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A weaning reaction to microbiota is required for resistance to immunopathologies in the adult

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SUMMARY

Microbes colonize all body surfaces at birth and participate in the development of the immune system. In newborn mammals, the intestinal microbiota is first shaped by the dietary and immunological components of milk, and then changes upon the introduction of solid food during weaning. Here we explored the reactivity of the mouse intestinal immune system during the first weeks after birth and into adulthood. The intestinal microbiota at weaning induced a vigorous immune response - a “weaning reaction” - that was programmed in time. Inhibition of the weaning reaction led to pathological imprinting and increased susceptibility to colitis, allergic inflammation and cancer later in life. Prevention of this pathological imprinting was associated with the generation of ROR γ ⁺ regulatory T cells, which required bacterial and dietary metabolites - short chain fatty acids and retinoic acid. Thus, the weaning reaction to microbiota is required for immune ontogeny, the perturbation of which leads to increased susceptibility to immunopathologies later in life.

KEYWORDS

Microbiota; short chain fatty acids; neonatal period; weaning; mucosal immunity; regulatory T cells; colitis; inflammatory pathology.

INTRODUCTION

The mammalian intestine is home to one of the densest community of bacteria on earth, the number of which exceeds the number of eukaryotic cells composing its host. This bacterial community is involved in the metabolism of food, the production of essential compounds such as vitamins, short chain fatty acids (SCFAs) and secondary bile acids, and plays a role in direct and host-mediated defence against pathogens (Round and Mazmanian, 2009; Sonnenburg and Backhed, 2016). It is proposed that the intestinal microbiota behaves like an organ, producing microbial components and metabolites that are involved in many processes of the animal host.

Shortly after birth, the colonizing microbiota is shaped by the dietary and immunological components of the milk (Gomez de Agüero et al., 2016; Le Doare et al., 2018), which includes mother-derived IgA, and follows a developmental process that is characterized by the presence of age-specific bacterial species (Subramanian et al., 2014). During weaning, the introduction of solid food leads to a new phase in the development of the microbiota, characterized by a large increase in bacterial numbers, and evolution towards a composition that is associated with adult individuals (Subramanian et al., 2014). The period between birth and weaning also witnesses important changes in the intestinal immune system. Population of fetus-derived T cells, innate lymphoid cells (ILCs) and myeloid cells are progressively replaced by cells generated in the bone marrow and thymus after birth (Bain et al., 2014; Sawa et al., 2010; Vantourout and Hayday, 2013). Furthermore, the colonizing microbiota induces the generation and expansion of effector and regulatory T cells (Tregs), ILCs, as well as the formation of isolated lymphoid follicles (ILFs) that produce anti-microbial IgA (Bouskra et al., 2008; Ohnmacht et al., 2015; Sanos et al., 2009; Sefik et al., 2015). Thus, important changes occur after birth and during weaning in the intestine, changes that link the development of the microbiota with the development of the immune system.

Perturbation of the microbiota before weaning leads to perturbation in the development of the immune system, and as a consequence, deregulation of immune responses later in life (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Prioult and Nagler-Anderson, 2005). In both mouse and man, the treatment of neonates with antibiotics is associated with increased susceptibility to allergy months or years later. The hygiene hypothesis proposes that decreased exposure to microbes is a cause for increased susceptibility to allergic inflammation (Bach, 2002), a hypothesis supported by observations that children raised close to animal farms, or given non-sterilized pacifiers, develop less allergies (Hesselmar et al., 2013; Riedler et al., 2001; von Mutius and Vercelli, 2010). In

experimental settings, germfree (GF) mice develop high levels of IgE (Cahenzli et al., 2013), whereas GF mice exposed to microbiota before weaning, but not after weaning, are protected from increased susceptibility to allergic inflammation (Olszak et al., 2012; Russell et al., 2012). In an allergy model involving IL-13-producing NKT cells, the microbiota induces the epigenetic modification of *Cxcl16*, a gene expressed by dendritic cells (DCs) that recruits NKT cells to the intestine. In the absence of microbiota before weaning, higher levels of *Cxcl16* are produced in the intestine, weeks after weaning (Olszak et al., 2012). Furthermore, regulatory T cells (Tregs) are generated before weaning that mediate tolerance to specific bacteria (Knoop et al., 2017; Scharschmidt et al., 2015). A similar phenomenon has been characterized in the lungs, where the exposure of DCs to microbiota before weaning induces a transient increase in PD-L1 required for the generation of Tregs (Gollwitzer et al., 2014).

In this work, we explored the reactivity of the mouse intestinal immune system during the first weeks after birth in reaction to microbial colonization. We observed a strong and broad immune reactivity during weaning, which did not occur after weaning, even when adult GF mice were exposed to microbiota. This “weaning reaction” protected mice from increased susceptibility to multiple types of inflammatory pathologies later in life. Such protective effect was achieved by the assembly of all necessary components to induce Tregs by microbiota, but could not be induced after weaning. We propose that the first vigorous reaction of the host to microbiota is programmed to occur during a pre-defined time window during weaning, a reaction that is necessary for the normal development of the immune system. If the weaning reaction does not occur, the immune system is imprinted with excessive reactivity, and as a consequence, develops increased susceptibility to multiple types of inflammatory pathology later in life.

RESULTS

The weaning reaction to microbiota

We observed that, after birth, an early bloom in intestinal γ -Proteobacteria was followed by the expansion of Lactobacillales that are best adapted to the milk environment before weaning (Figures 1A, S1A-B) (Subramanian et al., 2014). The weaning period was associated with a 10 to 100-fold relative expansion of Clostridia and Bacteroidia, which progressively lead to the bacterial composition of the adult microbiota. We performed a transcriptome analysis on the terminal ileum of GF or specific pathogen-free (SPF) mice that harboured a “normal” microbiota, at regular intervals from birth to adulthood, and observed a pronounced shift in expression pattern during weaning (Figures 1B, S1C-D). Genes induced at weaning by microbiota contributed to anti-microbial immunity, such as Reg3 defensins, CC and CXC chemokines and cytokines receptors, as well as for structural components of the epithelial barrier, such as mucins (Figure 1C), as previously reported (Mao et al., 2018; Rakoff-Nahoum et al., 2015). In particular, the expression of the pro-inflammatory cytokines TNF α and IFN γ , found mostly in T cells, was sharply induced by microbiota during weaning at 3 weeks of age, and receded to levels found in GF mice after weaning at 4 weeks of age (Figures 1D and 1E, S1E-H).

As expected, the expression of genes, such as *Tnfa* and *Ifng*, in response to microbiota at weaning was dependent on signalling induced by innate immune receptors or the inflammasome, as no expression was induced in mice deficient in Myd88 and Trif (Figures 1F, S1I). However, not all types of bacteria contributed equally to this “weaning reaction”. Mice treated with vancomycin or metronidazole, but not ampicillin or streptomycin, failed to develop a weaning reaction, indicating that Gram⁺ anaerobic bacteria were necessary (Figures 1G, S1J). Clostridia are the most prominent type of Gram⁺ anaerobic bacteria in the intestine. Among Clostridia, Segmented Filamentous Bacteria (SFB) are potent inducers of IgA and type 3 immunity (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Snel et al., 1997), and were sufficient to induce a weaning reaction in mono-colonized mice (Figures 1H). Members of Clostridia are also efficient fermenters of dietary fibers and producers of short chain fatty acids (SCFAs) (Narushima et al., 2014). The treatment of GF mice or antibiotic-treated mice with SCFA partially restored the weaning reaction to levels found in SPF mice (Figures 1I, S1K), independently of Myd88 and Trif (Figures S1L-M).

A critical time window for the weaning reaction

As the weaning reaction occurred in response to extensive reshaping and expansion of the intestinal microbiota, we reasoned that GF mice may react similarly to exposure to microbiota at any age. To test this hypothesis, GF mice were co-housed with SPF mice before weaning at 14 days of age, or after weaning at 28 days of age (Figures 2A, S2A). Strikingly, the induction of *Tnfa* and *Ifng* was observed when GF mice were exposed to SPF microbiota before weaning, but not after weaning, demonstrating the existence of a defined time window for the weaning reaction to occur. Accordingly, *Tnfa* and *Ifng* were not induced when GF mice were exposed to SPF microbiota in the adult (data not shown).

