatory T cells (Wekerle et al., 2013). Here we show that antibiotic treatment was sufficient to dampen neurological disease in the adoptive transfer EAE model and that blocking α4β7-integrin and its ligand MAdCAM-1 interactions was not be beneficial in the context of antibiotic treatment. Those results suggest an intrinsic relationship between CNS-specific Th17 cells and intestinal microbiota.
composition. However, the precise mechanisms of the gut flora-Th17 cell interactions remain to be studied.

In summary, CNS antigen-specific Th17 cells infiltrate the large intestine and depict high proliferating properties in this organ during EAE. Specifically targeting Th17 cell migration to the large intestine by blocking α4β7-integrin and its ligand MAdCAM-1 interactions attenuates EAE disease interdependently with the microbiota composition. Our results thus contribute to reevaluation of the gut as a target during CNS autoimmunity.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.09.002.

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Salmi, M., and Jalkanen, S. (2005). Lymphocyte homing to the gut: attraction,


Pappadakis, K.A., and Targan, S.R. (2000). Role of cytokines in the pathogen-


### STAR METHODS

#### KEY RESOURCES TABLE

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**Chemicals, Peptides, and Recombinant Proteins**

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(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Caroline Pot (Caroline.Pot-kreis@chuv.ch). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C57BL/6J mice were purchased from Charles River or bred in the animal facility. C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J (TCRMOG 2D2) and C57BL/6-Tg(TcraTcrb)425Cbn/Crl (OT-II) transgenic mice were purchased from Charles River. All mice used were females aged from 8 to 10 weeks and were maintained under specific-pathogen free conditions at Lausanne University Hospital. All experiments were performed in accordance with guidelines from the Cantonal Veterinary Service of state Vaud.

METHOD DETAILS

EAE induction and clinical evaluation

For induction of classical active EAE, mice were immunized with 100 μg myelin oligodendrocyte glycoprotein peptide 35-55 (MOG<sub>35-55</sub>) or PBS emulsified in complete Freund’s adjuvant supplemented with 5 mg/ml Mycobacterium tuberculosis H37Ra. A total of 200 μl emulsion was subcutaneously injected into four sites on the flanks of mice. At days 0 and 2 after initial peptide injections, animals received additional intravenously injection of 100 ng pertussis toxin. For induction of EAE by adoptive transfer, naive CD4<sup>+</sup> T cells from 2D2 mice were polarized in vitro in Th17 or Th1 cells as previously described (Jäger et al., 2009). Differentiation status was checked on day 5 and after 2 days of restimulation with anti-mouse CD3/CD28 antibodies (2 μg/ml), 8 × 10<sup>6</sup> CD4<sup>+</sup> T cells were injected intraperitoneal (i.p.) into wild-type C57BL/6J recipient mice. Mice were scored daily for clinical symptoms. The classical EAE symptoms were assessed according to the following score: score 0 – no disease; score 0.5 – reduced tail tonus; score 1 - limp tail; score 1.5 – impaired righting reflex; score 2 – limp tail, hind limb weakness; score 2.5 – at least one hind limb paralyzed; score 3 – both hind limbs paralyzed; score 3.5 – complete paralysis of hind limbs; score 4 – paralysis until hip; score 5 – moribund or dead. The atypical EAE symptoms were assessed according to the following score: score 0 – no disease; score 1 – head turned slightly (ataxia, no tail paralysis); score 2 – head turned more pronounced; score 3 – inability to walk on a straight
line; score 4 – laying on side; score 4.5 – rolling continuously unless supported; score 5 – moribund or dead. Combined score compiling typical and atypical scoring has also been applied and considers the highest score from the typical or atypical clinical signs (Peters et al., 2015). Mice were euthanized if they reached a score > 3.

**Antibody treatment**

For homing experiment, mice were injected i.p. with 400 µg of anti-mouse α4(7) (DATK 32) or isotype control antibodies (IgG2a isotype control, 2A3) one day before TCRMOG2D2 Th17 cell injection and every three days post-injection until the development of disease or as indicated.

