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Supplemental Information

The Short Isoform of the CEACAM1 Receptor in Intestinal T Cells Regulates Mucosal Immunity and Homeostasis via Tfh Cell Induction

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Inventory of Supplemental Information

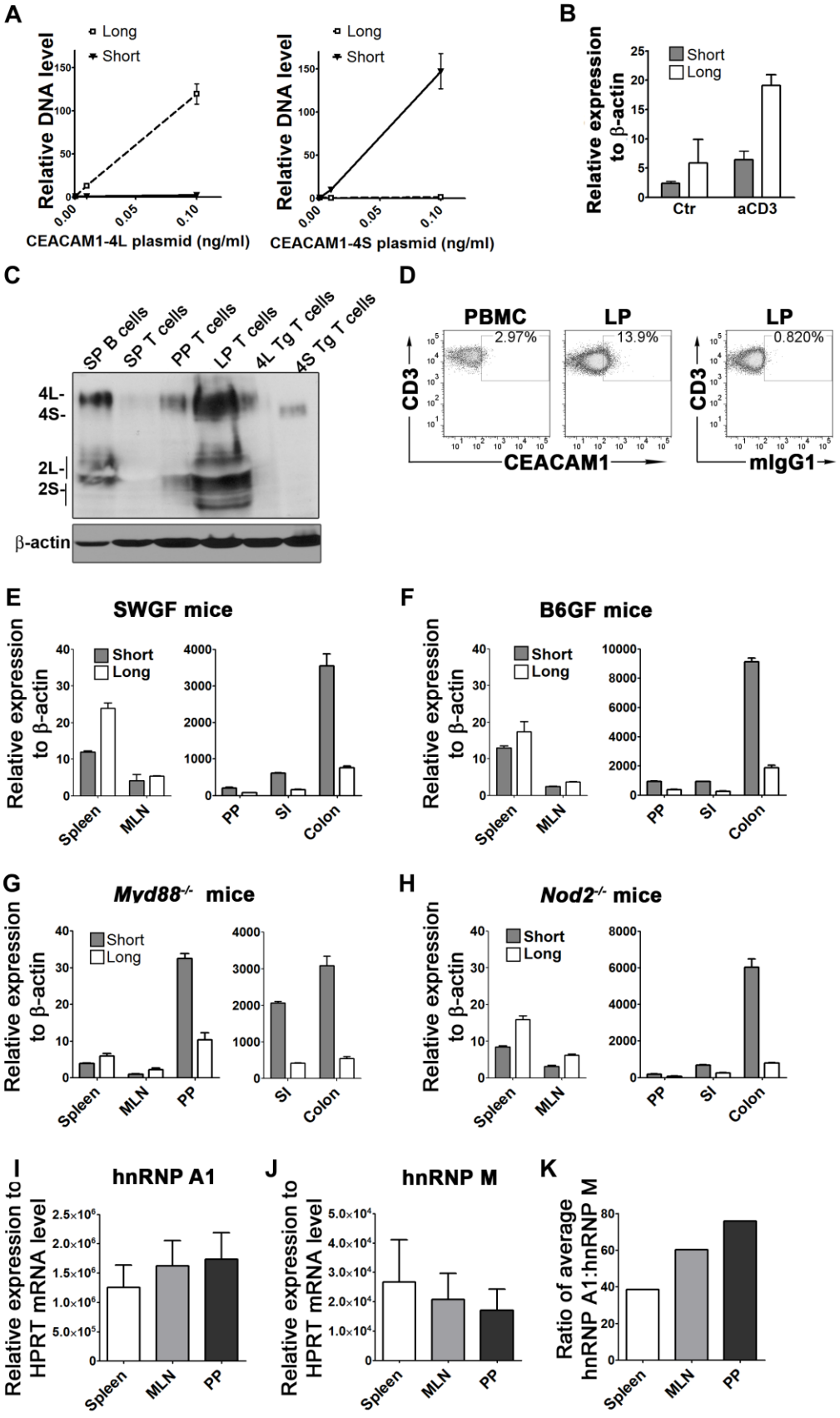
A
Long
Short
Relative DNA level
CEACAM1-4L plasmid (ng/ml)
CEACAM1-4S plasmid (ng/ml)
B
Short
Long
Relative expression to β-actin
Ctr
aCD3
C
SP B cells
SP T cells
PP T cells
LP T cells
4L Tg T cells
4S Tg T cells
4L-
4S-
2L-
2S-
β-actin
D
PBMC
LP
LP
2.97%
13.9%
0.820%
CD3
CEACAM1
mIgG1
E
SWGF mice
Short
Long
Relative expression to β-actin
Spleen
MLN
PP
SI
Colon
F
B6GF mice
G
Myd88-/- mice
H
Nod2-/- mice
I
hnRNP A1
Relative expression to HPRT mRNA level
J
hnRNP M
K
Ratio of average hnRNP A1:hnRNP M