Weaning is the gradual switch from a diet composed exclusively of milk to a diet that includes other foods. We therefore assessed whether milk was a determinant of the time window for the weaning reaction. In accordance with this hypothesis, a delay in weaning, forced by a prolonged diet composed exclusively of milk, delayed the loss of Lactobacilaceae and the expansion of Clostridiaceae, as well as the weaning reaction (Figures 2B, S2B-C). However, no weaning reaction could be elicited if the milk-only diet was imposed until 4 weeks of age, after normal weaning, indicating that milk blocked the weaning reaction, but did not define the end of the time window. Furthermore, not all milks were equal. Pups given milk produced by mothers 10 days after delivery blocked the weaning reaction, whereas milk produced by mothers 23 days after delivery, i.e. during weaning, did not, irrespective of the age of the pups (Figures 2C, S2D). Thus, a component of the early milk inhibited the weaning reaction. Milk contains several anti-inflammatory components, such as epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor β 2 (TGF β 2) (Chatterton et al., 2013), and the levels of EGF are highest at birth (Moran et al., 1983). We find that the high level of EGF present in milk at birth decreased 10-fold until weaning (Figure S2E). Exposure of mice to EGF during weaning effectively inhibited the weaning reaction (Figure 2D), whereas blocking the receptor for EGF prevented the inhibitory effect of milk before weaning (Figures 2E, S2F), revealing early reactivity to microbiota (Figure S2G). In summary, milk determines the opening of the time window for the weaning reaction, whereas closing of this time window is independent of microbiota or milk, and is thus intrinsic to the weaning mouse.

The weaning reaction prevents pathological imprinting

The existence of a time window during which the colonizing microbiota induces a weaning reaction indicates that this reaction is programmed in the life of the growing mammal and has a unique function in the development of the immune system. We therefore assessed whether the weaning reaction has a long-term impact on the immune system, and measured the susceptibility of adult mice, having developed a weaning reaction or not, to intestinal inflammation. In a first series of experiments, the weaning reaction was induced by the exposure of GF mice to SPF microbiota before or after weaning (Figures 3A-C, S3A-E). All groups of mice were co-housed as soon as they were exposed to microbiota in order to avoid divergence in individual microbiotas (Ubeda et al., 2012). At 9 weeks of age, mice were treated with two rounds of dextran sodium sulfate (DSS) in their drinking water to induce colitis. Mice exposed to microbiota before weaning (at 2 weeks of age), similar to SPF mice, developed a mild form of colitis. In contrast, mice exposed to microbiota after weaning, similar to GF mice, developed severe colitis (Figures 3A, S3A-D), characterized by increased intestinal permeability, myeloperoxidase activity and shortened colonic length, which translated into higher disease indexes and increased mortality. In agreement with increased susceptibility to colitis, mice exposed to microbiota after weaning expressed increased levels of TNF α and IFN γ protein and transcripts during DSS challenge (Figures 3B, S3E). Unexpectedly, these mice also expressed higher levels of the type 2 effector cytokines IL-4, IL-5 and IL-13 (Figures 3C, S3E), indicating that the absence of a weaning reaction lead to a broad deregulation of different types of immunity.

In a second series of experiments, the weaning reaction was prevented by the treatment of mice with a cocktail of antibiotics. Mice were co-housed after antibiotics treatment and challenged with DSS at 9 weeks of age (Figures 3D, S3F-J). Mice that were treated with antibiotics until 2 weeks of age, or from 4 weeks of age until challenge, developed a mild form of colitis similar to SPF mice. In contrast, mice exposed to antibiotics during weaning developed severe colitis, similar to GF mice (Figures 3D, S3F-J). As observed in GF mice recolonized with microbiota after weaning, mice treated with antibiotics during weaning expressed higher levels of type 1 (IFN- γ), type 2 (IL-4, IL-5, IL-13) and type 3 (TNF- α , IL-1 β) effector cytokines during DSS challenge (Figures 3E-F). Similar conclusions were obtained when these mice were exposed to DSS at 14 (Figure S3K-N) or 18 weeks of age (Figure S3O-R). In accordance with the role of Gram⁺ anaerobic bacteria in the induction of the weaning reaction, mice treated during weaning with vancomycin or

metronidazole developed increased susceptibility to DSS colitis (Figures S4A-I). Finally, blocking the weaning reaction by the administration of EGF during weaning similarly lead to increased susceptibility to DSS colitis in the adult (Figures 3G-H, S4J-N).

The broad deregulation of effector cytokines in mice that did not experience a weaning reaction suggested that such mice were also more susceptible to other types of challenges. To assess their susceptibility to allergic inflammation, mice treated with antibiotics during different periods of time were challenged at 12 weeks of age with oxazolone, which induces an IL-13-dependent intestinal inflammation (Heller et al., 2002). In this context again, mice that were treated with antibiotics during weaning developed more severe oxazolone-induced colitis, and expressed higher levels of all types of effector cytokines (Figure 4A-D).

We also assessed whether the increased susceptibility to inflammatory pathology of mice that did not experience a weaning reaction translated into increased susceptibility to colorectal cancer. SPF mice treated with antibiotics during different periods of time, or GF mice exposed to SPF microbiota before or after weaning, were treated at 9 weeks of age with the carcinogen azoxymethane (AOM) and three cycles of low doses of DSS. Mice that were not exposed to microbiota during weaning developed larger and higher numbers of colon tumours, expressed higher levels of *Tnfa*, *Ki67* (associated with proliferation) and the anti-apoptotic factor *Bcl-2*, and showed high mortality rates (Figures 4E-H). These data show that the weaning reaction, induced by the microbiota, is necessary and sufficient to prevent a pathological imprinting of the immune system that lasts into adulthood. In the absence of a weaning reaction, mice develop increased susceptibility to inflammatory pathology driven by a broad deregulation of the immune system.

Microbiota after weaning does not alter pathological imprinting

As shown above, microbiota introduced to GF mice after weaning did not reduce pathological imprinting (Figures 3A-C, S3A-D). In addition, SPF mice treated with antibiotics after weaning until challenge remained protected from pathological imprinting (Figures 3D-F, S3F-R, S4F-I), indicating that microbiota does not modify imprinting after weaning. To further test whether microbiota could modify imprinting after weaning, a strong reaction to microbiota was induced after weaning in GF mice. As exposure of GF mice to microbiota after weaning elicits only a weak immune response (Figure 2A), adult GF mice were exposed to microbiota together with a low dose of DSS in the drinking water for 5 days. The combination of microbiota and DSS induced an immune reaction that was comparable to the weaning reaction, at least in term of *Tnfa* and *Ifng* expression levels (Figures 5A). Nevertheless, when

challenged 8 weeks later with high doses of DSS, these mice still developed severe colitis (Figures 5B-C, S5A-C). Thus, the absence of a weaning reaction could not be rescued by an immune reaction to SPF microbiota later in life. Furthermore, exposure of adult GF mice to microbiota isolated from 17 days old mice, thus involved in the weaning reaction, did not modulate pathological imprinting either (Figures 5D-E, S5D-F), and no significant dysbiosis could be detected at different taxonomic levels in adult mice that carried pathological imprinting (Figure 5F-G, S5G-I). Altogether, these data indicate that microbiota in the adult does not modulate pathological imprinting, and is not a determinant of disease susceptibility.

The weaning reaction prevents pathological imprinting through the induction of Tregs

We have shown that, in the absence of a weaning reaction, mice develop increased susceptibility to inflammatory pathology driven by a broad deregulation of the immune system. Importantly, even though TNF α and IFN γ are reliable components and markers of the weaning reaction, these cytokines are not involved in the prevention of pathological imprinting, as their neutralization during weaning has no impact on colitis severity in the adult (Figure S6A-E). On the other hand, regulatory T cells (Tregs) are key modulators of the immune system, in the absence of which all types of immune responses are exacerbated (Bilate and Lafaille, 2012; Josefowicz et al., 2012). In order to assess whether CD4⁺CD25⁺ Tregs were required during the weaning reaction to protect from pathological imprinting, SPF mice were treated during weaning with cell-depleting antibodies to CD4 or CD25, and challenged at 9 weeks of age with DSS (Figures 6A-C, S6F-G). In the absence of CD4⁺ or CD25⁺ cells during weaning only (Figure 6D), mice developed severe colitis in the adult, even though the number of Tregs was not affected in the long term and normal before DSS challenge (Figure 6E). Similar results were obtained by transiently depleting Foxp3⁺ Tregs during weaning in “depletion of regulatory T cells (DEREG)” mice (Figure 6F-J, S6H-I). As the weaning reaction is induced by microbiota, we assessed whether microbiota-induced Tregs, which express the transcription factor ROR γ t and start to expand at weaning (Ohnmacht et al., 2015; Sefik et al., 2015) (Figure S6J), were involved. *Foxp3*^{Cre} x *Rorc(gt)*^{Fl} mice that specifically lack ROR γ ⁺ Tregs, developed severe DSS-induced colitis (Figures 6K-M), in agreement with a fundamental role for microbiota-induced Tregs in the protection from pathological imprinting.

