Title: A diet-dependent enzyme from the human gut microbiome promotes Th17 accumulation and colitis

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Abstract (125 words)

Aberrant activation of Th17 cells by the gut microbiota contributes to autoimmune and chronic inflammatory disease; however, the mechanisms responsible and their diet-dependence remain unclear. Here, we show that the disease-associated gut Actinobacterium *Eggerthella lenta* increases intestinal Th17 cells and worsens colitis. *E. lenta*-induced disease and Th17 accumulation was *Rorc*-dependent and strain variable. Comparative genomics revealed a single genomic locus predictive of Th17 accumulation and a gene within this locus, encoding the Cgr2 enzyme, was sufficient to increase Th17 cells. Increased dietary arginine prevented *E. lenta* mediated Th17 accumulation and ameliorated colitis suggesting that diet can modulate microbial associated disease. These results expand the mechanisms through which bacteria shape mucosal immunity and demonstrate the feasibility of identifying the causal species, genes, and enzymes that contribute to autoimmune disease.

Main text:

Intestinal immune responses are linked to the trillions of microorganisms that colonize the gastrointestinal tract (*1*, *2*). Therefore, inter-individual variations in the gut microbiome could contribute to diseases associated with dysregulated immune responses such as autoimmunity (*3*–*5*). Consistent with this hypothesis, multiple disease models are ameliorated in germ-free (GF) or antibiotic-treated mice (*6*–*8*). Microbiome-wide association studies have identified bacteria enriched in autoimmune diseases, including rheumatoid arthritis (RA) (*3*), inflammatory bowel disease (IBD) (*4*), and multiple sclerosis (*5*). However, whether or not these human gut bacterial strains play a causal role in disease remains largely unknown. Furthermore, the observation that host diet significantly impacts the structure and function of the gut microbiome (*9*–*11*) raises the question of whether or not diet-mediated shifts in the microbiome impact bacterial immune modulation and downstream disease phenotypes.

The mechanisms whereby the gut microbiome influences autoimmune disease and the role of diet in these interactions are even more mysterious. Perhaps the most well-studied mechanism is the bacterial accumulation of T helper 17 (Th17) cells which are involved in maintaining the intestinal barrier and coordinating immune responses to extracellular pathogens and whose aberrant activation drives autoimmunity (*12*). Seminal work revealed that segmented filamentous bacteria (SFB) increase Th17 cells in mice (*13*, *14*). Likewise, the composition and structure of the human gut microbial community significantly impact Th17 levels and severity of disease models following transfer into germ-free mice (*14*, *15*). The prevalent human gut Actinobacterium *Bifidobacterium adolescentis*, which is enriched in IBD patients, increases Th17 cells and worsens arthritis in a mouse model (*16*). Similar to SFB, the physical adhesion of *B. adolescentis* to the intestinal epithelium has been implicated in the mechanism of Th17

accumulation; however, it remains unclear if other gut bacterial species regulate Th17 cells through adhesion-independent mechanisms.

Eggerthella lenta, another prevalent gut Actinobacterium found in >80% of individuals (*17*), is enriched in patients with autoimmune diseases (*3*, *5*), but the impact of this bacterium on host immunity and disease phenotypes remains unexplored. Here, we show that *E. lenta* is sufficient to increase Th17 cells and exacerbate murine colitis. Through a combination of gnotobiotics, comparative genomics, *in vitro* assays, and bacterial genetics we identify a single strain-variable gene (cardiac glycoside reductase 2, *cgr2*) which is necessary and sufficient to increase Th17 cells. These data provide the first physiological function for Cgr2, which was previously implicated in xenobiotic metabolism, inactivating the cardiac drug digoxin and other toxic plant compounds within the cardenolide family (*11*, *18*). Finally, by leveraging the knowledge that Cgr2 activity is abrogated by high levels of dietary arginine (*11*, *18*) we show that dietary arginine prevents *E. lenta*-dependent Th17 accumulation and associated colitis emphasizing that diet can affect the capacity of specific bacterial strains to modulate immune responses and downstream disease.

We began by investigating the impact of *E. lenta* colonization of germ-free (GF) mice on Th17 levels. GF mice gavaged with *E. lenta* strain DSM 2243 (2243) had increased percentages and numbers of IL-17a+ CD4+ Th17 cells in the small intestine and colon but not spleen (**Figure 1A-C, Sup. Fig 1A and B**). There was no effect on intestinal IFNγ+ CD4+ T cells (**Sup. Fig 1C and D**) and qPCR on ileal tissue confirmed an increase in IL-17F without significant changes in Th1 (IFNγ, Tbet), Treg (FoxP3), or other inflammatory markers, with the exception of a slight increase in BCL6, which could indicate an increase in T follicular helper cells (**Figure 1D**). Interestingly, small intestinal IL17a+ TCRγδ+ T cells trended upwards in *E. lenta*

monocolonized mice but Lineage- Rorγt+ CD90+ IL17+ group 3 innate lymphoid cells (ILC3) were not altered (**Sup. Fig 1E and F**). To test if *E. lenta* could augment IL-17 production in the context of a more complex microbiota, we gavaged SPF mice with *E. lenta* or a media control (BHI) every other day for two weeks and observed a similar accumulation of Th17 cells with *E. lenta* in the colon and trend towards increased levels in the small intestine (**Figure 1E, Sup. Fig 1G and H**).

To investigate whether *E. lenta* colonization impacts chronic inflammatory and autoimmune mouse models, we induced DSS colitis in GF and *E. lenta* monocolonized mice. Mice were colonized 2 weeks prior to disease induction to stimulate Th17 accumulation to assess the impact of this accumulation on disease. *E. lenta* colonized mice developed more severe disease than their GF counterparts in both disease scores and colon shortening (**Figure 2A and 2B**), and colonic Th17 levels were elevated, correlating with colon shortening severity (**Fig. 2C and Sup. Fig 2A-C**). To determine if *E. lenta* mediated Th17 accumulation was the root of this worsened disease phenotype, we compared *Rorc*$^{-/-}$ and WT SPF mice gavaged with *E. lenta* or media control every other day for 2 weeks preceding induction of DSS colitis. *E. lenta* colonized *Rorc*$^{-/-}$ mice had no differences in disease severity compared to the media control while WT mice colonized with *E. lenta* developed worsened disease compared to control WT mice (**Figure 2D and 2E**). Colon shortening severity correlated to elevated Th17 levels (**Figure 2F-H**). These results suggest that Th17 accumulation by *E. lenta* contributes to worsened colitis severity in *E. lenta* colonized mice, although it is important to note that Th17 cells are not the only cell type missing from *Rorc*$^{-/-}$ mice as ILC3 cells are also lacking. However, as previously noted we did not see differences in the ILC3 population in *E. lenta* colonized mice (**Sup. Fig. 1F)**, consistent with the hypothesis that Th17 accumulation is responsible for the observed phenotype.