Figure S1. A Novel CEACAM1 Isoform-Discriminating qPCR Assay Demonstrated that Predominant Expression of CEACAM1-S Isoforms in Intestinal T Cells Is Not Determined by or Dependent on the Commensal Microbiota

(A-D) Validation of method to quantitatively determine the transcriptional levels of CEACAM1-L or S isoforms in mouse T cells. (A) The specificity and efficiency of qPCR primers for amplification of mouse CEACAM1-L and -S isoforms were verified using mCEACAM1-4L and 4S plasmids. (B) CEACAM1-L isoform expression predominated over CEACAM1-S isoforms in mouse spleen $CD3^+$ T cells (Ctr), and both were increased upon anti-CD3 stimulation (aCD3). (C) T cells were purified from spleen (SP), PP, colon lamina propria (LP) of WT mice and the spleen of 4S Tg and 4L Tg mice while B cells were isolated from the spleen of WT mice. Lysates prepared from each of these populations were immunoblotted with the mCEACAM1 specific antibody, CC1, or β-actin as a loading control. (D) Isotype control staining for cell surface expression of CEACAM1 (5F4 antibody) on $CD3^+$ T cells from human peripheral blood or colon LP. (E-K) CEACAM1-S isoform predominance in intestinal T cells did not depend on the commensal microbiota.

(E-H) qPCR analysis for the transcriptional abundance of CEACAM1-L and -S in $CD3^+$ T cells isolated from spleen, MLN, PP or segments of intestinal LP from (E) Swiss-Webster (SW) germ-free (GF), (F) C57BL/6 (B6) GF, (G) SPF *Myd88*$^{-/-}$ or (H) SPF *Nod2*$^{-/-}$ mice. The expression of CEACAM1-S and CEACAM1-L was similar to those observed in WT SPF mice.

(I-K) Transcripts of (I) hnRNP A1, (J) hnRNP M for T cells isolated from spleen, MLN and PP of SPF WT mice, and (K) the ratio of hnRNP A1 : hnRNP M which gradually increased and correlated with the ratio of CEACAM1- L:S.