ROR γ ⁺ Tregs are induced by microbial antigens, SCFAs and retinoic acid (RA) (Ohnmacht et al., 2015). These different and necessary components should therefore all be

critical during weaning to induce ROR γ^+ Tregs and protect from pathological imprinting. In accordance with this hypothesis, SCFAs protected antibiotic-treated SPF mice from pathological imprinting when administered during weaning (Figures 7A-C, S7A-D). As expected, the protective effect of SCFAs during weaning was dependent on ROR γ^+ Tregs (Figures 7D-I, S7E-I). Contrary to antibiotic-treated mice in which some bacteria are still present (data not shown), SCFAs did not protect from pathological imprinting when administered to GF mice during weaning (Figure S7J-L), as bacterial antigens are required to induce ROR γ^+ Tregs. Conversely, GF mice mono-colonized before or after weaning with SFB, Gram⁺ bacteria that do not produce SCFAs, were not protected from pathological imprinting, unless SCFAs were administered during weaning (Figure 7J-L). Furthermore, in accordance with the absence of effect on colitis severity of microbiota after weaning (Figures 3D-F, S3F-R, S4F-I), SCFAs had no effect if administered after weaning (Figures 7A-C and J-L, S7A-C). Finally, as the conversion of dietary vitamin A into RA by host cells is also required to generate ROR γ^+ Tregs (Ohnmacht et al., 2015), the administration of an inhibitor of the RA receptor during weaning significantly diminished the protective effect of the weaning reaction (Figures 7M-O). Altogether, these data demonstrate that the weaning reaction, induced by the expanding intestinal microbiota during a defined time window, prevents pathological imprinting through the induction of ROR γ^+ Tregs. In the absence of microbiota or Tregs during weaning, mice develop broad pathological imprinting leading to increased susceptibility to inflammatory pathology in the adult, which is not reversed by microbiota after weaning.

DISCUSSION

Here, we show that the immune reaction to the expanding intestinal microbiota at weaning, a reaction we term the “weaning reaction”, is an essential phase in the ontogeny of the immune system after birth. It is defined in time partly by milk-derived factors, and does not occur before or after weaning. If the weaning reaction is absent or prevented, mice develop a “pathological imprinting” that translates into increased susceptibility to multiple types of inflammatory pathologies later in life. The generation of microbiota-induced ROR γ ⁺ Tregs is essential during the weaning reaction to prevent pathological imprinting, and requires bacterial antigens, diet-derived metabolites, as well as RA produced by the host’s tissues.

The impact of microbiota early in life on susceptibility to allergy later in life has been reported previously both in human and mouse. Exposure to antibiotics in newborns increases susceptibility to allergic inflammation in the adult (Prioult and Nagler-Anderson, 2005), whereas exposure to microbes or microbial metabolites decreases such susceptibility (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Hesselmar et al., 2013; Olszak et al., 2012; Riedler et al., 2001; Russell et al., 2012; von Mutius and Vercelli, 2010). Furthermore, an increased incidence in pediatric IBD is observed in children that had been treated with antibiotics earlier in life (Kronman et al., 2012). Our data now show that early exposure to microbiota was necessary not only to decrease susceptibility to allergy later in life, driven by type 2 immune responses, but more generally, to modulate the reactivity of the immune system to different types of immune responses. In the absence of microbiota early in life, we observed exacerbated oxazolone-induced colitis, driven by type 2 responses, as well as exacerbated DSS-induced colitis, which is driven by type 1 and type 3 responses, and larger and more numerous tumors in a model of colorectal cancer that is driven by a chronic type 3 response. The hygiene hypothesis proposes that exposure to microbes is necessary to mitigate the progression of inflammatory pathology, including allergy and autoimmunity (Bach, 2002). We now propose that exposure to microbiota early in life, during weaning, is necessary for the normal development of the immune system, the perturbation of which leads to long-term and broad deregulation of inflammation.

The existence of a time window for exposure to microbiota in order to prevent pathological imprinting was demonstrated in the intestine, the skin and the lung. In the intestine, exposure must occur before weaning, i.e. 3 weeks after birth in mice (Olszak et al., 2012; Russell et al., 2012), whereas in the lung, the first 2 weeks after birth appear critical (Gollwitzer et al., 2014). More recently, Knoop *et al.* have shown that a “window of opportunity” opens 10 days after birth and closes shortly after weaning, 3-4 weeks after birth

(Knoop et al., 2017). Goblet cell-associated antigen passages, or GAPs, open once the EGF level in milk recedes below a threshold, and close in response presumably to microbiota. Antigens delivered through GAPs induce the generation of Tregs against bacteria present during this time window. Such Tregs then maintain long-term tolerance against similar bacteria. In accordance with Knoop and colleagues, we show that EGF blocked the weaning reaction induced by microbiota. However, we show that microbiota was not required to close the window of opportunity, as GF mice failed to develop a significant reaction to microbiota when exposed after weaning at 4 weeks of age. Furthermore, mice closed the window of opportunity even if fed formula milk beyond normal weaning age, a regimen that limits bacterial expansion, suggesting that closure is encoded by yet unknown mechanisms in the weaning mouse. We also show that bacteria were not necessary after weaning to prevent pathological imprinting, indicating that the antigen-specificity of Tregs induced during weaning is not a determinant to prevent pathological imprinting.

Therefore, a more general mechanism may operate during weaning to prevent pathological imprinting into adulthood. Different mechanisms have been reported for the prevention of pathological imprinting. In the case of oxazolone-induced colitis that is driven by IL-13, the microbiota alters the epigenetic code of *Cxcl16* before weaning, and thereby limits the number of pathogenic IL-13⁺ NKT cells that are recruited to the intestine later in life (Olszak et al., 2012). In the lung, an early peak in PD-L1 expression by dendritic cells (DCs) induces the early generation of Tregs associated with long-term protection from allergy (Gollwitzer et al., 2014). In the neonatal skin, CCL20 expression by hair follicles directs the early recruitment of Tregs (Scharschmidt et al., 2015), whereas SCFAs given to pregnant mothers protect pups from developing allergy as adults (Thorburn et al., 2015). We demonstrate that ROR γ ⁺ Tregs, induced during weaning, were required to prevent pathological imprinting in the intestine. The induction of ROR γ ⁺ Tregs requires the activation of a type 3 immune pathway involving the transcription factor STAT3, bacterial antigens presented by MHC class II on DCs, SCFAs produced by certain types of bacteria from dietary fibers, and RA converted from dietary vitamin A by the host's cells (Ohnmacht et al., 2015). All these components needed to be present during weaning to induce the protective ROR γ ⁺ Tregs. The treatment of mice with various antibiotics demonstrated that Gram⁺ anaerobic bacteria were necessary for protection from pathological imprinting, consistent with the role of Clostridia in the generation of SCFAs and the induction of Tregs (Narushima et al., 2014). This interpretation is also consistent with earlier data showing that a low diversity microbiota

devoid of Clostridia, early in life, leads to high serum levels of pro-allergic IgE in the adult (Cahenzli et al., 2013).

It remains to be understood how the generation of ROR γ ⁺ Tregs during the weaning reaction prevent pathological imprinting into adulthood. In the case of IL-13⁺ NKT cell-mediated colitis, it is the epigenetic modification of *Cxcl16* in DCs that carries the memory of early life encounter with microbiota (Olszak et al., 2012). In addition, bacteria-specific Tregs generated by antigen delivery through GAPs before and during weaning carry antigen-specific memory into adulthood (Knoop et al., 2017), and the epigenetic modification by SCFAs of *FoxP3* in the lung epithelial cells of the fetus is proposed to regulate lung inflammation and Treg activity in the adult (Thorburn et al., 2015). It is possible that several mechanisms concur to carry memory of early exposure to microbiota in the intestine, which include antigen-specific memory, as well as epigenetic modification, in different cell types including epithelial and stromal cells (Schuijs et al., 2015), of multiple genes involved in the recruitment, activation and regulation of immune cells. Furthermore, in our hands, pathological imprinting in the intestine cannot be reversed by microbiota after weaning, indicating that the mechanisms preventing pathological imprinting are limited to the weaning period. However, ROR γ ⁺ Tregs can be induced after weaning (Sefik et al., 2015), and therefore, an element in addition to the generation of ROR γ ⁺ Tregs during weaning must be evoked to explain the existence of this restricted time window. Along this idea, but in the context of thymic-derived Tregs, it has been shown that the perinatal processing and presentation of antigens by stromal cells in the thymus induces the generation of a set of Tregs carrying a distinct TCR repertoire (Yang et al., 2015).

