**Revised manuscript GASTRO-D-12-00265**

**Supplemental Materials and Methods**

*Histological examination*

Histological examination was performed as described previously, and the mean degree of inflammation was calculated using a modification of a previously described scoring system.

*Flow cytometry and antibodies*

For intracellular cytokine staining, cells were incubated for 12 h with 50 ng/mL phorbol 12-myristrate 13-acetate (PMA; Sigma, St. Louis, MO), 1,000 ng/mL ionomycin (Sigma), and 1 μL/mL GolgiPlug (BD Pharmingen, San Diego, CA) at 37 °C. For IL-23 stimulation, cells were incubated for 3 h with mouse recombinant IL-23 (40 ng/mL; R&D Systems, Minneapolis MN). For the final 1.5 hr, 1 μL/mL GolgiPlug was added to the culture medium.

Surface staining was performed with the corresponding cocktail of FITC-, PE-, PerCP-Cy5.5-, APC-, PE-Cy7-, APC-Cy7- or Alexa Fluor 647-conjugated monoclonal antibodies (mAbs) for 20 min at 4 °C. After staining surface molecules, the cells were resuspended in Fixation/Permeabilization solution (BD Pharmingen), and intracellular staining was performed. For Foxp3 staining, the cells were fixed and permeabilised according to manufacturer’s instructions (eBiosciences) and stained. Standard four- or six-color flow cytometry analyses were obtained using a FACS Calibur™ or FACSCanto™ II (Becton
Dickinson, NJ, USA) and analyzed by FlowJo (Tree Star, Inc., Ashland, OR). The following mAb were obtained for purification of cell populations and flow cytometry analysis; CD3 mAb (145-2C11), CD4 mAb (RM4-5), CD45RB mAb (C363.16A), IL-17A mAb (TC11-18H10), Ly5.1 mAb (A20) and Ly5.2 mAb (104), TCRγδ mAb (GL3), CD62L mAb (MEL-14), c-kit mAb (2B8), NK1.1 mAb (PK136), CD11c mAb (HL3), CD45R/B220 mAb (RA3-6B2), TNF-α mAb (MP6-XT22), CTLA-4 mAb (UC10-4F10-11), and GITR mAb (DTA-1) (BD Pharmingen); IL-7Rα mAb (A7R34), NKp46 mAb (29A1.4), CD44 mAb (IM7), TCRβ mAb (H57-597), IFN-γ mAb (XMG1.2), IL-17A mAb (eBio17B7), CD25 mAb (PC61.5), CD103 mAb (2E7), Foxp3 mAb (FJK-16s), and IL-17F mAb (eBio18F10), (eBioscience); CCR6 mAb (140706) and IL-22 mAb (140301) (R&D Systems).

Cells isolations

Single cell spleen suspensions were aseptically prepared by mechanical mashing. Single cell suspensions of intestinal lamina propria mononuclear cells (LPMCs) were prepared as previously described.4 Briefly, the intestines (SI and colon) were removed and placed in Ca²⁺, Mg²⁺-free Hanks’ BSS (HBSS, Nacalai Tesque, Japan. After removal of residual mesenteric fat tissue, Peyer’s patches were carefully excised, and the intestine was opened longitudinally. The intestines were thoroughly washed in HBSS and cut into small pieces. The dissected mucosa was incubated with HBSS containing 1 mM DTT (Sigma) and 5 mM EDTA (Gibco, Carlsbad, CA) for 30 min at 37 °C to remove the epithelial layer. The pieces of intestine were
washed and placed in digestion solution containing 1.5% FBS and 1.0 mg/mL collagenase A (Roche Diagnostics GmbH, Germany) for 1 h at 37 °C. Intestine supernatants were washed and resuspended in the 40 % fraction of a 40:75 Percoll gradient, and overlaid on 75% fraction. Percoll gradient separation was performed by centrifugation at 840 × g for 20 min at room temperature. Mononuclear cells were collected at the interphase of the Percoll gradient, washed, and resuspended in FACS buffer or RPMI-1640 (Sigma) containing 10% FBS and penicillin/streptomycin (Nacalai Tesque, Japan). GFP+ LP CD4+ cells were isolated from the SI and colon of RORγt^{gfp/+} mice or from the colon of colitic RAG-2−/− mice previously transferred with CD4+CD45RB^{high} T cells of RORγt^{gfp/+} mice, then stained with CD4 mAbs. Cell sorting was performed on a FACSARia™ to obtain a pure population of GFP+CD4+ cells as RORγT+ T cells.