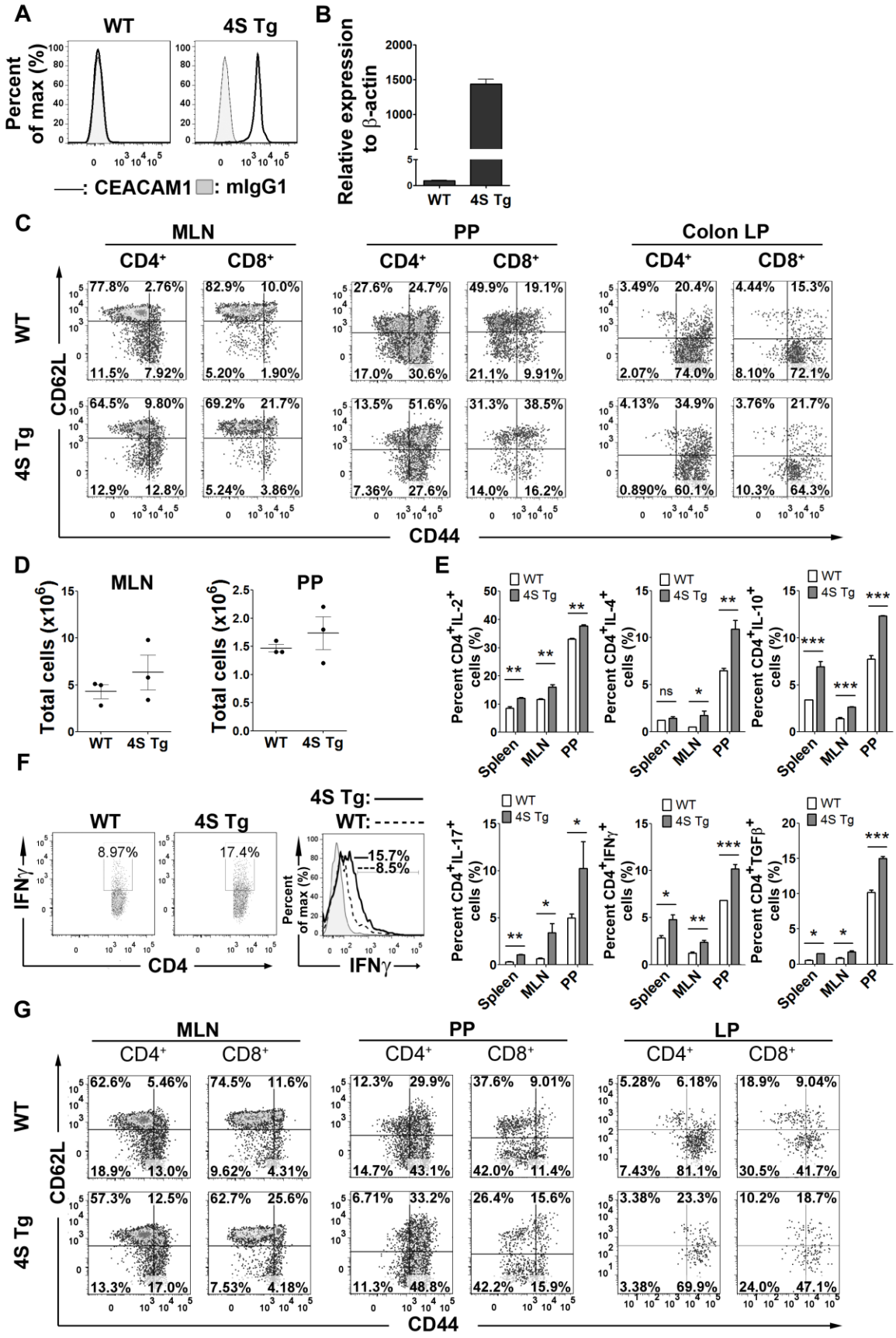
A
WT
4S Tg
Percent of max (%)
—: CEACAM1 ▒: mIgG1
B
Relative expression to β-actin
WT
4S Tg
C
MLN
PP
Colon LP
CD4+
CD8+
WT
4S Tg
CD62L
CD44
77.8% 2.76%
11.5% 7.92%
82.9% 10.0%
5.20% 1.90%
27.6% 24.7%
17.0% 30.6%
49.9% 19.1%
21.1% 9.91%
3.49% 20.4%
2.07% 74.0%
4.44% 15.3%
8.10% 72.1%
64.5% 9.80%
12.9% 12.8%
69.2% 21.7%
5.24% 3.86%
13.5% 51.6%
7.36% 27.6%
31.3% 38.5%
14.0% 16.2%
4.13% 34.9%
0.890% 60.1%
3.76% 21.7%
10.3% 64.3%
D
MLN
PP
Total cells (x10^6)
WT
4S Tg
E
WT
4S Tg
Percent CD4+IL-2+ cells (%)
Percent CD4+IL-4+ cells (%)
Percent CD4+IL-10+ cells (%)
Spleen
MLN
PP
ns
F
WT
4S Tg
4S Tg: ——
WT: ------
IFNγ
CD4
8.97%
17.4%
Percent of max (%)
15.7%
8.5%
Percent CD4+IL-17+ cells (%)
Percent CD4+IFNγ+ cells (%)
Percent CD4+TGFβ+ cells (%)
G
MLN
PP
LP
CD4+
CD8+
WT
4S Tg
CD62L
CD44
62.6% 5.46%
18.9% 13.0%
74.5% 11.6%
9.62% 4.31%
12.3% 29.9%
14.7% 43.1%
37.6% 9.01%
42.0% 11.4%
5.28% 6.18%
7.43% 81.1%
18.9% 9.04%
30.5% 41.7%
57.3% 12.5%
13.3% 17.0%
62.7% 25.6%
7.53% 4.18%
6.71% 33.2%
11.3% 48.8%
26.4% 15.6%
42.2% 15.9%
3.38% 23.3%
3.38% 69.9%
10.2% 18.7%
24.0% 47.1%

Figure S2. Characterization of CEACAM1-4S Tg and *Ceacam1*$^{-/-}$-4S Tg Mice

(A) The demonstration that transgenic mice with forced expression of CEACAM1-4S in T cells exhibited increased CEACAM1 expression on T cells as shown by flow cytometry using the CC1 monoclonal antibody and anti-CD3 antibody in cells isolated from spleen. Histograms represent expression on $CD3^+$ cells.

(B) The overexpression of CEACAM1-S isoforms on T cells in 4S Tg mice was defined by qPCR analysis confirming the dominance of this isoform in the Tg mice.

(C) In 4S Tg mice, cells isolated from MLN, PP or colon LP exhibited lower percentages of $CD62L^{hi}CD44^{low}$ naïve T cells, and higher percentages of $CD62L^{low}CD44^{hi}$ effector memory or $CD62L^{hi}CD44^{hi}$ central memory T cells when compared to WT mice.

(D) Total cell count numbers in the PP and MLN of WT and 4S Tg mice were not significantly different, consistent with selective depletion of T cells under conditions of 4S overexpression.

(E) Intracellular cytokine staining of the $CD4^+$ T cells isolated from spleen, MLN and PP also revealed that IL-2, IL-4, IL-10, IL-17, IFN-γ and TGF-β were all increased in the T cells of 4S Tg mice when compared to WT littermates. (Mean ± SEM) *, $p < 0.05$ (two-tailed Student's *t* test).