Altogether, our data demonstrate the importance of early life exposure to microbiota for the development of a normal and equilibrated immune system. The protective role of SCFAs also shows an important contribution of diet, and its metabolism by microbiota and host, in preventing pathological imprinting. Modern life is associated with increased hygiene and a broad usage of antibiotics, which limit exposure to microbiota, as well as increasingly high fat and low fiber diets. Therefore, we suggest that this lifestyle, while protecting efficiently from infectious diseases, concomitantly leads to increased susceptibility to inflammatory pathologies, including allergy, autoimmunity and IBD, as well as to the consequences of chronic inflammation, such as type 2 diabetes, cancer and possibly dementia. It is therefore important to elaborate strategies to prevent pathological imprinting early in life by adjusting diets to the breast-feeding mother and the baby, providing corrective bacteria, as

well as to elaborate strategies to reverse pathological imprinting later in life. Our data show that reaction to microbiota after weaning is not sufficient to reverse pathological imprinting, but different types of “therapeutic” immune reactions, induced by pathogens for example, remain to be tested.

STAR★METHODS

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

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GF mice colonization with SPF microbiota or SFB

Mice cross-fostering and formula milk

Models of colitis

Model of colorectal cancer

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Flow cytometry

Microbial DNA extraction and sequencing

Quantitative PCR

RNA sequencing and analysis

QUANTIFICATION AND STATISTICAL ANALYSIS

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AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Validation, Z.A.N. and G.E.; Investigation and Data Analysis, Z.A.N., S.D., R.M., C.C., S.A.B., F.D. and M.B.; Formal Analysis, Z.A.N.; Resources, T.S., M.B., and N.C.-B.; Writing – Original draft, Z.A.N. and G.E.; Funding Acquisition, Z.A.N. and G.E.; Supervision, G.E.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

REFERENCES

- Al Nabhani, Z., Lepage, P., Mauny, P., Montcuquet, N., Roy, M., Le Roux, K., Dussaillant, M., Berrebi, D., Hugot, J.P., and Barreau, F. (2016). Nod2 Deficiency Leads to a Specific and Transmissible Mucosa-associated Microbial Dysbiosis Which Is Independent of the Mucosal Barrier Defect. *J Crohns Colitis* *10*, 1428-1436.
- Bach, J.F. (2002). The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* *347*, 911-920.
- Bain, C.C., Bravo-Blas, A., Scott, C.L., Perdiguero, E.G., Geissmann, F., Henri, S., Malissen, B., Osborne, L.C., Artis, D., and Mowat, A.M. (2014). Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol* *15*, 929-937.
- Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., and Salzman, N. (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect Immun* *76*, 907-915.
- Bilate, A.M., and Lafaille, J.J. (2012). Induced CD4⁺Foxp3⁺ regulatory T cells in immune tolerance. *Annu Rev Immunol* *30*, 733-758.
- Bouskra, D., Brezillon, C., Berard, M., Werts, C., Varona, R., Boneca, I.G., and Eberl, G. (2008). Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* *456*, 507-510.
- Cahenzli, J., Koller, Y., Wyss, M., Geuking, M.B., and McCoy, K.D. (2013). Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe* *14*, 559-570.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* *7*, 335-336.
- Chatterton, D.E., Nguyen, D.N., Bering, S.B., and Sangild, P.T. (2013). Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* *45*, 1730-1747.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., *et al.* (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* *37*, D141-145.
- Gaboriau-Routhiau, V., Rakotobe, S., Lecuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G., *et al.* (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* *31*, 677-689.
- Gensollen, T., Iyer, S.S., Kasper, D.L., and Blumberg, R.S. (2016). How colonization by microbiota in early life shapes the immune system. *Science* *352*, 539-544.

- Gollwitzer, E.S., and Marsland, B.J. (2015). Impact of Early-Life Exposures on Immune Maturation and Susceptibility to Disease. *Trends Immunol* 36, 684-696.
- Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., Sherburn, R., McCoy, K.D., Nicod, L.P., Lloyd, C.M., and Marsland, B.J. (2014). Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat Med* 20, 642-647.
- Gomez de Agüero, M., Ganal-Vonarburg, S.C., Fuhrer, T., Rupp, S., Uchimura, Y., Li, H., Steinert, A., Heikenwalder, M., Hapfelmeier, S., Sauer, U., *et al.* (2016). The maternal microbiota drives early postnatal innate immune development. *Science* 351, 1296-1302.
- Heller, F., Fuss, I.J., Nieuwenhuis, E.E., Blumberg, R.S., and Strober, W. (2002). Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* 17, 629-638.
- Hesselmar, B., Sjöberg, F., Saalman, R., Aberg, N., Adlerberth, I., and Wold, A.E. (2013). Pacifier cleaning practices and risk of allergy development. *Pediatrics* 131, e1829-1837.
- Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., *et al.* (2009). Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* 139, 485-498.
- Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30, 531-564.
- Knoop, K.A., Gustafsson, J.K., McDonald, K.G., Kulkarni, D.H., Coughlin, P.E., McCrate, S., Kim, D., Hsieh, C.S., Hogan, S.P., Elson, C.O., *et al.* (2017). Microbial antigen encounter during a preweaning interval is critical for tolerance to gut bacteria. *Sci Immunol* 2.
- Kronman, M.P., Zaoutis, T.E., Haynes, K., Feng, R., and Coffin, S.E. (2012). Antibiotic exposure and IBD development among children: a population-based cohort study. *Pediatrics* 130, e794-803.
- Le Doare, K., Holder, B., Bassett, A., and Pannaraj, P.S. (2018). Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. *Front Immunol* 9, 361.
- Li, W., and Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22, 1658-1659.
- Mao, K., Baptista, A.P., Tamoutounour, S., Zhuang, L., Bouladoux, N., Martins, A.J., Huang, Y., Gerner, M.Y., Belkaid, Y., and Germain, R.N. (2018). Innate and adaptive lymphocytes sequentially shape the gut microbiota and lipid metabolism. *Nature* 554, 255-259.
- Moran, J.R., Courtney, M.E., Orth, D.N., Vaughan, R., Coy, S., Mount, C.D., Sherrell, B.J., and Greene, H.L. (1983). Epidermal growth factor in human milk: daily production and diurnal variation during early lactation in mothers delivering at term and at premature gestation. *J Pediatr* 103, 402-405.

- Narushima, S., Sugiura, Y., Oshima, K., Atarashi, K., Hattori, M., Suematsu, M., and Honda, K. (2014). Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia. *Gut Microbes* 5, 333-339.
- Ohnmacht, C., Park, J.H., Cording, S., Wing, J.B., Atarashi, K., Obata, Y., Gaboriau-Routhiau, V., Marques, R., Dulauroy, S., Fedoseeva, M., *et al.* (2015). The microbiota regulates type 2 immunity through ROR γ t+ T cells. *Science* 349, 989-993.
- Olszak, T., An, D., Zeissig, S., Vera, M.P., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L., *et al.* (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336, 489-493.
- Prioult, G., and Nagler-Anderson, C. (2005). Mucosal immunity and allergic responses: lack of regulation and/or lack of microbial stimulation? *Immunol Rev* 206, 204-218.
- Rakoff-Nahoum, S., Kong, Y., Kleinstein, S.H., Subramanian, S., Ahern, P.P., Gordon, J.I., and Medzhitov, R. (2015). Analysis of gene-environment interactions in postnatal development of the mammalian intestine. *Proc Natl Acad Sci U S A* 112, 1929-1936.
- Riedler, J., Braun-Fahrlander, C., Eder, W., Schreuer, M., Waser, M., Maisch, S., Carr, D., Schierl, R., Nowak, D., von Mutius, E., *et al.* (2001). Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 358, 1129-1133.
- Round, J.L., and Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 9, 313-323.
- Russell, S.L., Gold, M.J., Hartmann, M., Willing, B.P., Thorson, L., Wlodarska, M., Gill, N., Blanchet, M.R., Mohn, W.W., McNagny, K.M., *et al.* (2012). Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep* 13, 440-447.
- Sanos, S.L., Bui, V.L., Mortha, A., Oberle, K., Heners, C., Johner, C., and Diefenbach, A. (2009). ROR γ and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol* 10, 83-91.
- Sawa, S., Cherrier, M., Lochner, M., Satoh-Takayama, N., Fehling, H.J., Langa, F., Di Santo, J.P., and Eberl, G. (2010). Lineage relationship analysis of ROR γ t+ innate lymphoid cells. *Science* 330, 665-669.
- Sawa, S., Lochner, M., Satoh-Takayama, N., Dulauroy, S., Berard, M., Kleinschek, M., Cua, D., Di Santo, J.P., and Eberl, G. (2011). ROR γ t+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol* 12, 320-326.
- Scharschmidt, T.C., Vasquez, K.S., Truong, H.A., Gearty, S.V., Pauli, M.L., Nosbaum, A., Gratz, I.K., Otto, M., Moon, J.J., Liese, J., *et al.* (2015). A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity* 43, 1011-1021.
- Schuijjs, M.J., Willart, M.A., Vergote, K., Gras, D., Deswarte, K., Ege, M.J., Madeira, F.B., Beyaert, R., van Loo, G., Bracher, F., *et al.* (2015). Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. *Science* 349, 1106-1110.