To test whether *E. lenta* colonization contributes to more severe disease in other models of colitis, we gavaged *E. lenta* strain 2243 or a media control three times a week into *IL-10*$^{-/-}$ SPF mice and weight was tracked. *E. lenta* gavage resulted in lower percentage of initial weight which correlated with intestinal Th17 levels (**Figure 2I and J, Sup. Fig D-F)** and prolapse incidence was increased compared to media control (**Figure 2K**) suggesting that *E. lenta* colonization contributes to more severe disease.

We next wanted to investigate if *E. lenta* mediated Th17 accumulation was a strain-variable phenotype. Therefore, we monocolonized mice with *E. lenta* strains DSM 2243 (2243), FAA 1-3-56 (1356), DSM 15644 (15644), or heat-killed *E. lenta* 2243 (see Table S5 for strain information). Viable *E. lenta* 2243 was the only group which elevated Th17 accumulation compared to GF mice (**Figure 3A and 3B**) in both percentage and total number (**Sup. Fig. 3)** indicating *E. lenta* mediated Th17 accumulation is strain variable and requires live bacteria. Next, we tested whether monocolonization with a strain of *E. lenta* that did not increase Th17 levels would impact DSS colitis. While colonization with *E. lenta* 2243 again induced more severe colitis, colonization with *E. lenta* 1356 did not induce more severe disease (**Figure 3C and 3D**), consistent with the inability of this strain to increase intestinal Th17 cells to the same extent as *E. lenta* 2243 (**Figure 3E and 3F**).

To more systematically assess the variation between *E. lenta* strains and their capacity to increase Th17 cells, we turned to an *in vitro* cell culture assay. Splenic CD4+ T cells were cultured in Th17 skewing conditions with the addition of conditioned media from *E. lenta* strains or media controls. Recapitulating our *in vivo* findings, conditioned media from *E. lenta* 2243 but not 1356 or 15644 significantly increased IL-17a levels compared to media control as assessed by IL-17a ELISA (**Figure 4A**). Interestingly, media itself (BHI) decreased IL-17a production,

suggesting there may be an uncharacterized factor in our bacterial culture media inhibiting its production.

Having validated our *in vitro* assay, we tested a broader panel of 10 *E. lenta* strains (*17*) for their Th17 accumulation capacity. This screen revealed 6 *E. lenta* strains that did not increase IL-17a production and 4 strains that significantly increased IL-17a production (**Figure 4B**). Using the ElenMatchR comparative genomics tool (*17*), we determined that a single genomic locus (orthologous gene cluster 4725-4731), was present in all of the strains that increased IL-17a production and missing from all of the strains that did not induce IL-17a (**Figure 4B**). Interestingly, we had previously implicated this same locus, referred to as the cardiac glycoside reductase (cgr)-associated gene cluster, in the metabolism of the cardiac drug digoxin and other plant toxins from the cardenolide family (*18*). These results indicated that one or more of the genes in this locus plays a role in shaping host immunity.

Based on prior evidence that digoxin inhibits Th17 cell differentiation (*19*, *20*), we hypothesized that the enzyme sufficient for digoxin reduction, Cgr2, may be capable of increasing Th17 cells due to the reduction of an endogenous substrate found in the mouse gut lumen and our cell culture media. A key prediction of this hypothesis is that Cgr2 should be sufficient to promote IL-17a production in our *in vitro* assay. We used constructs in which WT Cgr2 or a partial loss-of-function natural variant of Cgr2 (Y333N) were expressed in *Rhodococcus erythropolis* under control of a thiostrepton-inducible promoter (*18*). Expression of the WT but not variant form of Cgr2 resulted in a significant increase in IL-17a levels compared to uninduced controls (**Figure 4C**). To confirm that Cgr2 expression is also sufficient to increase Th17 cells *in vivo*, we supplied conditioned media from *R. erythropolis* strains with WT or Y333N Cgr2 in the presence or absence of the thiostrepton inducer. Only conditioned media

from *R. erythropolis* with induced WT Cgr2 expression resulted in a significant increase in small intestinal Th17 cells (**Figure 4D and Sup. Fig. 4**).

Prior studies have shown that elevated protein intake, likely due to the increased concentration of arginine in the gut lumen, inhibits the ability of Cgr2 to reduce the cardiac drug digoxin (*11*, *18*). We hypothesized that arginine would have a similar ability to prevent *E. lenta* mediated Th17 accumulation and associated disease phenotypes. GF, *E. lenta* strain 2243 (*cgr+*), or 15644 (*cgr-)* monocolonized mice were fed a 1% or 3% arginine diet. The 1% and 3% arginine diets had no impact on Th17 levels in the GF or 15644 colonized mice. *E. lenta* 2243 colonized mice had elevated Th17 cells on the low arginine diet but those levels were decreased back to baseline levels on the high arginine diet (**Figure 5A and 5B**). To determine if the effect of arginine levels on *E. lenta* mediated Th17 accumulation results in altered colitis outcome, we gavaged *E. lenta* 2243 (*cgr+*), or 15644 (*cgr-)* into SPF mice on a 1% or 3% arginine diet three times a week for two weeks and then induced DSS colitis. Mice gavaged with *E. lenta* 2243 (*cgr+*) on a high arginine diet had decreased Th17 cells and developed less severe disease than their counterparts on a low arginine diet similar to disease severity in mice given *E. lenta* strain 15644 (*cgr-*) on either a high or low arginine diet (**Figure 5C-E, Sup. Fig 5**). This demonstrates that, in the context of colonization with a *cgr+ E. lenta* strain, increased arginine in the diet decreases both Th17 accumulation and colitis severity.