**Antibiotic treatment**

Mice were treated with 2.5 mg/ml enrofloxacin in drinking water for 2 weeks, followed by 0.8 mg/ml of amoxicillin and 0.114 mg/ml clavulanic acid in drinking water for 2 further weeks (Zaiss et al., 2015) prior to TCRMOG2D2 Th17 cell injection. Treatment with amoxicillin and clavulanic acid was then continued throughout the experiment.

**Isolation of immune cells**

Mice were perfused through cardiac ventricle with Phosphate-buffered saline (PBS) 1 x. Whole colon, 15 cm-long pieces of terminal ileum and whole lung were excised and washed in PBS 1 x. Gut was opened longitudinally. Washed gut and lung pieces were cut into 2 cm pieces and incubated for 20 min at 37°C in HBSS containing 10 mM EDTA under gentle agitation (80 rpm). Tissues were washed by vortexing with PBS 1 x. Organ pieces were then incubated 2 times at 37°C for 20 min in a dissociation mix composed of 5 mL HBSS, Liberase TL (1 Wünsch unit/mL) and DNase I recombinant (1 U/mL) and 2% fetal calf serum (FCS). The remaining tissue pieces were mechanically disaggregated on a 70 µm cell strainer using a syringe plunger. For the preparation of CNS mononuclear cells, brain and spinal cord were cut into pieces and digested 45 min at 37°C with collagenase D (2.5mg/ml) and DNase I (1mg/ml) followed by 70%/37% Percoll gradient centrifugation. For the preparation of lymph nodes, organs were removed and single cell suspensions were prepared by digestion of the tissues through a 70 µm cell strainer. The cellular suspensions were washed and filtered through 40 µm cell strainer and resuspended in culture medium for further analysis.

**Flow cytometric analysis**

Single-cell suspensions in PBS 1 x were stained with fixable viability dye eFluor™ 660. For cell surface stainings, cells were preincubated with anti-CD16/32 for 10 min to block Fc receptors and stained in FACS buffer (PBS containing 1% BSA) with directly labeled monoclonal antibodies for 30 min. For intracellular cytokine staining, cells were activated for 4 h with 50 ng/ml PMA, 1 µg/ml ionomycin in the presence of 10 mg/ml brefeldin A. After surface staining, cells were fixed and permeabilized using Foxp3/transcription factor staining buffer set and stained intracellularly with directly labeled monoclonal antibodies for 30 min. Data were acquired on a LSR II cytometer and all data were analyzed using FlowJo software. Fluorochrome-conjugated antibodies were purchased from several commercial sources indicated below and listed in the Key Resources Table. Antibodies against CD45 (30-F11), α4(7) (DATK32), CD45.1 (A20) were from Biolegend; CD3 (145-2C11), CD4 (GK1.5), IL-17 (ebio17B7), T-bet (eBio4B10), Foxp3 (clone FJK-16 s), TCRVβ3.2 (RR3-16), CD29 (30-D11), IL-12 (ebio12G), IFN-γ (XM16.2), TNF-α (ebio5H10), IL-10 (ebio10E7), VEGF (ebio28H), RORγT (Q31-378) was from BD Biosciences and CD49d (a4) (PS/2) was from Biorad.

**Tetramer staining**

Cellular suspensions from colon and CNS were prepared as previously described from immunized and non-immunized mice at the peak of the disease. Cells were stimulated 4h with 10 µg/ml of MOG35-55 peptide in the presence of hIL-2 (50U/mL). I-A<sup>b</sup> MOG<sub>35-55</sub> tetramer-positive CD4<sup>+</sup> T cells were detected by flow cytometry after surface staining with corresponding directly labeled I-A<sup>b</sup> MOG<sub>35-55</sub> tetramer.