Sefik, E., Geva-Zatorsky, N., Oh, S., Konnikova, L., Zemmour, D., McGuire, A.M., Burzyn, D., Ortiz-Lopez, A., Lobera, M., Yang, J., *et al.* (2015). Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. *Science* 349, 993-997.

Snel, J., Bakker, M.H., and Heidt, P.J. (1997). Quantification of antigen-specific immunoglobulin A after oral booster immunization with ovalbumin in mice mono-associated with segmented filamentous bacteria or *Clostridium innocuum*. *Immunol Lett* 58, 25-28.

Sonnenburg, J.L., and Backhed, F. (2016). Diet-microbiota interactions as moderators of human metabolism. *Nature* 535, 56-64.

Subramanian, S., Huq, S., Yatsunenkov, T., Haque, R., Mahfuz, M., Alam, M.A., Benezra, A., DeStefano, J., Meier, M.F., Muegge, B.D., *et al.* (2014). Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 510, 417-421.

Tanaka, T., Kohno, H., Suzuki, R., Yamada, Y., Sugie, S., and Mori, H. (2003). A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* 94, 965-973.

Thorburn, A.N., McKenzie, C.I., Shen, S., Stanley, D., Macia, L., Mason, L.J., Roberts, L.K., Wong, C.H., Shim, R., Robert, R., *et al.* (2015). Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. *Nature communications* 6, 7320.

Ubeda, C., Lipuma, L., Gobourne, A., Viale, A., Leiner, I., Equinda, M., Khanin, R., and Pamer, E.G. (2012). Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *J Exp Med* 209, 1445-1456.

Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol* 13, 88-100.

von Mutius, E., and Vercelli, D. (2010). Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* 10, 861-868.

Yang, S., Fujikado, N., Kolodin, D., Benoist, C., and Mathis, D. (2015). Immune tolerance. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance. *Science* 348, 589-594.

FIGURE LEGENDS

Figure 1. The weaning reaction to microbiota

(A) Representative frequency of the main bacterial classes in the ileum of SPF mice. Data are representative of 3 individual experiments.

(B) Transcriptomic profile in total ileum of SPF mice (n=1). Data are representative of 2 individual experiments.

(C) Heatmap of the SPF/GF transcriptional profile ratio at different age (n=2/group/week).

(D) Quantitative RT-PCR for the indicated genes in the ileum of SPF and GF mice.

(E) Number of IFN γ ⁺ lymphoid cells in small intestine of SPF and GF mice. CD45⁺ were gated on live cells, CD3⁺ T cells were gated on CD45⁺ cells, CD4⁺ and CD8a⁺ T cells were gated on CD3⁺CD45⁺ cells, and innate lymphoid cells shown as CD3⁻CD127⁺ cells were gated on CD45⁺CD3⁻ cells. Each dot represents 1 mouse. Data are representative of 2 individual experiments.

(F-I) Quantitative RT-PCR for the indicated genes in the ileum of (F) Myd88- and Trif-deficient mice and littermate wild-type mice, (G) mice treated in their drinking water with the indicated antibiotics or a cocktail of these antibiotics (ABX), (H) GF mice mono-colonized with SFB, and (I) GF or SPF mice treated between 2 weeks and 4 weeks after birth with SCFAs by i.p., vehicle is saline. Data are representative of 2 individual experiments (n=3-6/group).

Statistical analysis: (D; E; F, H) Mann-Whitney U test, or (G; I) one-way ANOVA. *p<0.03, **p<0.002 and ***p<0.0002 vs control group. ns, not significant. Mean \pm SEM.

See Figure S1.

Figure 2. A critical time window for the weaning reaction

(A-B) Experimental scheme and quantitative RT-PCR for the indicated genes in the ileum of (A) GF mice exposed to microbiota before or after weaning, or (B) SPF mice exposed to formula milk for different periods of time, the end of which is indicated by an arrow.

(C-E) Quantitative RT-PCR for the indicated genes in the ileum of (C) mice obtained by synchronised or non-synchronised cross-fostering, (D) SPF mice exposed to EGF or to vehicle (PBS) control during weaning (2-4 weeks), (E) SPF 10 days old mice exposed to EGF inhibitors or vehicle.

Data are representative of 2 individual experiments. n=3-6/group, each dot represents 1 mouse.

Statistical analysis: (A; B and D) one-way ANOVA or (C and E) Mann-Whitney U test. * $p < 0.03$ and ** $p < 0.002$ vs GF or indicate group && $p < 0.002$ vs SPF + vehicle group. ns, not significant. Mean \pm SEM.

See Figure S2.

Figure 3. The weaning reaction prevents pathological imprinting for colitis

(A-C) DSS-induced colitis in adult GF mice exposed to microbiota at the indicated age, as measured by weight loss (A) and expression of cytokine genes in the colon (B-C).

(D-F) DSS-induced colitis in adult SPF mice exposed to an antibiotic cocktail (ampicillin, metronidazole, streptomycin and vancomycin) in the drinking water before conception until the indicated age, as measured by weight loss (D) and expression of cytokine genes in the colon (E-F).

(G-H) DSS-induced colitis in adult SPF mice treated with EGF or saline between 2 and 4 weeks after birth, as measured by weight loss (G) and expression of cytokine genes in the colon (H).

Colitis was induced at 9-10 weeks of age in all groups. Data are pooled from 2-3 individual experiments (n=3-33/group). Each dot represents 1 mouse.

Statistical analysis: (A-F and H) one-way ANOVA or (G) Mann-Whitney U test. * $p < 0.03$, ** $p < 0.002$, *** $p < 0.0002$ and **** $p < 0.0001$ vs SPF control group. ns, not significant. Mean \pm SEM.

See also Figure S3-4.

Figure 4. The weaning reaction prevents pathological imprinting for allergy and cancer

(A-D) Oxazolone-induced allergic inflammation in adult SPF mice exposed to an antibiotic cocktail (ampicillin, metronidazole, streptomycin and vancomycin) in the drinking water before conception until the indicated age, as measured by weight loss (A), colon length (B) and expression of cytokine genes in the colon (C-D). Colitis was induced at 9 weeks of age. Data are pooled from 2 individual experiments (n=5-10/group). Each dot represents 1 mouse.

(E-H) AOM/DSS-induced colon carcinomas in SPF mice treated with an antibiotic cocktail (as above) before conception to the indicated age (E and G) (data are pooled from 2 individual experiments (n=5-20/group); Each dot represents 1 mouse), or in GF exposed to microbiota at the indicated age (F, H) (data are pooled from 2 individual experiments (n=3-9/group); Each dot represents 1 mouse). Measured are weight loss and mouse survival (E and F), as well as number of polyps, size of tumours and expression of the indicated genes (G and H).

Statistical analysis: One-way ANOVA. * $p < 0.03$, ** $p < 0.002$, *** $p < 0.0002$ and **** $p < 0.0001$. ns, not significant. Mean \pm SEM.

Figure 5: Microbiota after weaning does not alter pathological imprinting

(A-C) Exposure of 8-10 weeks old GF mice to microbiota and low level DSS (0.5%), and expression of the indicated genes measured by quantitative RT-PCR 7 days after initiation of exposure (A). Data are representative of 2 individual experiments (n=3-6/group). Mice were challenged 6-8 weeks later with DSS (2.5%) to induce colitis, and measure weight loss (B) and expression of the indicated genes (C). Data are representative of 3 individual experiments (n=3-8/group). Each dot represents 1 mouse.

(D-E) Weight loss (D) and quantitative RT-PCR for the indicated genes in the colon (E) of adult GF mice exposed at 8 weeks of age to microbiota isolated from the caecum of 17 days or 8 weeks old mice, exposed to DSS at 14 weeks of age and measured 21 days later. Data are representative of 2 individual experiments (n=4-5/group). Each dot represents 1 mouse.

(F-G) Principal component analysis (F) and composition (G) of the fecal bacterial microbiota, at the phylum level, of 9 weeks old GF mice colonized at the indicated age by exposure to SPF microbiota. Data are representative of 2 individual experiments. Each dot represents 1 mouse.

Statistical analysis: (A-E) One-way ANOVA, or (F) Monte Carlo rank test. * $p < 0.03$, ** $p < 0.002$, *** $p < 0.0002$ and **** $p < 0.0001$. ns, not significant. Mean \pm SEM.

See Figures S5.