(F) Representative data for the intracellular IFN-γ staining of the $CD4^+$ T cells isolated from PP of WT or 4S Tg mice. All data are representative of ≥ 3 experiments with n = 4-6 mice per group.

(G) Characterization of *Ceacam1*$^{-/-}$-4S Tg mice. Cells isolated from MLN, PP or colon LP of *Ceacam1*$^{-/-}$-4S Tg mice exhibited lower percentages of $CD62L^{hi}CD44^{low}$ naïve T cells, and higher percentages of $CD62L^{low}CD44^{hi}$ effector memory or $CD62L^{hi}CD44^{hi}$ central memory T cells when compared to *Ceacam1*$^{-/-}$ mice. Data are representative of 5 pairs of animals.

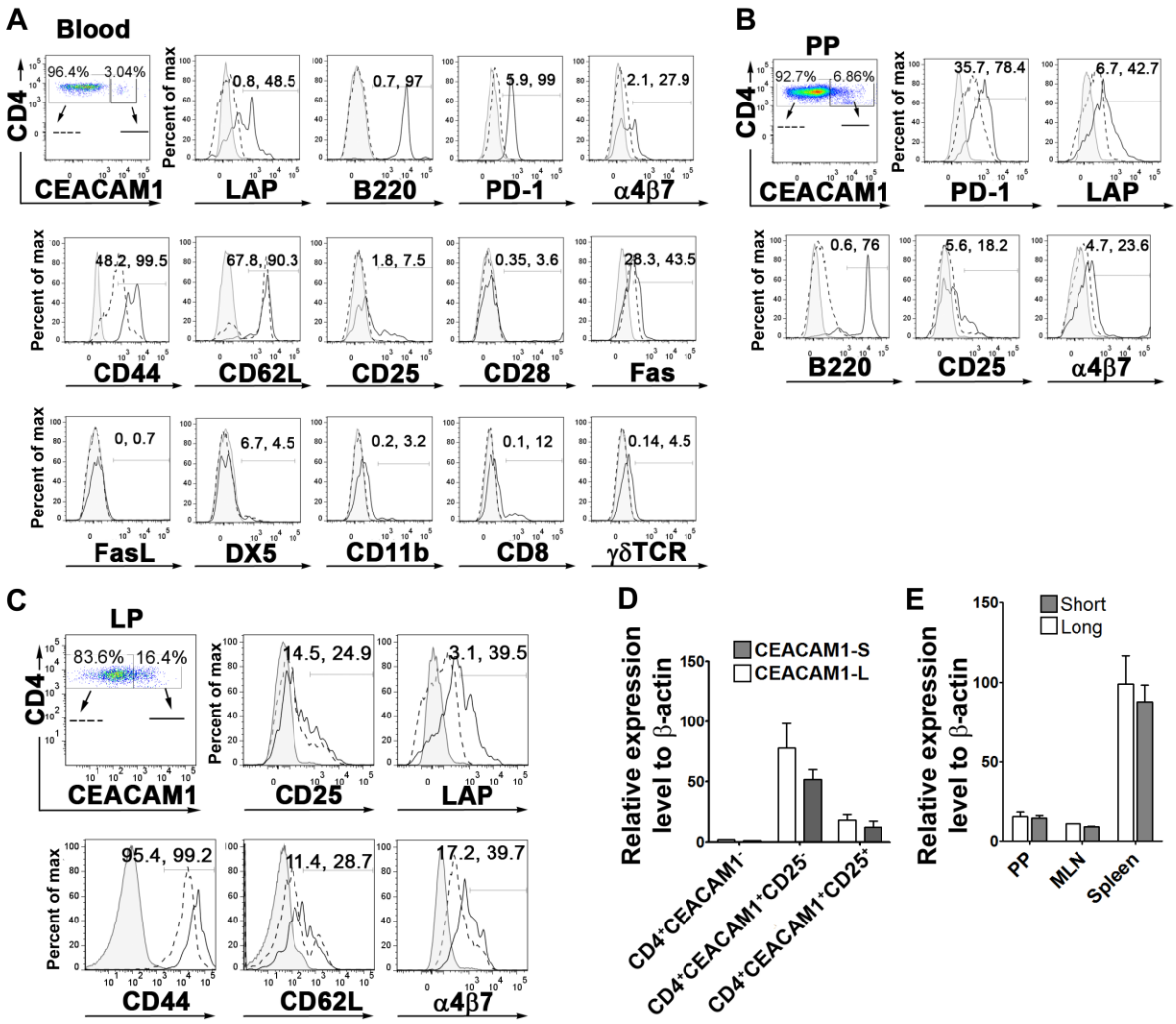

Figure S3. CEACAM1-S Expression on CD4$^+$ T Cells Is Associated with the Expression of Molecules, Which Characterize Specific Subsets of T Cells

(A-C) Characterization of cell surface molecule expression on CD4$^+$CEACAM1$^+$ and CD4$^+$CEACAM1$^-$ cells in (A) blood, (B) PP and (C) LP of WT mice. Percentages on histogram gates indicate the frequency of cells positive for the given marker in the CD4$^+$CEACAM1$^-$ and CD4$^+$CEACAM1$^+$ cell populations, respectively. Representative data from 3 independent experiments with n = 5-6 mice per group.