Discussion

The immunomodulatory effects of members of the human gut microbiota has long been appreciated and the field is now making efforts to explore the variety of mechanisms whereby these microbes influence a host's immune response. Previous studies have identified bacteria

capable of increasing Th17 levels and have implicated bacterial adherence to the gut epithelial in this activation (*14*, *16*). While we do not rule out that *E. lenta* activates Th17 cells through an epithelial adhesion mechanism in addition to the effect of Cgr2 it entirely possible that a single microbe could increase Th17 cells through multiple mechanisms as seen with SFB (*14*, *21*). With recent studies profiling the immunomodulatory properties of a range of microbes spanning multiple phyla (*16*, *22*) we have gained insight into how these associations could be leveraged for therapy (*23*). However, it is important to note that we may be missing many microbe-immune interactions due to the strain-level differences in immune alterations. For example, rat-derived SFB does not increase Th17 cells in mice but mouse-derived SFB does (*14*). This concept also applies to *Bifidobacterium* where *B. infantis* monocolonization increases Th17 cells but *B. longum* monocolonization does not (*16*). *B. infantis* is a subspecies of *B. longum;* therefore these two bacteria share a significant overlap in their genomic content (over 97% percent identity in overlapping regions and between 9%-26% genomic content differences (BLAST)) suggesting that something in the unshared genetic material is involved in Th17 accumulation. This could be narrowed further by comparing other members of these subspecies. Thus, strain-level variations in altered immune response could provide insight into the genetic factors underlying that phenotype as seen with our findings where *E. lenta* strains have different capabilities to promote Th17 accumulation leading to the identification of the Cgr2 protein's involvement in this process.

Cgr2 has been previously studied for its role in reducing the heart medication digoxin into a less active by-product, dihydrodigoxin (*11*). Digoxin is also a potent Th17 inhibitor and binds directly to the master transcription factor of Th17 cells, Rorγt (*19*, *20*) while dihydrodigoxin is a less potent inhibitor (*24*). However, digoxin is not normally present in the

gastrointestinal tract and so the question arises whether Cgr2 is acting on an endogenous substrate. The substrate specificity of Cgr2 was recently explored and suggested that Cgr2 acts exclusively on the cardenolide class of molecules (*18*). Therefore, our current hypothesis is that Cgr2 acts on an endogenous Th17 inhibitory compound that resembles digoxin such as an endogenous cardenolide (*25*).

One of the major environmental factors impacting the microbiota and microbial-mediated immune modulation is the host diet. Alterations in diet significantly impact gut microbiota composition and function (*9*, *10*) and the idea that the diet could be leveraged to manipulate the microbial immune modulation has been proposed by many researchers (*26*). However, it is often difficult to assess the direct impact of dietary alterations on immune diseases from microbiota-mediated effects. This is the case with dietary arginine where previous studies have shown that arginine supplementation improves disease severity in a DSS model in an iNOS dependent manner, suggesting a host mechanism of improved disease under arginine supplementation (*27*). Additionally, arginine has been well characterized to have broad impacts on immune response where the route of arginine metabolism by macrophages determines the regulatory versus inflammatory skewing of macrophage response and in T cells arginine promotes proliferation and cytokine production in response to T cell receptor (TCR) stimulation (*28*, *29*). However, arginine can also be metabolized by the microbiota into polyamides (*30*), which have immunomodulatory functions, and can alter microbial metabolism (*11*). By comparing the impact of differential arginine levels on GF and mice monocolonized with *E. lenta* strains we can begin to tease apart the microbial mediated versus direct impact of arginine on Th17 accumulation. Our finding that increased dietary arginine decreases *E. lenta*-mediated Th17 accumulation and corresponding colitis severity suggests that dietary alterations to microbial

metabolism could be used to tune microbial immune modulation. However, as arginine has well established roles in inflammation and can play a pro- or anti-inflammatory role depending on its metabolism caution and careful consideration of the additional direct effects of dietary interventions must be considered.

Materials and Methods

Experimental Design. The objectives of this study were to investigate whether and how a prevalent member of the human gut microbiota, which is associated with autoimmune diseases, *Eggerthella lenta,* modulates T helper 17 (Th17) responses and mouse models of chronic inflammation and autoimmunity in a diet dependent manner. To investigate these questions we utilized a combination of gnotobiotics, comparative genomics, *in vitro* assays, and bacterial genetics, which are outlined below.

Mice. *Gnotobiotic mouse studies*. C57BL/6J mice (females and males, ages 6-16 weeks) were obtained from the UCSF Gnotobiotics core facility (gnotobiotics.ucsf.edu) and housed in gnotobiotic isolators for the duration of each experiment (Class Biologically Clean). Mice were colonized via oral gavage with turbid mono-cultures of *E. lenta* (10^9 CFU/ml, 200ul gavage) and colonization was confirmed via anaerobic culturing. Mice were colonized for 2 weeks. For the heat killed *E. lenta* preparation turbid *E. lenta* 2243 culture was incubated at 65°C for 15 min to kill bacteria. All mouse experiments were approved by the University of California San Francisco Institutional Animal Care and Use Committee.

SPF mouse studies. C57BL/6J mice (females and males, ages 6-10 weeks) were ordered from Jackson Labs. Mice were orally gavaged with turbid mono-cultures of *E. lenta* strains (10^9

CFU) every other day for 2 weeks and colonization was confirmed with qRT-PCR for *E. lenta* 16s specific marker (*17, 18*) (F- GTACAACATGCTCCTTGCGG, R- CGAACAGAGGATCGGGATGG). *Rorc-/-* (Rorctm2Litt) were generously given by the Baron lab. *IL-10-/-* mice were ordered from Jackson Labs and bred in house. *Il-10-/-* mice (females and males, ages 6-8 weeks at gavage start) were orally gavaged with turbid mono-cultures of *E. lenta* strains (10^9 CFU) 3 times a week for 6 or 10 weeks.

DSS disease model. For dextran sodium sulfate treatment (DSS) (Alfa Aesar, Cat no. 9011-18-1), mice were given 2% DSS (w/v) *ad libitum* in their drinking water for 7 days after mice were colonized with *E. lenta* for 2 weeks prior to disease (either once for monocolonized mice or every other day gavage for SPF animals). Mice were monitored for disease progression and weighed daily. Gross signs of toxicity, including hematochezia and weight loss greater than 15% were monitored in this study and mice showing these signed were immediately euthanized. Stools were scored as follows: 0 = normal stool consistency, 1 = soft stool, 2 = blood in stool, 3 = bloody rectum, 4 = prolapsed rectum, 5 = moribund/death (scoring based on (*31*)).