Figure 6. The weaning reaction prevents pathological imprinting through the induction of Tregs

(A-C) SPF mice treated with anti-CD4 or anti-CD25 cell-depleting antibodies between 2 to 4 weeks of age were challenged at 9 weeks of age with DSS, and measured for weight loss (A), colon length (B) and expression of *Tnfa* (C). Data are representative of 3 individual experiments (n=5-10/group). Each dot represents 1 mouse.

(D-E) Frequency of FoxP3⁺CD4⁺ T cells at 4 weeks (D) or 9 weeks of age (E) in SPF mice treated with anti-CD4 or anti-CD25 antibodies between 2 to 4 weeks of age. Each dot represents 1 mouse.

(F-G) Frequency of FoxP3⁺CD4⁺ T cells at 4 weeks (F) or 9 weeks of age (G) in SPF DEREK mice treated with diphtheria toxin (DT) between 2 to 4 weeks of age. Each dot represents 1 mouse.

(H-J) SPF DEREG mice treated with diphtheria toxin (DT) between 2 to 4 weeks of age were challenged at 9 weeks of age with DSS, and measured for weight loss (H), colon length (I) and expression of *Tnfa* (J). Data are representative of 2 individual experiments. Each dot represents 1 mouse.

(K-M) *Foxp3^{Cre}* x *Rorc(gt)^{Fl}* mice were exposed to DSS at 9 weeks of age and measured for weight loss (K), colon length (L) and expression of the indicated proteins in colon (M). Data are representative of 2 individual experiments (n=4-10/group). Each dot represents 1 mouse.

Statistical analysis: (A-E; H-J; L-M) One-way ANOVA, or (F, G, K) Mann-Whitney U test. *p<0.03, **p<0.002, ***p<0.0002 and ****p<0.0001. ns, not significant. Mean ± SEM.

See Figure S6.

Figure 7. Dietary metabolites prevent pathological imprinting through the induction of Tregs

(A-C) SPF mice exposed to a cocktail of antibiotics (ampicillin, metronidazole, streptomycin and vancomycin) were treated with short chain fatty acid (SCFAs) during the indicated periods of time, and exposed to DSS-induced colitis at 9-10 weeks of age. Measured were weight loss (A), colon length (B) and expression of *Tnfa* in the colon (C).

(D-F) SPF mice were exposed to antibiotics, SCFAs, as well as anti-CD4 or anti-CD25 cell-depleting antibodies during 2 to 4 weeks of age, challenged and monitored as in A-C.

(G-I) *Foxp3^{Cre}* x *Rorc(gt)^{Fl}* mice were treated with SCFAs between 2 and 4 weeks of age and challenged as in A-C. Measured were weight loss (G), colon length (H) and levels of TNFα in the colon (I).

(J-L) GF mice were re-colonized with Segmented Filamentous Bacteria (SFB) at 2 weeks (SFBn) or 5 weeks (SFBa) of age, and concomitantly treated with SCFAs, before being challenged as in A-C. Measured were survival (J), weight loss (K) and expression of Lipocalin-2 in the serum (L).

(M-P) SPF mice were treated with an inhibitor of RAR (RARi) during the indicated periods of time, then challenged and monitored for weight (M), disease activity (N), colon length (O) and gene expression (P). Vehicle is saline.

Data are pooled from 2 individual experiments (n=3-5/group). Each dot represents 1 mouse. Statistical analysis: One-way ANOVA. *p<0.03, **p<0.002, ***p<0.0002 and ****p<0.0001. ns, not significant. Mean ± SEM.

See figures S7.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor® 700-conjugated anti-CD8a (clone 53-6.7)	Sony Biotechnology	1103650
APC-conjugated IFN γ (clone REA638)	Miltenyi	130-109-723
Brilliant Violet 650-conjugated anti-CD19 (clone 6D5)	BioLegend	115541
eFluor450-conjugated anti-CD25 (clone PC61.5)	eBioscience	48-0251
FITC-conjugated anti-CD4 (clone RM4-5)	BD Biosciences	553047
FITC-conjugated anti-CD45.2 (clone 104)	BD Biosciences	553772
Horizon V500-conjugated anti-CD4 (clone RM4-5)	BD Biosciences	560782
Horizon V500-conjugated anti-CD45.2 (clone 104)	BD Biosciences	562129
InVivoPlus anti-mouse CD4 (clone GK1.5)	Bio X Cell	BP0003
InVivoPlus anti-mouse CD25 (clone PC-61.5)	Bio X Cell	BP0012
InVivoPlus rat IgG2b isotype control, anti-keyhole limpet hemocyanin	Bio X Cell	BP0090
InVivoPlus rat IgG2a isotype control (clone 2A3)	Bio X Cell	BP0089
PE-CF594-conjugated anti-CD3e (clone 145-2C11)	BD Biosciences	562286
PE-conjugated TNF α (clone MP6-XT22)	Miltenyi	130-102-386
PE-Cy7-conjugated CD127	eBioscience	25-1271
PerCP-Cy5.5-conjugated Foxp3 (clone FJK-16s)	eBioscience	45-5773
Commercial kits		
Fixable Blue Dead Cell Stain Kit	Invitrogen™	L23105
Fixation-permeabilization buffer	eBioscience	00-5521-00
Mouse EGF ELISA Kit	R&D System	MEG00
Mouse IFN-gamma ELISA	R&D System	DY485-CP
Mouse Il-1b ELISA	BD Biosciences	559603
Mouse Il-4 ELISA	BD Biosciences	555232
Mouse Il-5 ELISA	BD Biosciences	555236
Mouse IL13 ELISA	R&D System	DY413-CP
Mouse Lipocalin-2 ELISA Kit	R&D System	MLCN20
Mouse Myeloperoxidase ELISA	R&D System	DY3667
Mouse TNF-alpha ELISA	R&D System	DY410-CP
Chemicals, Peptides, and Proteins		
Acetate	Sigma-Aldrich	S5636
Ampicillin	Sigma-Aldrich	A9518

Azoxymethane	Sigma-Aldrich	A5486
Butyrate	Sigma-Aldrich	303410
Dextran sulfate 40 sodium salt BioChemic	PanReacAppliChem	A3261
EDTA 0.5M, pH 8.0	Invitrogen™	AM9262
EGF	Sigma-Aldrich	E5160
Fluorescein isothiocyanate–Carboxymethyl–Dextran	Sigma-Aldrich	68059
Formula milk	www.tvn.fr	G.T.I.N. 03700454503702
Metronidazole	Sigma-Aldrich	M3761
pan-retinoic acid receptor (RAR) inverse agonist BMS493	Tocris abiotechne brand	3509
Pepinh-MYD	Invitrogen	tlrl-pimyd
Percoll	Sigma-Aldrich	17-0891
Propionate	Sigma-Aldrich	P1880
Streptomycin	Sigma-Aldrich	S6501
Tyrphostin AG 1478	Sigma-Aldrich	T4182
Vancomycin	Sigma-Aldrich	V1150
4-ethoxymethylene-2-phenyl-2-oxazoline-5-one	Sigma-Aldrich	E0753
RNA analysis		
MultiMACS cDNA synthesis Kit	Miltenyi Biotec	130-094-408
SsoAdvanced Universal SYBR Green Supermix	BioRad	1725275
Primers		
<i>Ifng</i>	Qiagen	PPM03121A
<i>Il1b</i>	Qiagen	PPM03109F
<i>Il4</i>	Qiagen	PPM03013F
<i>Il5</i>	Qiagen	PPM03014F
<i>Il13</i>	Qiagen	PPM03021B
<i>Tnfa</i>	Qiagen	PPM03113G
Experimental Models: Organisms/Strains		
Mouse C57BL/6J	Charles River	
Mouse C57BL/6J Germ-free	Institut Pasteur	N/A
Mouse <i>Foxp3</i> ^{Cre} x <i>Rorc(gt)</i> ^{Fl}	Institut Pasteur	N/A
Mouse Myd88- and Trif-deficient	Institut Pasteur	N/A
Software and Algorithms		
Flow Jo-v10	FlowJo	www.flowjo.com
QIIME	(Caporaso et al., 2010)	http://qiime.org
Prism 7	Prism-Graphpad	www.graphpad.com
R-3.1.0	R	www.r-project.org
Qlucore	Qlucore	www.qlucore.com

CONTACT FOR REAGENT AND RESOURCE SHARING

All unique reagents and resources will be made readily available upon request to the Lead Contact, Gérard Eberl (gerard.eberl@pasteur.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All animal experiments were approved by the committee on animal experimentation of the Institut Pasteur and by the French Ministry of Research. SPF females and males C57BL/6 mice were purchased from Charles River and exposed to the environment of the local animal facility at Institut Pasteur at least for 2 weeks before mating. Germ-free C57BL/6 mice were generated at the Gnotobiology Platform of the Institut Pasteur. Myd88/Trif-double deficient mice were obtained from Ivo Gomperts Boneca (Institut Pasteur). *Foxp3^{Cre} x Rorc(gt)^{Fl}* DEREK mice were maintained on a C57BL/6 background. All mice were weaned 28 days after birth. Co-housed littermates were used in all experiments in order to avoid cage-specific divergence in the composition of the gut microbiota (Ubeda et al., 2012); experimental group were both age- and sex-matched. The age of mice at the beginning of each experiment is indicated in the legend to the figures.