**Parabiosis**

To assess the hemodynamics and development of naturally-occurring Th17 cells and LTi-like cells, we carried out parabiosis surgery between sex-matched Ly5.2+ LTα−/− and Ly5.1+ C57BL/6 WT mice as previously described.4

**Generation of BM chimeras**

Recipient mice were irradiated with 5 Gy and were injected with BM from donors the next day. After 6 wk, we assessed four groups: Gr. 1, WT mice transplanted with WT BM cells; Gr. 2, LTα−/− mice transplanted with WT BM cells; Gr. 3, WT mice transplanted with LTα−/− BM
Adoptive transfer

Adoptive transfer colitis experiments were performed as previously described. Briefly, WT or LTα−/− CD4+CD45RBhigh (3 × 10⁵ cells/mouse) were intraperitoneally injected into RAG-2−/− or LTα−/−xRAG-2−/− mice. Mice were sacrificed 6 wk after transfer. GFP+ LP CD4+ cells (3 × 10⁵ cells/mouse) from RORγtgfp/− mice or from colitic RAG-2−/− mice previously transferred with CD4+CD45RBhigh T cells of RORγtgfp/+ mice were intraperitoneally injected into RAG-2−/− mice. For the LTi-like cells adoptive transfer experiment, SI LPMCs were stained with CD3e, CD11c, CD11b, NK1.1, B220, CD45.2 and NKp46 antibodies. Cell sorting was performed on a FACSARia™ to obtain a pure population of Lin (CD3e, CD11c, CD11b, NK1.1 and B220)CD45.2+NKp46EGFP+ cells (LTi-like cells) as recently reported.

For adoptive transfer experiments using LTi-like cells, 2.5 × 10⁵ cells of the sorted LTi-like cells were transferred into LTα−/− mice.

In vitro induction of Th17 cells

Naïve splenic CD4+CD62L+ T cells were isolated by magnetic sorting according to the manufacturer’s instructions (Miltenyi). Naive CD4+CD62L+ T cells (1 × 10⁵/well) were cultured in 96-well plates containing plate bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml), supplemented with 5 ng/ml rhTGF-β1 (R&D Systems), 30 ng/ml rmIL-6 (Peprotech), 10 μg/ml anti-IFN-γ (clone XMG1.2), and 10 μg/ml anti-IL-4 (clone 11B11).
After 3 d, CD4⁺ T cells were collected and stimulated with PMA (50 ng/ml; Sigma) and ionomycin (1,000 ng/ml; Sigma) for 5 hr in the presence of Golgi Plug (BD).

**In vitro proliferation assay**

Proliferation assay was performed as previous report⁶. Responder CD4⁺ T cells were prepared from Ly5.1 splenocytes by anti-CD4 MACS beads and were labeled with 1 μM CFSE (Molecular Probes) for 10 min at 37 °C. Splenic CD4⁺CD25⁺ regulatory T cells were isolated from WT (Ly5.2) splenocytes according to the manufacturer’s instructions (Miltenyi). LP naturally-occurring Th17 (GFP⁺ CD4⁺) cells were prepared using FACS Area. As antigen-presenting cells (APCs), 1 x 10⁷ CD4 depleting cells were prepared from WT (Ly5.2) splenocytes and were treated with 0.1 mg/mL mitomycin C for 20 min at 37 °C. CD4⁺ responder T cells cells (2.0 x 10⁴) and APC (5.0 x10⁵) were cultured with or without CD4⁺CD25⁺ regulatory T cells (2.0 x 10⁴) or CD4⁺GFP⁺ naturally-occurring Th17 (2.0 or 1.0 x 10⁴) for 3 days in 96-well round-bottomed plates in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), 50 μM 2-ME, 100 μM MEM non-essential amino acids, 10 mM HEPES and 1 mM sodium pyruvate. Anti-CD3 mAb (145-2C11) at a final concentration 0.5 μ g/ml was added to the cultures for stimulation. Proliferation of Ly5.1⁻CD4⁺ Tresp cells was assessed by CFSE dilution.