Diets. Custom diets with 1% (TD.170862) or 3% (TD.170863) Arg were purchased from Envigo. Otherwise, standard chow diet (Lab Diet 5058) is used for SPF mice and standard autoclaved chow diet (Lab Diet 5021) was used for the gnotobiotic mice. Diets used for gnotobiotic experiments were either autoclaved or irradiated and vacuum sealed to ensure sterility.

Bacterial culturing. *E. lenta* strains were cultured at 37°C in an anaerobic chamber (Coy Laboratory Products) (2-3% H_2, 20% CO_2, and the balance N_2). Culture media was composed of brain heart infusion (BHI) media supplemented with *L*-cysteine-HCl (0.05%, w/v), resazurin (0.0001%, w/v), hemin (5 µg/mL), Vitamin K (1 µg/mL), and arginine (1%) (BHI CHAVR). *E.*

lenta strains were previously isolated and sequenced (*17*). *R. erythropolis* strain L88 was cultured in aerobic conditions at 30°C with 200 rpm in media specified below.

Heterologous expression. Cgr2 was heterologously expressed in *R. erythropolis* strain L88 as previously described (*18*). In short, competent *R. erythropolis* were electroporated (2.5 kV pulse (time constant ~4.8)) with pTipQC plasmid carrying the WT (Cgr2 WT) or Y333N mutated *cgr2* gene (Y333N Cgr2) and transformed cells were selected on LB chloramphenicol (17ug/ml) plates. Liquid cultures were grown in BHI CHAVR with 34 µg/mL chloramphenicol to .6OD and then treated with or without Thiostrepton (.1ug/ml) to induce expression. Conditioned media was harvested 48 hours later, centrifuged at 2500rpm for 10 min to pellet the cells and debris then passed through a .2um syringe filter and used for cell culture assays. Expression induction was verified to be similar levels in the WT and Y333N plasmids via qRT-PCR for cgr2 (F-TGCGCTGGTCGCAAGGTCTG, R- CGGCGCGCTTTTTCAGCGTT) relative to dinB control for total *R. erythropolis* normalization (*32*) (F - GACTCCGGCCAACTCCAC, R-GGATGTCGTTGTATTCGGTTC). For mouse experiments 200ul *R. erythropolis* conditioned media or media control (BHI CHAVR +Thiostrepton (.1ug/ml)) was gavaged every other day for 2 weeks from the *R. erythropolis* strains described above (WT Cgr2 or Y333N Cgr2). Liquid cultures were prepared as above.

Cell culture. *Th17 skewing assay.* RBC lysed mouse splenic T cells were isolated via Dynabeads untouched mouse CD4 isolation kit according to kit specifications. In a 96 well plate pre-coated with anti-CD3 (5mg/ml, overnight 37°C), equal cell numbers were plated and were treated with bacterial conditioned media or controls with pH adjusted to 7 at a concentration of 5% vol/vol. At the same time Th17 skewing conditions were supplied (anti-CD28 (10µg/ml), TGFβ

(.3ng/ml), IL-6 (20ng/ml), anti-IFNγ (2mg/ml), anti-IL-4 (2mg/ml) (*19*). Bacterial conditioned medias were harvested from 48 hour stationary cultures, bacterial cells were pelleted (2500rpm 10 min) and filtered through a .2µm filter to exclude cells from the conditioned media preparation. Isolated CD4+ T cells were developed in Th17 skewing conditions with bacterial conditioned media present for 4 days at 37°C and then re-stimulated with PMA (50 ng/ml) and ionomycin (1000 ng/ml) overnight, then supernatants were harvested for IL-17 quantification via ELISA.

ELISA. To measure the levels of secreted IL-17a from the Th17 cell culture assay we utilized the mouse IL-17a (homodimer) ELISA (ThermoFisher) according to the kit instructions.

Lamina Propria Lymphocyte Isolation. Lamina propria lymphocytes (LPLs) were isolated with modifications of previously described methods (*33–35*). Briefly, small intestinal (SI) Peyer's patches were excised and colons and SI tissue were splayed longitudinally with mucus removed and stored in complete RPMI (10% fetal bovine serum, 100 units per ml penicillin and streptomycin, β-mercaptoethanol, glutamax, sodium pyruvate, HEPES and non-essential amino acids). Media was removed by filtering through a 100µM filter, and remaining tissue incubated in 1X HBSS (without Ca^{2+} and Mg^{2+}) containing 5 mM EDTA and 1 mM DL-Dithiothreitol (DTT) for 45 min at 37°C on a shaker (200RPM). Supernatant were filtered through a 100 µM filter, and remaining tissue was incubated for 45 min (colon) or 35 min (SI) at 37°C on a shaker in a solution containing 1X HBSS containing 5% (v/v) fetal bovine serum (GIBCO heat inactivated), 1 U/ml Dispase (Sigma), 0.5 mg/ml Collagenase VIII (Sigma), and 20 µg/ml DNaseI (Sigma). The vortexed supernatant was filtered over a 40 µm cell strainer into 1X PBS. Cells were subjected to a Percoll (VWR) gradient (40%/80% [v/v] gradient) and spun at

2000RPM for 20 min with no brake and no acceleration. Cells at the interface were collected, washed 1X PBS and prepared for flow cytometry analysis.

Flow Cytometry. Lymphocytes were isolated from the colonic and small intestinal lamina propria as described above. Spleen cells were prepped through gentle mashing with a syringe plunger. Spleen cells were treated with 1X RBC Lysis Buffer (Biolegend) to lyse and remove red blood cells. Surface staining for lymphocytes was done in staining buffer (1X HBSS (Corning) supplemented with 10 mM HEPES (Fisher Scientific), 2 mM EDTA (Invitrogen), and 0.5% (v/v) fetal bovine serum (heat inactivated) for 20 min at 4°C. Cells were then washed twice in supplemented 1X HBSS and enumerated via flow cytometry. The following antibodies were used: anti-CD3 (17A2, Fisher Scientific), anti-CD4 (GK1.5, Biolegend). For intracellular staining, cells were first stimulated with ionomycin (1000 ng/ml), PMA (50 ng/ml), and Golgi Plug (1 μl/sample) (BD Bioscience) 4-6 hours or overnight at 37°C. Cells were surface stained, washed, and then fixed/permeabilized in 100μl Perm/Fix buffer (BD Bioscience). Cells were washed twice in Perm/Wash buffer (BD Bioscience) and then stained for intracellular cytokines with the following antibodies: anti-IFNγ (XMG1.2, Fisher Scientific), anti-IL17a (ebio17B7, Invitrogen), Roryt (B2D, ebioscience). Cells were washed twice in Perm/Wash buffer and then placed in staining buffer for flow cytometry analysis. Gating cell populations was done using isotype and single stain controls. These data were collected with a BD LSR Fortessa and analyzed with FlowJo software and a list of all antibodies are included in table S8.