METHOD DETAILS

Mice treatment with antibiotics, chemicals and antibodies

Mice were treated during the indicated periods of time with a cocktail of antibiotics in their drinking water, containing 2.5 mg/ml streptomycin, 1 mg/ml ampicillin, 0.5 mg/ml vancomycin and 0.5 mg/ml metronidazole. The antibiotic-containing drinking water was changed 2x/week. Once the treatment was terminated, treated and untreated mice were co-housed. To inhibit EGFR activation, mice were injected i.p. with 500µg/kg of tyrphostin AG1478; to activate EGFR, mice were injected with 1 µg of recombinant murine EGF in 20 µl PBS into the colon. For inhibition of the retinoic acid receptor, 250µg of the pan-retinoic acid receptor inverse agonist BMS493 was administered to adult mice and 125µg to young mice i.p. 2x/week during the weeks indicate on figure. For SCFAs, 50 µL of cocktail contains acetate (2 mg/mL), butyrate (1.65 mg/mL) and propionate (1.45 mg/mL) was administered i.p. 3x/week. To ablate lymphocyte subsets, mice were treated 3x/week with 100µg/mouse with a monoclonal antibody to CD4 (clone GK1.5) or to CD25 (clone PC-61.5), or their IgG2a and IgG2b isotypes, respectively. To bloc Myd88 signalling, 10 µg of 1mM of MyD88 inhibitory peptide Pepinh-MYD was administered i.p. 3x/week. Male DEREK mice, provided by Professor Tim Spawasser (TWINCORE, Hannover, Germany), were backcrossed to wild type C57BL6 at the animal facility of Institut Pasteur. All the offsprings were genotyped by

PCR with specific primers for GFP and DTR. Foxp3⁺ Treg cell depletion was achieved by intraperitoneal (i.p.) administration of 50 ng diphtheria toxin (DT) 3 times per week at weaning period (2 to 4 weeks of age).

GF mice colonization with SPF microbiota or SFB

GF mice were exposed to microbiota by co-housing with SPF mice, or gavaged with 0.5 ml of fecal homogenate from microbiota ceacal contents of 17 days or 8 weeks old mice. To generate SFB-monocolonized mice, GF mice were gavaged twice at a 24 hr-interval at D12-14, or at 6 -7 weeks of age, with 0.5 ml of fecal homogenate from previously SFB mono-associated mice (Gaboriau-Routhiau et al., 2009). The presence of SFB was verified by qRT-PCR conducted on ileal biopsies or fecal DNA by amplifying 16S rRNA genes using the following bacteria-specific primers (Barman et al., 2008): 5'-GACGCTGAGGCATGAGAGCAT-3' and 5'-GACGGCACGGATTGTTATTCA-3'. qPCR is performed on a Bio Rad CFX96 Real Time System with SYBR Green reagent. The PCR conditions include a pre-incubation step at 95°C for 5 minutes, 40 amplification cycles of 95°C for 10 s and annealing at 62°C for 45 s, and a melting curve.

Mice cross-fostering and formula milk

Asynchronously cross-fostering (CF) of mice was done by placing newborn day 1 (D1) pups with mothers having delivered 13 days earlier, and D13 pups with mothers having delivered 1 day earlier. Synchronously CF of mice was done by placing D1 or D13 pups with mothers having delivered the day of pups' birth. In brief, mothers were removed from their cages and placed in separate clean cages. The litter to be fostered was picked up gently and mixed with dirty bedding, nesting material, and other pups from the recipient dam's cage. This manipulation was done to transfer the recipient dam's scent. Pups were placed back in the recipient dam's nest, and the recipient mouse mother was returned to the cage. The cage was monitored visually every 15 min for the next 60 min. If there was evidence of rejection by the dam (for example, agitation, carrying the pups around), the pups were removed from the cage and euthanized. The cages were assessed at least once daily and not disturbed for the first 72 h after fostering to decrease potential cannibalism. The cages were then monitored daily.

Formula milk was purchased from TVM laboratory. Between 16 to 18 days after birth, mice received 15µL of formula milk by gavage (8x/day) using a sterilized syringe, while mothers were exposed to the milk in the sterile bottle. 18 days after birth, the pups were

separated from their mothers and exposed to formula milk delivered by sterile bottle. To avoid bacteria growth in milk, the bottle was changed every 5h. The pups were then weaned to normal show at the indicated times.

Models of colitis

To test susceptibility to DSS-induced colitis, adult mice with similar body weight received two cycles of 2.5% DSS (with a mol. wt. of approx. 40.000 g/mol) in their drinking water, for 7 days interrupted by 7 days of normal water. Control mice received drinking water without DSS. Progression of disease was monitored daily by weight and survival. Colitis severity was scored by daily observation of the following parameters: weight loss (0 point = No weight loss or weight gain, 1 point = 5–10% weight loss, 2 points = 11–15% weight loss, 3 points = 16–20% weight loss, 4 points = >21% weight loss); stool consistency (0 point = normal and well formed, 2 points = very soft and unformed, 4 points = watery stool); and blood in stool (0 point = normal color stool, 2 points = reddish color stool, 4 points = bloody stool). The disease activity index (DAI) was calculated as the combined scores of weight loss, bleeding, stool consistency, with a maximum score of 12. After 21 days, mice were euthanized, the length of colons was measured, and organs and blood were collected for biochemical, gene expression and cytometric analysis. To test susceptibility to oxazolone-induced colitis, adult mice were pre-sensitized with 200 µl of 2% (w/v) oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one) in 100% ethanol by subcutaneous injection. Five days later, mice were challenged intra-rectally, under anesthesia, with 150µl of 1.5% oxazolone in 50% ethanol, or only 50% ethanol as a control. Intrarectal injections were administered with a 3.5F polyurethane catheter. Mice were monitored every day for body weight. After 6 days, mice were euthanized, the length of colons was measured, and a small piece (0.2cm) of distal colon was taken for the analysis of gene expression.

Model of colorectal cancer

Colitis-associated colorectal cancer was induced as previously described (Tanaka et al., 2003). Briefly, nine weeks old mice were injected intraperitoneally with Azoxymethane (AOM; 10 mg/kg body weight) diluted in PBS. Five days later, mice were subjected to three cycles of DSS treatment, each cycle consisting of 1.5% DSS in the drinking water for 5 days interrupted by a 10-day recovery period with normal water. Mice were euthanized 10 days after the last DSS cycle. Mice were monitored every day for body weight and survival. After

50 days, mice were euthanized, and the colon was collected to determine polyp size and numbers, and further characterized by gene expression.

Gut permeability

In vivo permeability assays were performed using fluorescein isothiocyanate (FITC)–dextran 4kDa as a paracellular permeability tracer. FITC–dextran was given by gavage to adult mice 6 days after initiation of the second cycle of DSS in the DSS-induced colitis model. Mice were gavaged with FITC–dextran (5 mg/200 μ L/mouse) 4 h prior to sacrifice. Whole blood FITC–dextran concentration was determined by a spectrometry. FITC–dextran concentrations in serum were calculated from standard curves generated by serial dilution of FITC–dextran. *In vivo* permeability was expressed as the mean whole blood FITC–dextran concentration in ng/mL.

ELISA

Cytokines (IL-1 β ; IL-4; IL-5; IL-13; TNF- α and IFN- γ), Myeloperoxidase (MPO), Lipocalin-2 and Epidermal growth factor (EGF) were measured using ELISAs and following the manufacturers' recommendations. Luminal EGF was measured in stomach, small intestine and colonic contents; to isolate contents, the stomach was opened and contents were added to 1 mL PBS. For the small intestine and colon, 1 ml of PBS was injected to one open end of the small intestine or colon using a 24-gauge gavage needle. Contents were then collected in a tube from the other open end of the small intestine or colon.