Supplemental Figure Legends

Supplemental Figure 1
The percentage of naturally-occurring Th17 cells, but not CD3- IL-17A-expressing cells, is significantly reduced in the colon of LTα−/− mice compared to WT mice. (A) Expression of IL-17A and IFN-γ in spleen and colonic LP CD3+CD4+ T cells from WT and LTα−/− mice. (B) Mean percentages of IL-17A+CD3+CD4+ T cells in spleen and colonic LP CD3+CD4+ T cells of WT and LTα−/− mice. (C) Expression of IL-17A and CD3 in the spleen and colon LP mononuclear cells from WT and LTα−/− mice. (D) Mean percentages of IL-17A+CD3− in splenic and colon LP mononuclear cells from WT and LTα−/− mice. Data (B and D) represent the mean ± SEM (n = 3/group). *, P< 0.05. NS, not significant.

Supplemental Figure 2

The percentage of naturally-occurring Th17 cells are significantly reduced in the SI of LTα−/− mice compared with co-housed WT mice. (A) (Upper) Expression of IL-17A and IFN-γ in the SI LP CD3+CD4+ T cells of co-housed WT and LTα−/− mice. (Lower) Expression of IL-17A and CD3 in the SI LP mononuclear cells of co-housed WT and LTα−/− mice. (B) (Upper) Mean percentages of IL-17A−CD3+CD4+ T cells in total SI LP CD3+CD4+ T cells from WT and LTα−/− mice. (Lower) Mean percentages of IL-17A+CD3− in SI LP CD3− cells of co-housed WT and LTα−/− mice. Data (B) represent the mean ± SEM (n = 3/group). *, P< 0.05.
Supplemental Figure 3

**LTα expression of CD4⁺ T cells is not required for the development of *in vitro*-manipulated Th17 cells by TGF-β and IL-6 stimulation.** Expression of IL-17A and IFN-γ in splenic CD3⁺CD4⁺ T cells stimulated with TGF-β and IL-6 for 72 h. Cytokine production by splenic naïve CD4⁺CD62L⁺ T cells from the indicated mice after stimulation with anti-CD3/CD28 for 3 days with TGF-β and IL-6. Expression of IL-17A and IFN-γ in the differentiated T cells of the indicated mice.

Supplemental Figure 4

**LTα-dependent GALT structure is essential for the generation of intestinal naturally-occurring Th17 cells.**

(A) Scheme of the BM chimera study. (B) Expression of IL-17A and IFN-γ in CD3⁺CD4⁺ T cells in the SI LP of the indicated mice. (C) Mean percentages of IL-17A⁺ cells in SI LP CD3⁺CD4⁺ T cells. (D) Expression of IL-17A and CD3 in SI LPMCs of the indicated mice. (E) Mean percentages of IL-17A⁺ CD3⁻ cells in SI LPMCs. Data (C and E) represent the mean ± SEM (n = 5/group). *, P< 0.05. NS, not significant.
Supplemental Figure 5

LTα-dependent GALT structure is essential for the development of intestinal naturally-occurring Th17 cells. (A) Scheme of adoptive transfer of WT LTi-like cells into LTα<sup>-/-</sup> mice. We isolated Lin<sup>-</sup>CD45<sup>+</sup>NKp46<sup>-</sup> GFP (RORgt)<sup>high</sup> LTi-like cells from the SI of Rorc<sup>(gt)gfp<sup>+</sup></sup> reporter mice, and transferred them into LTα<sup>-/-</sup> mice. Age-matched WT and LTα<sup>-/-</sup> mice were used as controls. (B) Expression of IL-17A and IFN-γ in CD3<sup>+</sup>CD4<sup>+</sup> T cells in the SI LP of the indicated mice. (C) Mean percentages of IL-17A<sup>+</sup> cells in SI LP CD3<sup>+</sup>CD4<sup>+</sup> T cells. Data show represent the mean ± SEM (n = 5/group). *, P< 0.05. NS, not significant.