Comparative genomics. To identify shared genetic regions of IL-17a inducing *E. lenta* strains which were also excluded from non-IL-17 inducing *E. lenta* strains we utilized ElenMatchR, a comparative genomic tool developed by Jordan Bisanz (jbisanz.shinyapps.io/elenmatchr/) (*17*)

for hut gut Actinobacteria. Briefly, gene presence/absence is used as the input variable for a random forest classifier against user-provided phenotypes or traits, in this case induction or non-induction of IL-17a by *E. lenta* strains. With these classifications we performed comparative genomics using ElenMatchR with 80% coverage and 80% minimum identity to determine genomic regions shared between the 4 inducing strains and absent from the 6 non-inducing strains.

RNA isolation and qRT-PCR. Ileal segments from GF or *E. lenta* monocolonized mice were harvested and RNA was extracted with Direct-zol RNA MiniPrep kit (Zymo) according to manufacturer instructions for tissue RNA isolation. Tissue was homogenized with a mortar and pestle and DNase treatment was performed on columns. RT-PCR was performed with iScript (BioRad) using 300ng RNA and qPCR using SYBR select. Primers are listed in table S8.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism software (Version 7). ANOVA with Tukey's multiple comparison test was used for the parametric analysis of variance between groups, and unpaired Welch's *t* test were used for pairwise comparisons. Numbers (*N*) are either stated are displayed as individual points on plots.

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Supplementary Materials:

Materials and Methods

Figures S1-S5

Tables S1-S8

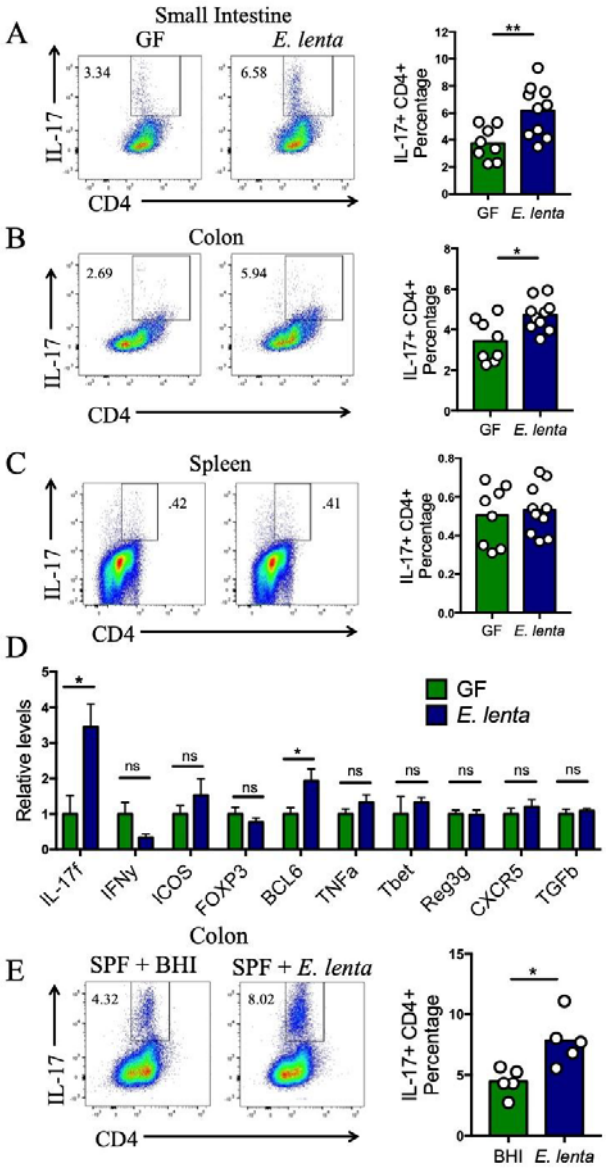

Figure 1. *E. lenta* colonization increases intestinal Th17 cells in the presence and absence of an intact gut microbiota. (A) IL-17a+ CD4+ Th17 levels were measured via flow cytometry in the small intestinal and (B) colonic lamina propria and (C) the spleen of germ-free (GF) mice or mice monocolonized with *E. lenta* strain 2243 (GF n = 8, *E. lenta* n = 10). Representative flow plots of the IL-17a+ CD4+ population are displayed and frequencies are quantified to the right. Data are pooled from two independent experiments with each point representing an individual mouse. (D) RT-qPCR panel of immune genes from GF or *E. lenta* monocolonized ileal samples (n = 4). Data are relative to GF which is set to 1 and represented as mean±SEM. (E) C57BL/6J SPF mice were gavaged every other day for 2 weeks with BHI media control or *E. lenta* strain 2243. Representative flow plots of colonic Th17 levels as measured by IL-17a+ CD4+ markers with frequencies plotted to the right. Each point represents an individual mouse. **P* <0.05; ***P* <0.01 (Welch's t-test).