(D) The relative expression of the L and S isoforms did not differ between CD4$^+$CEACAM1$^+$CD25$^-$ and CD4$^+$CEACAM1$^+$CD25$^+$ regulatory T cells with the L isoform predominating in both populations. The L:S ratio was 1.52 for CD4$^+$CEACAM1$^+$CD25$^-$ cells and 1.52 for CD4$^+$CEACAM1$^+$CD25$^+$ cells. Cells were isolated by flow cytometric sorting from pooled spleen and MLN. Representative data from 3 independent experiments with n = 5-6 mice per group.

(E) The L and S isoforms of CEACAM1 were equally expressed in PD-1$^+$CXCR5$^+$ Tfh cells isolated by flow cytometric sorting from spleen, MLN and PP of WT mice. The L:S ratios were 1.08, 1.22 and 1.13 for the PP, MLN and spleen, respectively. Data are representative of 3 independent experiments n = 3-4 mice per group.

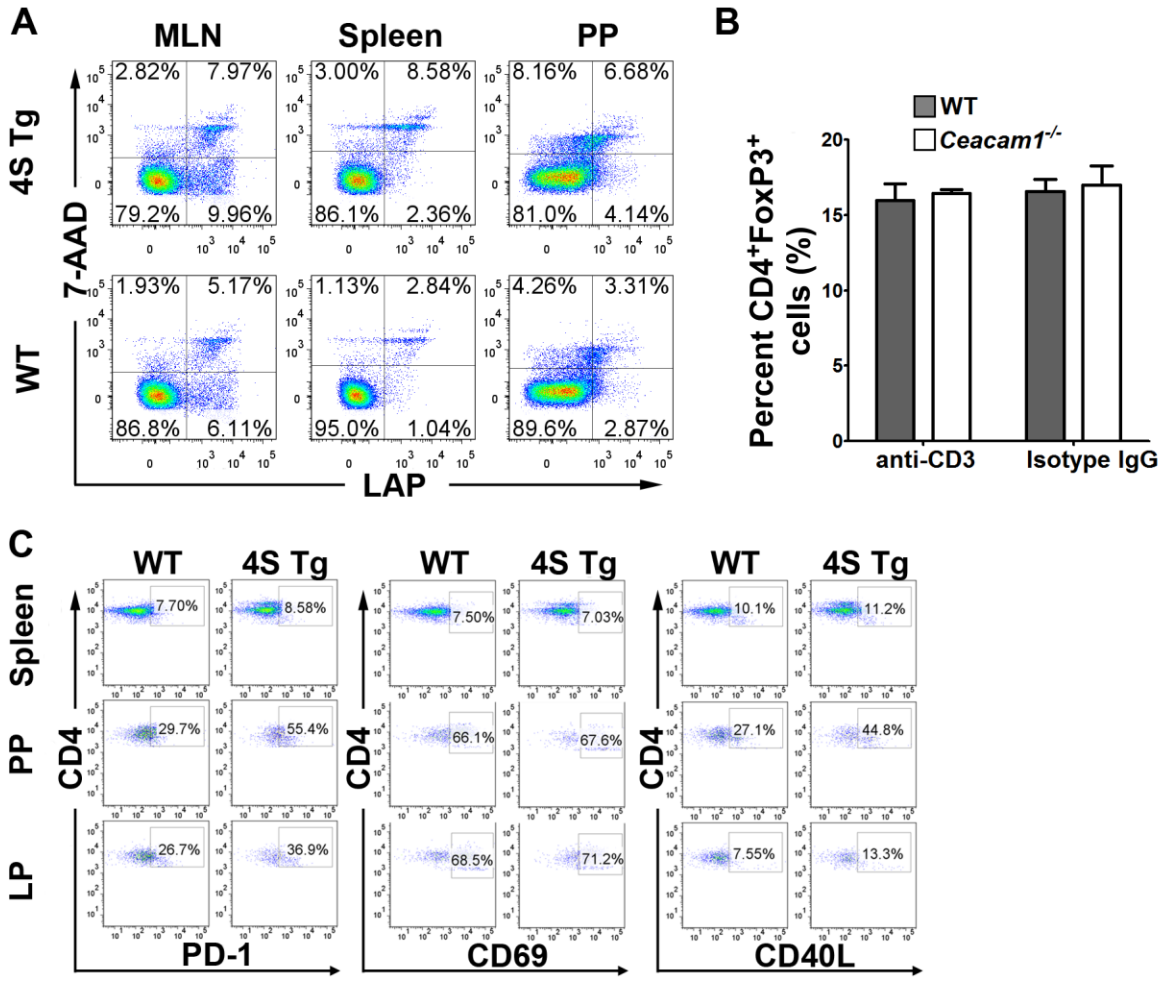

Figure S4. CEACAM1-S Determined the Generation and Accessory Phenotype of Specific T Cell Subsets