Supplemental Figure 6

RORγ<sub>intermediate</sub>, but not RORγ<sub>high</sub>, cells express CD3. (A) (Upper) GFP expression of Rorc(γ<sup>t</sup>)<sup>gfp<sup>+</sup></sup> SI LP CD4<sup>+</sup> cells. (Lower) GFP expression of LP CD4<sup>+</sup> cells from colitic RAG-2<sup>-/-</sup> mice previously transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from Rorc(γ<sup>t</sup>)<sup>gfp<sup>+</sup></sup> mice. (B) CD3 and CD4 expression in each indicated population. RORγ<sub>t</sub>-negative and positive cells from either Rorc(gt)<sup>gfp<sup>+</sup></sup> mice or colitogenic mice were CD3-expressing T cells. RORγ<sub>t</sub><sup>high</sup>
cells in \( \text{Rorc}(\gamma)t^{gfp/+} \) mice were CD3 negative. (C) Naturally-occurring Th17 cells do not respond to IL-23 to induce IFN-\( \gamma \) production. Sorted SI LP GFP\(^+\) CD4\(^+\) T cells from \( \text{Rorc}(\gamma)t^{gfp/+} \) mice and MACS-sorted colitic LP CD4\(^+\) T cells from colitic CD4\(^+\)CD45RB\(^{\text{high}}\) T cell-transferred mice were cultured with or without IL-23 (40 ng/ml) for 3 h. Mean percentages of IFN-\( \gamma \)^\( + \) cells in cultured T cells. Data represent the mean \( \pm \) SEM (n = 3/group).

\( *, P < 0.05 \). NS, not significant.

Supplemental Figure 7

Model of the development of naturally-occurring and colitogenic Th17 cells. (Upper) LT\( \alpha \)-dependent GALT is essential for the generation of protective naturally-occurring SI Th17 cells in the steady state, but the lack of those molecules results in a marked decrease of naturally-occurring Th17 cells and the increase of LTi-like cells. (Middle) Parabiosis experiments clarified that: 1) naturally-occurring SI Th17 cells, but not LTi-like cells, are well mixed in each parabiont partner; and 2) GALT residing normal LTi-like cells are required for the generation of naturally-occurring SI Th17 cells. (Lower) In sharp contrast to steady state conditions, the generation of colitogenic Th17 and Th17/Th1 cells under inflammation is not dependent on LT\( \alpha \)-expressing LTi-like cells.
Supplemental Figure 1.
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A

Spleen

Colon

B

C

Spleen

Colon

D

Supplemental Figure 1.
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Supplemental Figure 2.
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A Co-housed

WT LTα-/-

IL-17A

IFN-γ

IL-17A

CD3e

0
5
10
15
20
*
% IL-17+ CD3+CD4+

B

WT LTα-/-

% IL-17+ CD3-CD4+

*
Supplemental Figure 3.
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Supplemental Figure 4.
Ono, et al.
Supplemental Figure 5.
Ono, et al.

A

![Flow cytometry plots showing Lin, CD45, NKp46, RORγt-GFP, LTα−/−, and LTα−/− transfected LTα−/−.](image)

B

![Bar chart showing % IL-17+ CD3+CD4+ in WT, LTα−/−, and LTα−/− transfected LTα−/−.](image)

C

![Graph showing % IL-17+ CD3+CD4+ with error bars indicating statistical significance.](image)
Supplemental Figure 6.
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A

Naturally-occurring RORγt

Colitogenic RORγt

B

RORγt Negative

RORγt Positive

RORγt Super High

CD4

CD3e

C

% LP CD3+CD4+

IFN-γ+

NS

*
**Steady state condition**

WT

- **GALT**
  - WT Naïve T
  - WT Th17 ‘protective’

LTα−/−

- **GALT**
  - LTα−/− Naïve T
  - LTα−/− Th17

**Parabiosis**

WT

- **GALT**
  - LTα−/− Naïve T
  - WT Naïve T
  - LTα−/− Th17
  - WT Th17

LTα−/−

- **GALT**
  - LTα−/− Naïve T
  - WT Naïve T
  - LTα−/− Th17
  - WT Th17

**Inflammatory condition**

RAG-2−/−

- **GALT**
  - Naïve T
  - Th17
  - Th17/Th1
  - ‘pathogenic’

RAG-2−/− LTα−/−

- **GALT**
  - Naïve T
  - Th17
  - Th17/Th1
  - Th1
  - ‘pathogenic’