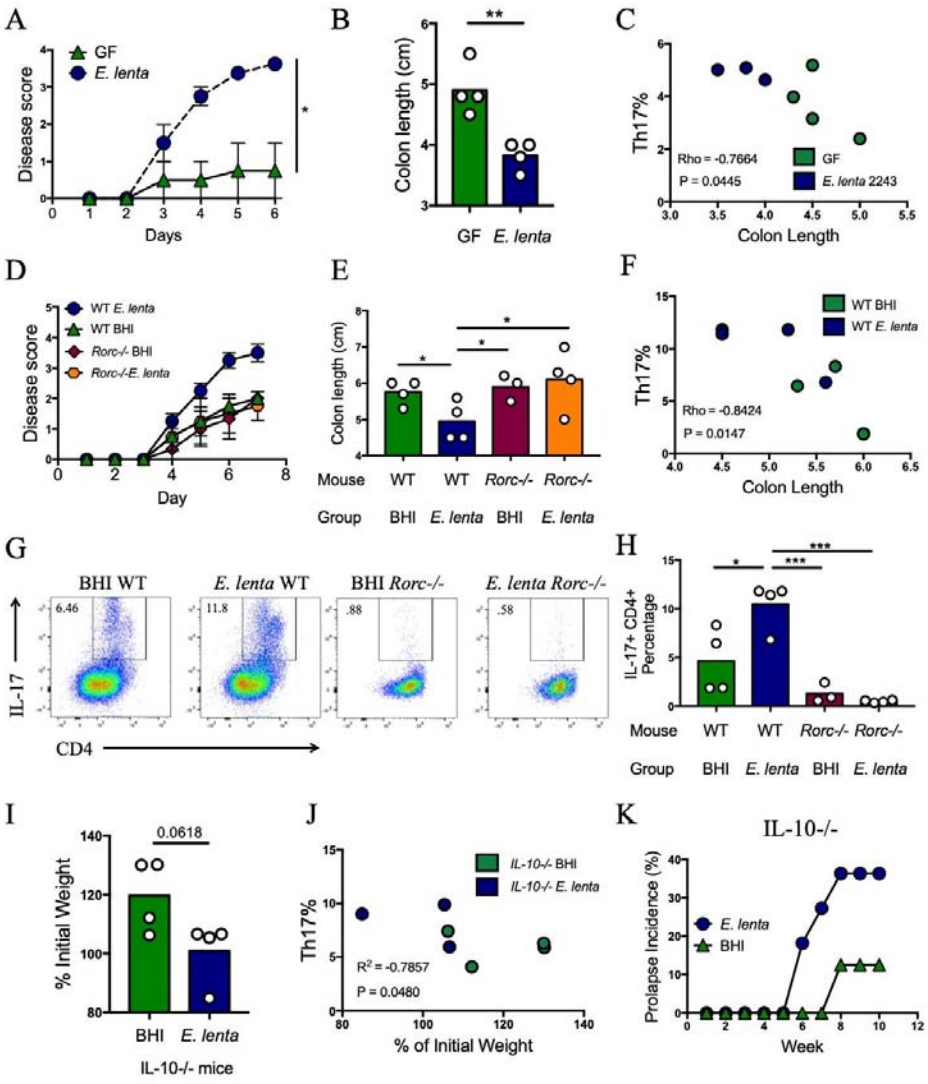

Figure 2. *E. lenta* colonization contributes to worsened colitis in an Rorc-dependent manner. (A) Germ-free (GF) or *E. lenta* strain 2243 monocolonized mice were treated with 2% DSS for 6 days and disease was scored based on published scoring metrics (*31*). **P* <.05 Welsh's t-test of final day score differences. Mean±SEM is displayed. (B) Colon shortening was assayed on day 6. ***P*<0.01 (Welch's t-test). (C) Correlation between colonic Th17 percentage and colon length (cm). Spearman Rho and *P* value are listed. (D) SPF WT or *Rorc*$^{-/-}$ mice were gavaged with a BHI media control or *E. lenta* 2243 every other day for 2 weeks then treated with 2% DSS for 7 days and disease was tracked. **P*<0.05 one-way ANOVA with Tukey multiple comparison test (WT *E. lenta* vs. WT BHI and WT *E. lenta* vs. *Rorc*-/- BHI) ***P*<0.01 (WT *E. lenta* vs. *Rorc*-/- *E. lenta*) of day 7 disease scores. Mean±SEM is displayed. (E) Day 7 colon lengths. (F) Correlation between colonic Th17 percentage and colon length (cm). Spearman Rho and *P* value are listed. (G) Th17 levels in the small intestinal lamina propria were assessed via flow cytometry and representative flow of the IL-17a+ CD4+ live cell population is shown and (H) quantified to the right. (I) *IL-10*$^{-/-}$ mice were gavaged with a BHI media control or *E. lenta* 2243 three times a week for 6 weeks and disease was tracked by percentage of initial weight. *P*-value listed is Welch's t-test of final week (week 6). (J) Correlation between intestinal Th17 percentage and percentage of initial weight (week 6). Spearman Rho and *P* value are listed. (K) *IL-10*$^{-/-}$ mice were again gavaged with BHI or *E. lenta* 2243 three times a week for 10 weeks and rectal prolapse was tracked (n=9 (BHI), n=11 (*E. lenta*)). **P*<0.05; ***P*<0.01, ****P*<0.001, or stated (one-way ANOVA with Tukey multiple comparison test unless otherwise noted).