**RNA isolation and quantitative PCR analysis**

RNA was extracted from tissue samples using RNeasy Mini Kit following the manufacturer’s instructions. cDNA was produced from equivalent amounts of RNA with the Superscript II RT (Invitrogen) and the PCR products were amplified with the PowerUp SYBR Green Master Mix. Samples were detected on the StepOne Real-Time PCR System. RNA was extracted from tissue samples using RNeasy Mini Kit following the manufacturer’s instructions. cDNA was produced from equivalent amounts of RNA with the Superscript II RT (Invitrogen) and the PCR products were amplified with the PowerUp SYBR Green Master Mix. Samples were detected on the StepOne Real-Time PCR System. 378–390.e1–e4, October 8, 2019

**Whole-mount immunostaining**

Whole-mount immunostaining was performed as previously described (Bernier-Latmani and Petrova, 2016). Briefly, mice were perfused with 4% paraformaldehyde and intestines were fixed in picric acid fixation buffer. Colon immunostaining was performed
with DAPI (1:4000) and the following primary and secondary antibodies: CD3ε (145-2C11, 1:1000), VEGFR2 (1:100), LYVE1 (1:500), Ki67 (1:200), Vx3.2-APC (1:100), goat anti-Armenian hamster DyLight 488 (1:500) and donkey anti-goat AlexaFluor 555 (1:500). After immunostaining the colon was cut into ~0.5 mm thick strips which were cleared and mounted in RIMS Buffer (Yang et al., 2014a) on a microscope slide fitted with 0.1 mm spacers (Molecular Probes). Image acquisition was performed on a Zeiss LSM 880 confocal microscope and image analysis and 3D reconstruction was performed with Imaris (Bitplane) and Fiji.

Microbiome Analysis

Two weeks prior the experiment, mice were randomized in cages of two mice. A fecal pellet (9–50 mg) was mixed with 550 μL GT buffer (RBS Bioscience) and homogenized in a Nucleospin Bead Tube (Machery-Nagel, Düren, Germany) for 20 min at maximum speed on a Vortex-Genie 2 with a horizontal tube holder (Scientific Industries). After addition of 1 μL of 50 mg/mL RNaseA, samples were incubated at room temperature for 5 min and centrifuged for 2 min at 11,000 × g. DNA was extracted from 400 μL of the supernatant using the MagCore Genomic DNA Tissue kit on a MagCore HF16 instrument and eluted in 100 μL of 10 mM Tris-HCl pH 8. Two negative extraction controls were processed in parallel with fecal pellets by omitting addition of biological material in the GT buffer. Purified DNA was stored at −20°C. The V3–4 region of the bacterial 16S rRNA genes was amplified using 2 ng of extracted DNA (or 5 μL of the eluate from negative extraction control) as described before (Bouillaguet et al., 2018), except 30 PCR cycles were used. The amplicon barcoding/purification and MiSeq (2 × 300) sequencing were performed at LGC Genomics (Berlin, Germany) as previously described (Lazarevic et al., 2016). After removal of adaptor remnants and primer sequences from demultiplexed fastq files using proprietary LGC Genomics software, sequencing data were submitted to European Nucleotide Archive (ENA) database. Clustering of quality filtered merged reads into OTUs and taxonomic assignments of representative OTUs using mothur (Schloss et al., 2009) and EzBioCloud database (Yoon et al., 2017) were performed following a pipeline described previously (Bouillaguet et al., 2018), with modified command options in PEAR (Zhang et al., 2014) (-m 450 -t 250) and USEARCH (Edgar, 2010) (-usearch_global -wordlength 30). Principal Coordinates Analysis (PCoA) of Bray–Curtis similarity, based on the square-root transformed relative abundance of OTUs was performed in PRIMER (Primer-E Ltd., Plymouth, UK). Shannon diversity index (H’ = − Σ (pi ln pi)) was calculated in PRIMER from the relative abundance of OTUs (pi).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analyses and graphs were performed using GraphPad Prism 7.0 software. P values < 0.05 were considered significant. Results are displayed as mean and SEM or mean and SD, as described in the figure legends. Differences in microbiota between before and after adoptive transfer EAE were assessed using PERmutational Multivariate Analysis Of Variance (PERMANOVA) (PRIMER) with 9,999 permutations. Wilcoxon signed-rank test was used for statistical comparisons of individual taxa, with a confidence level set at 95% (p < 0.05).

DATA AND CODE AVAILABILITY

The fastq files containing sequencing reads generated during this study are available at European Nucleotide Archive (ENA) database under study number PRJEB 29544; https://www.ebi.ac.uk/ena/data/view/PRJEB29544.

This study did not generate/analyze code.