(A) The induction of AICD by CEACAM1-S overexpression was not restricted by LAP expression, as indicated by increases in apoptosis seen in both $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells from the MLN, spleen and PP of 4S Tg mice compared to their littermate controls.

(B) Oral feeding of anti-CD3 antibody did not affect the induction of $CD4^+Foxp3^+$ Treg cells in either WT or *Ceacam1*$^{-/-}$ mice. Mice were fed anti-CD3 (5 μg/mouse/day) or an isotype control for 5 consecutive days and tissues were analyzed after 7 days for analysis of regulatory T cell frequency.

(C) $CD4^+$ T cells within the PP or LP, but not in the spleen of 4S Tg mice exhibited increased levels of CD69, PD-1 and CD40L relative to their WT littermate controls. All data are representative of 3 independent experiments with n = 5-6 mice per group.

Table S1. An Elevation of Genes Associated with Ca^{++}- Nuclear Factor of Activation (NFAT) Signaling in Affymetrix cDNA Microarray Analysis on Primary $CD4^+$ T Cells Isolated from MLN of WT or 4S Tg Mice

Gene name	Expression ratio: 4STg/WT
Il2	1.263939
Ifng	1.731262
Il4	3.267857
Cd40l	1.216321
Nfatc1	1.38471
Nfatc2	1.21496
Camk2b	1.373985

Table S2. Quantification for the $B220^-IgA^+$ and $B220^+IgA^+$ Cells in PP and LP of WT and 4S Tg Mice as Determined by Flow Cytometric Staining

	Genotype	$B220^-IgA^+$	$B220^+IgA^+$
PP	**WT**	1.3±0.15%	4.3±0.48%
	4S Tg	2.7±0.31%**	3.7±0.22%
LP	**WT**	16.8±0.47%	2.8±0.41%
	4S Tg	25.8±1.7%**	3.6±0.84%

** $P < 0.005$ (compared to WT counterparts)

Table S3. Cultured Species, Conditions and Methods Used for Identification

Phylum > Order	Species	Selective Culture Conditions	Biochemical ID method
Bacteroidetes > Bacteroidales	*Parabacteroides loescheii*	LKV, anaerobic	MIDI, ANA
	Prevotella tannerae	LKV, anaerobic	MIDI, ANA
Firmicutes > Bifidobacteriales	*Bifidobacterium pseudolongum*	CNA and ROG, anaerobic	MIDI, ANA
Firmicutes > Clostridiales	*Eubacterium* species	CNA, anaerobic	MIDI
Firmicutes > Lactobacilliales	*Lactobacillus acidophilus* group	ROG, anaerobic	MIDI
	Lactobacillus brevis	ROG, anaerobic	MIDI
	Lactobacillus curvatus	ROG, anaerobic	MIDI
	Lactobacillus farcimus	ROG, anaerobic	MIDI
	Lactobacillus leichmanii	ROG, anaerobic	MIDI
	Lactobacillus murinus	ROG, anaerobic	MIDI
Firmicutes > Lactobacilliales	*Enterococcus faecalis*	CNA, aerobic	API-20Strep
	Gemella morbillorum	CNA, anaerobic	API-20Strep
	Streptococcus intermedius	CNA, anaerobic and aerobic	API-20Strep

LKV: Laked Blood, Kanamycin, Vancomycin Agar; CAN: Colistin Nalidixic Acid Agar; ROG: Rogosa Agar; ID: identification; MIDI: the long chain fatty acid analysis on the Microbial Identification System (MIS) (MIDI Inc., Newark, DE); ANA:Rapid ANA Panel

Supplemental Experimental Procedures

Antibodies and Reagents

A mAb specific for mouse CEACAM1, CC1, was previously described (Dveksler et al., 1993). 5F4 is a mouse anti-human CEACAM1 specific mAb (Morales et al., 1999). Caspase-3 and NFAT1 antibodies were purchased from Cell Signaling. GAPDH and β-actin antibodies were obtained from Sigma-Aldrich. Biotinylated anti-LAP antibody was from R&D Systems (Ochi et al., 2006). All other antibodies used for flow cytometry or immunohistochemistry staining were purchased from BD Biosciences, eBioscience or Biolegend. Human rIL-2 for the stimulation of mouse primary T cells was provided by the National Institutes of Health.