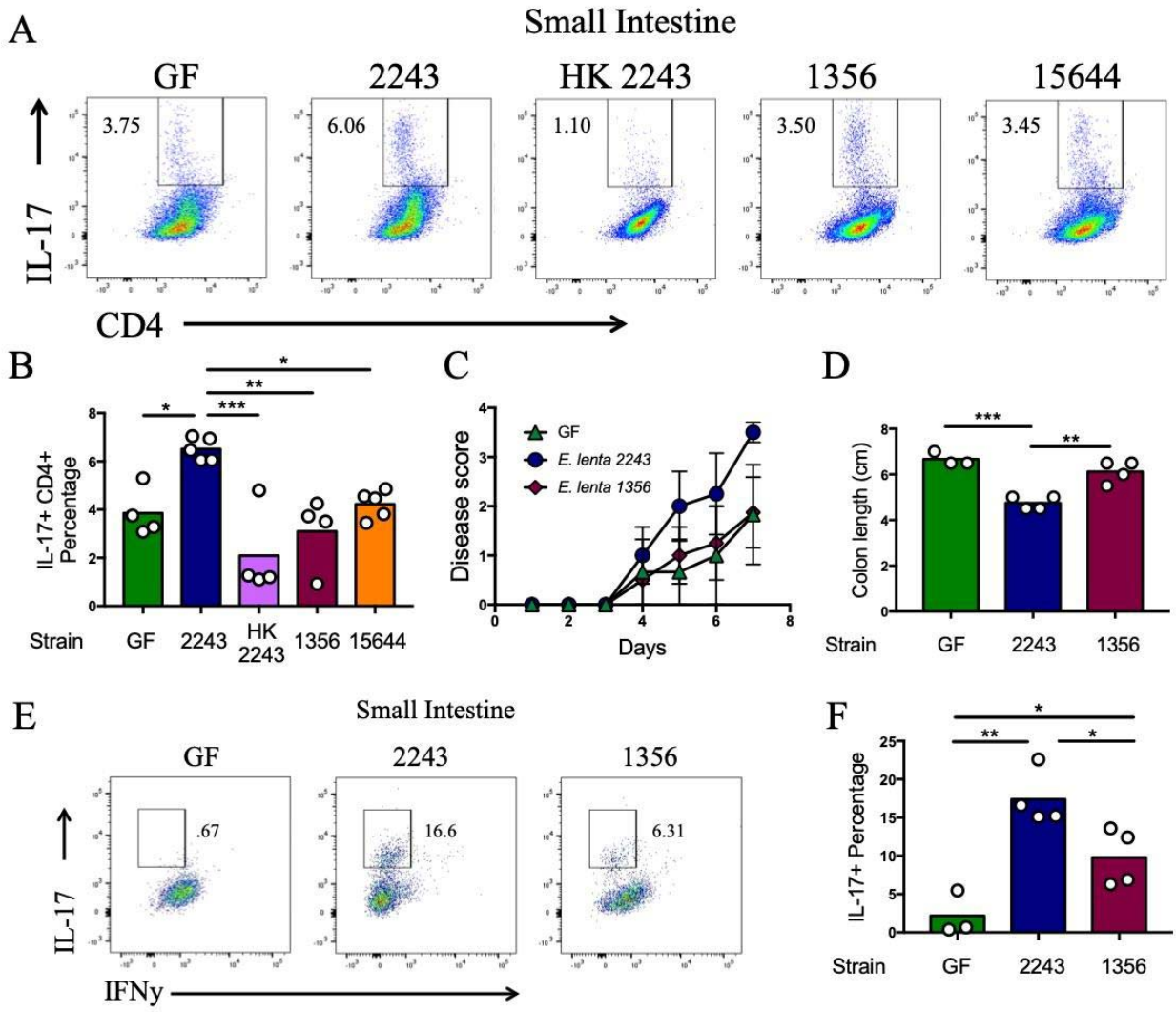

Figure 3. Th17 induction and colitis severity varies between *E. lenta* strains. (A) Th17 levels were analyzed in germ-free (GF) mice or mice monocolonized with live *E. lenta* 2243 (2243), heat-killed *E. lenta* 2243 (HK 2243), *E. lenta* 1356 (1356), or *E. lenta* 15644 (15644) as measured by IL-17a+ CD4+ live cell population in the small intestinal lamina propria. Representative flow plots are shown and (B) quantified. (C) GF, mice or monocolonized with *E. lenta* 2243, or *E. lenta* 1356 for 2 weeks were treated with 2% DSS and disease was tracked for 7 days. (D) Day 7 colon lengths. Mean±SEM is displayed. (E) Th17 levels were measured in the small intestinal lamina propria and representative flow plots of IL-17a+ cells within the live CD3+ CD4+ population and (F) these frequencies were quantified. Each point represents an individual mouse. **P*<0.05; ***P*<0.01, ****P*<0.001 (one-way ANOVA with Tukey multiple comparison test).