Preparation of Human Samples

Human leukopacks were obtained from Kraft Family Blood Donor Center of at Dana-Farber Cancer Institute, and Brigham and Women's Hospital. Human normal colon samples were obtained under informed consent from the Tissue Banks of Brigham and Women's Hospital. Experiments were performed under Brigham and Women's Hospital Review Board approval. Biopsies from specimens of normal colon tissue (distant from the colon cancer) were collected and stored in HBSS on ice. For isolation of LPL, the colon tissue was rinsed with PBS and treated with HBSS containing dithiothreitol (DTT) 100 μg/ml (Sigma), 10% fetal bovine serum (FBS) for 30min at RT. Then the mucosal pieces were treated with three 1 hour washes of HBSS containing 0.75 mM EDTA to remove the epithelial cells at RT. Tissues were further digested in RPMI 1640 containing 2% FBS, purified collagenase (50 μg/ml, Sigma) and DNase I (10 μg/ml, Sigma) in an incubator at 37ºC for 10 hr. Cells were then layered on a 40–75% Percoll gradient (GE Healthcare) and lymphocyte-enriched populations were isolated from the cells at the 40–75% interface. After washing, LPL single cell suspensions were prepared.

Murine Lymphocyte Isolation

Single cell suspensions of mononuclear cells from spleen, lymph nodes, blood, Peyer's patches (PP) (Iwasaki and Kelsall, 1999) or intestinal lamina propria (LP) were prepared as previously described (Nakajima et al., 2002). PP or intestines (removed PP, rinsed with PBS and cut in small pieces) were treated with media containing dithiothreitol 100μg/ml (Sigma), 25 mM Hepes, 10% fetal bovine serum (FBS), and 5 mM EDTA in RPMI1640 for 30 min at 37 ºC to remove epithelial cells, and were washed extensively with RPMI1640. Tissues were further digested in RPMI 1640 containing 2% FBS, collagenase D (400 U/ml, Roche) and DNase I (0.1 mg/ml, Sigma) in a shaking incubator at 37 ºC for 30 min. Cells were then layered on a 40–75% Percoll gradient (GE Healthcare) and lymphocyte-enriched populations were isolated from the cells at the 40–75% interface. After washing, single cell suspensions were prepared. Cells were directly used for flow staining, or were stained with PE conjugated anti-CD3 antibody and followed by anti-PE MicroBeads according to the instructions provided by Miltenyi Biotech to further isolate $CD3^+$ T cells.

Single-cell isolations from spleen and mesenteric lymph nodes were performed with a 40 μm cell strainer (BD Bioscience) and red blood lysis buffer (Sigma) for depletion of red blood cells. $CD3^+$ or $CD4^+$ T cells were isolated from spleen and/or lymph nodes of mice by pan T cell isolation kit or $CD4^+$ T cell isolation kit for mouse (Miltenyi Biotech) according to the manufacturer's instructions.

Quantification of Peyer's Patches

The entire small intestines were removed from mice. Serosal fat on the surface was carefully removed, and then the number and size of PPs were determined by macroscopic observation. The PP score was established by combining the number and size, and calculated in a blinded fashion, as described previously (Barreau et al., 2007). For absolute quantification of PP numbers, SI were opened along the mesenteric border, washed by shaking in dH_2O for one hour and fixed in 5% glacial acetic acid overnight. After washing in dH_2O for another hour, tissues were stained by briefly incubating in Unna's polychrome methylene blue stain (31.3mM methylene blue, 72.4mM potassium carbonate). Tissues were washed once more, then left in dH_2O for 48h to allow differentiation of the stain.

Cell Culture, Proliferation and Determination of Cytokine Production

Cells were resuspended in complete RPMI 1640 medium containing 10% FBS, 1% penicillin and streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 20 mM HEPES, and 1% nonessential amino acids (all from Invitrogen) at a density of 1×10^6 cells/ml, and cultured in 96-well flat bottomed plates with the indicated concentrations of plate-bound anti-CD3 antibodies (eBioscience). Culture supernatants were harvested at 24 h and cytokine production determined by ELISA (BD Biosciences) according to the manufacturer's instructions. Proliferation assays were performed by addition of [^{3}H]-thymidine (1.0 μCi/well) at 48 hours for 8 hours.

Protein Isolation and Western Blotting

Spleen and MLN mononuclear cells were washed twice in cold PBS. Total protein was isolated with RIPA buffer as previously described (Chen et al., 2008). The nuclear and cytoplasmic protein was isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). The concentration of proteins in the supernatants was assessed using the BAC Protein Assay (Thermo Scientific). Immunoblotting was performed as previously described by using specific antibodies as indicated in the results and legends (Chen et al., 2008).

Real-Time Quantitative PCR

RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions, and genomic DNA was removed with DNase I. qPCR was performed as described previously using SYBR Green quantitative PCR master mix (Roche) (Nagaishi et al., 2006). All runs were accompanied by two internal control genes, β-actin and GAPDH. Samples were normalized using a $\Delta\Delta$ cycle threshold-based algorithm to provide arbitrary units representing relative expression levels between samples. The following primers were used for mouse CEACAM1: CEACAM1-L, 5'-GCGAGATCTCACAGAGCACA-3' (forward) and 5'-GCTGGGAATTGAAGTTCAGG-3' (reverse); CEACAM1-S, 5'-CTGGCATCGTGATTGGAGTT-3' (forward) and 5'- CAGAAGGAGCCAGATCCG-3' (reverse). hnRNP expression was determined by qPCR, as described previously (Dery et al., 2011).

Isolation of Microorganisms and Determination of Microbiologic Counts

Samples of terminal ileum (2 cm from the ileocecal valve) were sterilely dissected from mice, placed in cryovials and immediately snap frozen in liquid nitrogen. Samples were delivered to the Harvard Digestive Disease Center's (HDDC) Microbiome and Gnotobiotics Core facility on dry ice and stored at -80°C until processed. The frozen tissue sample was placed into a sterile pre-weighed tube and re-weighed to determine the weight of the sample. Immediately following this step, samples were placed into an anaerobic chamber containing an atmosphere of 10% hydrogen, 10% carbon dioxide and 80% nitrogen (Coy Laboratory Products, Grass Lake, MI) for

processing. Per the mass of the tissue, samples were diluted 1:10 with sterile, pre-reduced phosphate-buffered-saline (PBS). Using a hand held tissue grinder, samples were manually macerated until the material was completely dispersed in the PBS. A 0.1mL aliquot of each dilution from 10^{-3} to 10^{-8} was plated on enrichment or selective agar media and the aliquots spread over the surface of the medium. Media for recovery of obligate anaerobes included pre-reduced Brucella base blood agar containing 5% sheep's blood, hemin and menadione (BMB, enrichment medium for the isolation of fastidious obligate anaerobes; Remel, Norcross, GA), Brucella base blood agar containing 5% laked sheep blood, 100 ug/mL of kanamycin, and 7.5 ug/mL vancomycin (LKV, for the isolation of *Bacteroides*, *Fusobacterium, Prevotella* and *Porphyromonas*), Rogosa Selective agar (ROG for the isolation of *Lactobacillus* and *Bifidobacterium* species) and Brucella base blood agar containing colistin-naladixic acid (CNA, to inhibit facultative Gram-negative bacilli and select for Gram-positive organisms). Facultative organisms were isolated using Tryptic Soy base with 5% sheep blood agar (TSA), CNA, Bile Esculin Azide agar (BEA, for the isolation of *Enterococci*), and MacConkey agar (MAC), for the isolation of *Enterobacteriaceae.* For the enumeration and cultivation of spore-forming *Clostridia*, an equal volume of 95% ethanol was added to an aliquot of the initial 1:10 dilution of sample in PBS and mixed gently. The tube was removed from the hood and incubated at room temperature for 30 minutes and then placed back into the anaerobic chamber. Serial 10-fold dilutions were then made in sterile PBS and 0.1mL aliquots of each dilution plated onto BMB. Media for the isolation of anaerobes (BMB, LKV, ROG and one set of CNA) were incubated at 37^{o} in the anaerobic chamber for 72 and 96 hours before enumeration by trained microbiologists. Media for the culture of facultative and aerobic species - TSA, BEA, MAC, and the second set of CNA - were removed from the anaerobic chamber and incubated at 37^{o} in a 5% CO2 incubator in ambient air for 24-48 hours before enumeration. All quantitative counts were recorded as colony forming units (c.f.u.) per gram of specimen, from which the log10 c.f.u./g was calculated. Representative isolates from anaerobic culture of each mouse was re-passaged into BMB plates incubated anaerobically and aerobically at 37°C in a 5% CO2 atmosphere to determine aero-tolerance. Isolates were also inoculated into BHI or chopped meat broth for passage, preparation of frozen stocks and for identification.

Identification of Microorganisms

Following incubation under appropriate atmospheric conditions, colonies were enumerated on the various media and individual colony types selected for identification from each cohort of mice, based on colony morphology, Gram stain and additional microbiological phenotypes. Additional phenotypic characterization of obligate anaerobic isolates for species identification was performed using long chain fatty acid analysis on the Microbial Identification System (MIS) (MIDI Inc., Newark, DE) and/or the RapID ANA II System (Remel Inc., Norcross, GA). Streptococci were further typed using API-20Strep panels (Biomerieux, Durham, NC).

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