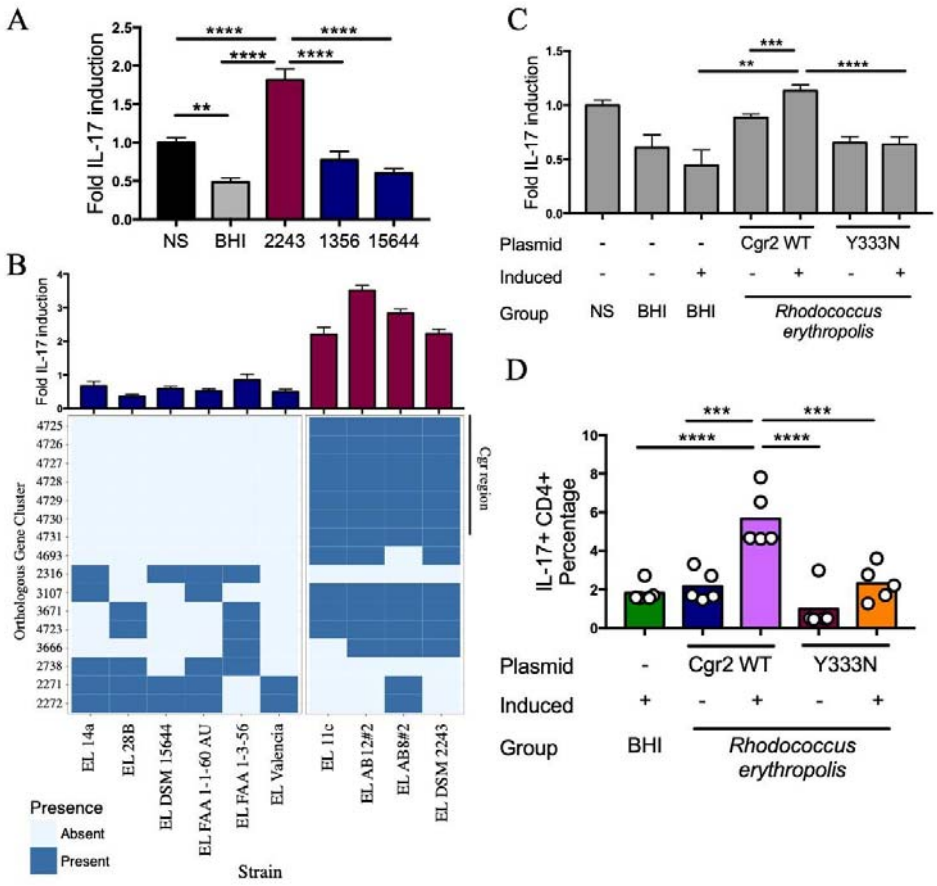

Figure 4. A single strain-variable genomic loci is predictive and sufficient for Th17 induction. (A) Splenic T cells were treated with Th17 skewing conditions and conditioned media from *E. lenta* strains (2243, 1356, 15644), a BHI media control, or no treatment (NS). IL-17a levels in the supernatant were measured via ELISA after 4 days of skewing followed by overnight PMA ionomycin restimulation (n=18-20 biological replicates (wells)/group). Levels of IL-17a are relative to the no treatment control which is set to 1. (B) In the same experimental design as A, 10 strains of *E. lenta* were screened and levels of IL-17a are relative to the no treatment control which is set to 1. Strains were classified into non-IL-17a inducers (fold IL-17a levels below 1) or high (fold IL-17a levels above 1). With these classifications we performed comparative genomics using ElenMatchR with 80% coverage and 80% minimum identity (*17*) to determine genomic regions shared between the 4 inducing strains and absent from the 6 non-inducing strains (n=8-12 biological replicates (wells)/group). Orthologous gene cluster numbers 4725-31 correspond to the cardiac glycoside reductase (*cgr*)-associated gene cluster (*18*). (C) With our *in vitro* Th17 system we tested *Rhodococcus erythropolis* stains with induced (+Thiostrepton) or uninduced (-Thiostrepton) expression of WT Cgr2 (n=14), a natural partial loss-of-function variant Y333N Cgr2 (n=12) with no treatment (NS) (n = 14), BHI (+/-Thiostrepton) (n=12 and n=8) as controls. Levels of IL-17a are relative to the no treatment control which is set to 1. ***P*<0.01, ****P*<0.001; *****P*<0.0001 Welch's t-test. (D) C57BL/6J SPF mice were gavaged with conditioned media from *R. erythropolis* strains with induced (+Thiostrepton) or uninduced (-Thiostrepton) expression of WT Cgr2, Y333N Cgr2, or a BHI media control (with Thiostrepton). Percentage of CD4+ IL-17a+ live small intestinal lamina propria lymphocytes are displayed. Each point represents an individual mouse. Data represents a combination of at least two independent experiments for A-C and one for D and mean±SEM is displayed. **P*<0.05; ***P*<0.01, ****P*<0.001; *****P*<0.0001 (one-way ANOVA with Tukey multiple comparison test unless otherwise noted).

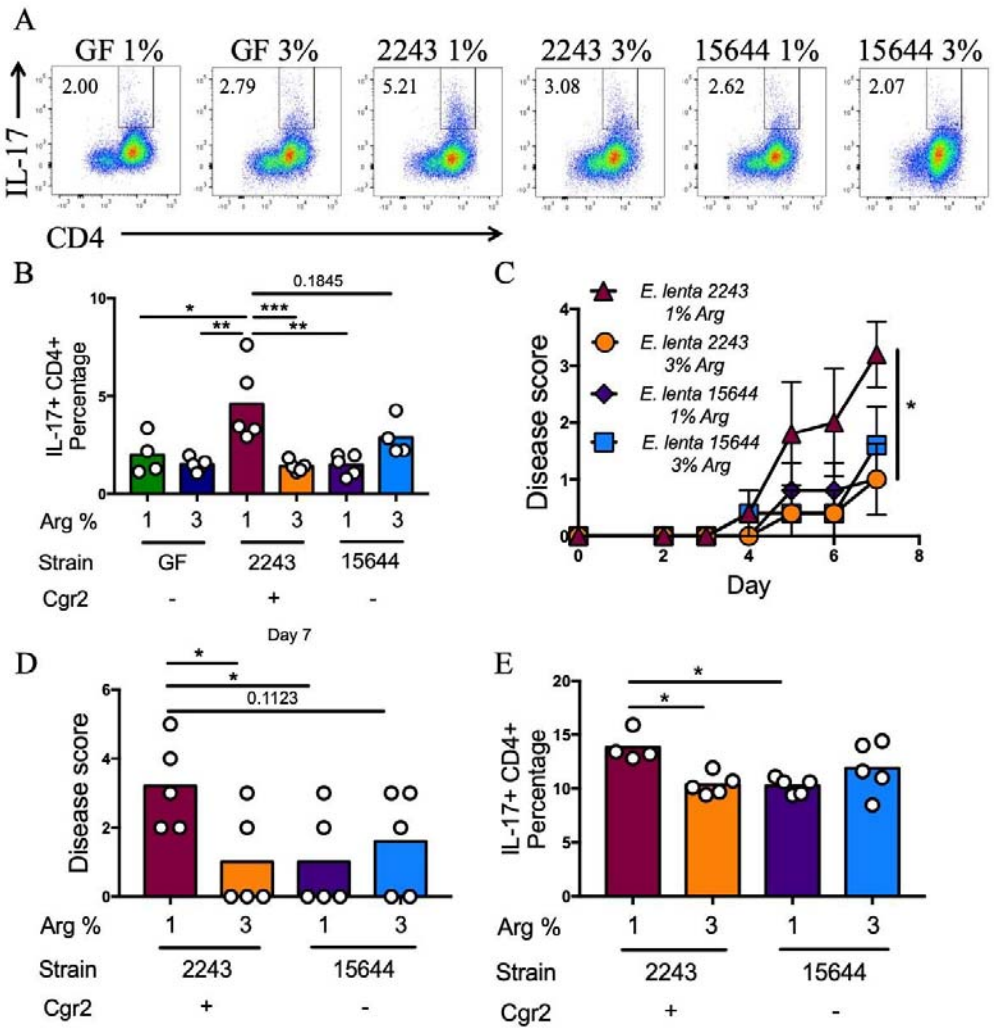

Figure 5. A high arginine diet specifically inhibits Th17 accumulation and colitis induction by a *cgr*+ *E. lenta* strain. (A) Representative flow of Th17 (CD4+ IL-17a+ cells within the live lymphocyte population) from the small intestinal lamina propria of germ-free (GF), *E. lenta* 2243 (2243), or *E. lenta* 15644 (15644) monocolonized mice and (B) frequencies of this population are quantified. (C) SPF mice on a 1% or 3% arginine (Arg) diet were gavaged three times a week with either *E. lenta* 2243 or *E. lenta* 15644 for two weeks and then 2% DSS was administered and disease was track via scoring over 7 days. **P*<0.05 Welch's t-test day 7 disease score (1% vs. 3% 2243). Mean±SEM is displayed. (D) Day 7 disease scores from experiment in C. (E) Levels of CD4+ IL-17a+ cell percentages from experiment C . Each point represents an individual mouse. **P*<0.05; ***P*<0.01, ****P*<0.001 or stated (one-way ANOVA with Tukey multiple comparison test).