Flow cytometry

Small intestinal and colonic *lamina propria* cells were isolated as described previously (Sawa et al., 2011). Briefly, Peyer's patches were removed, and whole small intestine and colon were opened longitudinally, cut into pieces and incubated for 30 minutes in 30mM EDTA, washed extensively, and incubated for several rounds in 0.7mg/ml collagenase D. These preparations were then grinded through a 100 μ m filter to generate single cell suspensions. Cells were separated by a 40/80% (w/v) Percoll density gradient and washed prior to staining. Cells were stained 5 minutes at 4°C with LIVE/DEAD fixable blue dead stain kit (Invitrogen), in order to differentiate live cells from dead cells. Cells were further pre-incubated with Fc-Block for 10 minutes and stained for 20 minutes with antibodies to surface markers. For intracellular stainings, cells were fixed and permeabilized with a commercially available fixation/permeabilization buffer. Intracellular staining was performed with PerCP-Cy5.5-conjugated Foxp3 (clone FJK-16s) or with APC-conjugated IFN γ (clone REA638) and

PE-conjugated TNF α (clone MP6-XT22). For intracellular cytokine staining, cells were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 hours in RPMI 1640 (Invitrogen) containing and 10% FCS, 25 mM HEPES, 0.05 mM 2-mercaptoethanol, and 100 U/ml of penicillin and streptomycin.

Microbial DNA extraction and sequencing

Total DNA was extracted from feces or ileal contents as previously described (Al Nabhani et al., 2016), using both physical and chemical lysis. DNA concentrations were determined by spectrophotometry using a Nanodrop (Thermo Scientific). Microbial composition was assessed by 454 pyrosequencing (GS FLX Ti technology) targeting the V3-V4 region of the bacterial 16S rDNA region (V3fwd: 5'TACGGRAGGCAGCAG3', V4rev: 5'GGACTACCAGGGTATCTAAT3'). Sequences were further clustered into OTUs (Operational Taxonomic Units) at 97% of identity using QIIME (Caporaso et al., 2010) and Cd-hit (Li and Godzik, 2006). OTU representative sequences were assigned to the different taxonomic levels using RDP Seqmatch (RDP database, release 11, update 1) (Cole et al., 2009). Relative abundance of each OTU and other taxonomic levels (from phylum to genus) was calculated for each sample in order to take into account different sampling levels across multiple individuals. After trimming, numbers of sequences clustered within each OTU (or other taxonomic levels) were converted to relative abundances. All statistical analyses were performed using R (ade4 package). Principal components analysis (PCA) was computed based on bacterial taxonomic composition and statistically assessed by Monte Carlo rank test. Robustness of each clustering result was assessed using Monte Carlo rank test using R (ade4 package).

Quantitative analysis of commensal bacteria

The terminal ileum was cut longitudinally. After removal of intestinal contents, the tissues were vigorously washed several times in PBS. The supernatants from these washes were used to quantify the bacterial content of the intestinal biofilm. DNA was extracted and quantitative PCR for 16S rDNA was performed on a Bio Rad CFX96 Real Time System with SYBR Green reagent and normalized to total bacterial DNA. The presence of commensal bacteria was conducted using the primers that have been adapted from Bouskra et al. (Bouskra et al., 2008).

Quantitative PCR

Frozen tissue samples were dissociated in lysing/binding buffer of Multi-MACS cDNA kit (Miltenyi) with 0.5% antifoam using the gentleMACS™ Octo Dissociator (Miltenyi). RNA isolation, cDNA synthesis and cDNA purification were performed using the MultiMACS M96thermo Separation Unit following the manufacturer's instructions of the MultiMACS™ cDNA Synthesis Kit (Miltenyi). Real-time quantitative PCR cDNA was performed using SybrGreen (BioRad) and Qiagen primers. Ct values were normalised to the mean Ct obtained with the three housekeeping genes Gapdh, Hsp90 and Hprt in each sample.

RNA sequencing and analysis

C57BL/6J mice were euthanized at 1, 2, 3, 4, 5 and 6 weeks of age, and the terminal ileum was frozen for subsequent RNA extraction. Total RNA was prepared using RNeasy micro kit (Qiagen) according to the manufacturer's instructions. RNA library construction and sequencing reactions were conducted at GENEWIZ Inc (South Plainfield, NJ). Libraries were constructed with TruSeq Stranded mRNA sample Prep Kit (Illumina). In brief, poly(A)⁺ mRNA was enriched with oligo d(T) beads and fragmented. cDNA fragments were synthesized, end repaired and adenylated at 3' ends, followed by universal adaptor ligation, index addition, and library enrichment with limited cycle PCR. Sequencing delivered 40 Millions reads per sample in average. Sequence reads were trimmed to remove possible adapter sequences and reads of poor quality (error rate > 0.05), and sequences reads shorter than 40 nucleotides were discarded. Remaining reads were aligned to the *Mus musculus* GRCm38 reference genome with CLC Genomics Server program v. 9.0.1. Total gene hit counts were measured and RPKM values were calculated. All samples contained high quality data. Gene expression comparison was performed using Qluore. For the generation of heatmaps, the ratio of genes expression SPF/GF was calculated across all time points.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed in GraphPad Prism statistical software (version 7). p values were calculated using the test indicated in the legends to the figures.

Figure 2:

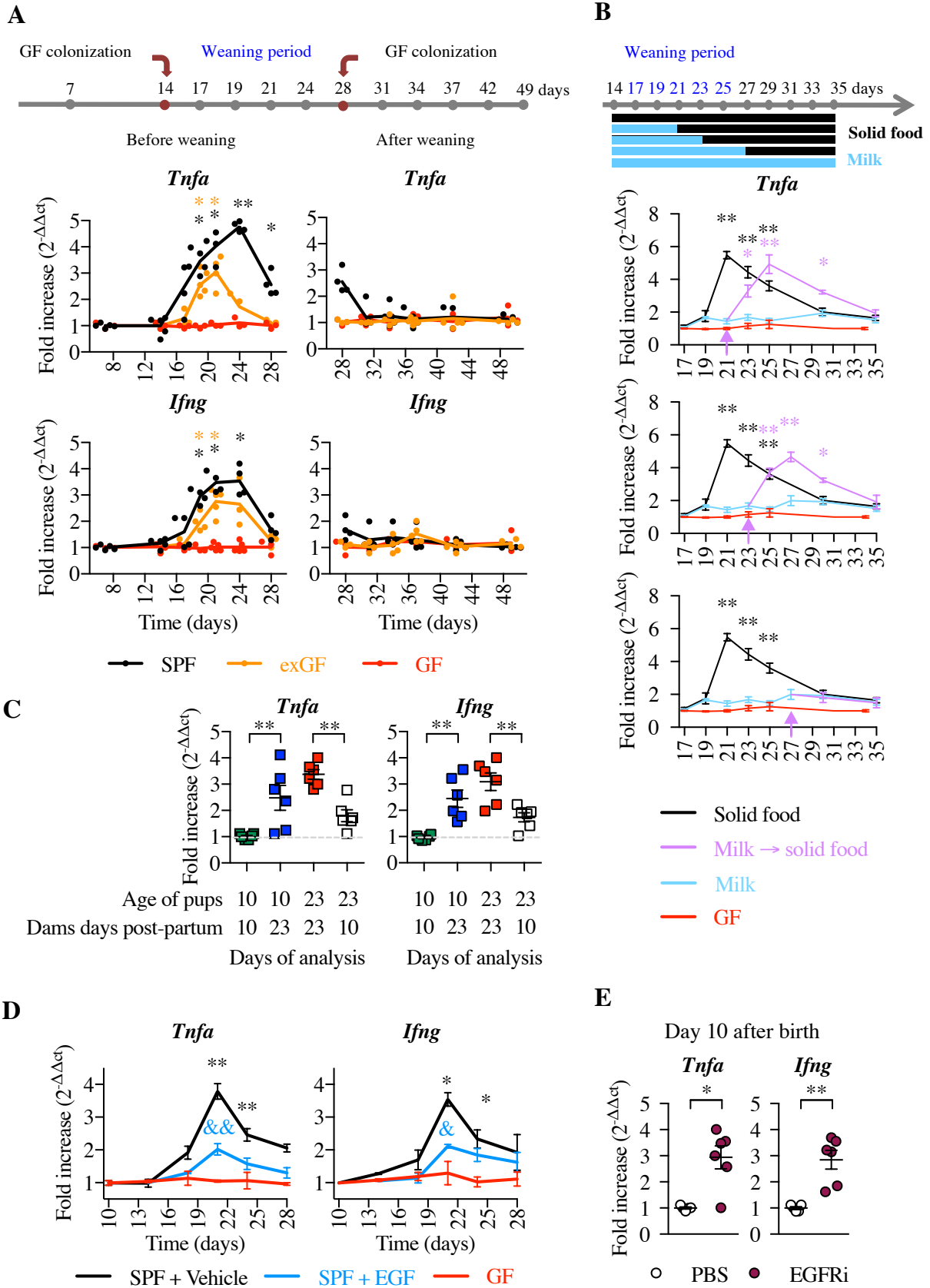


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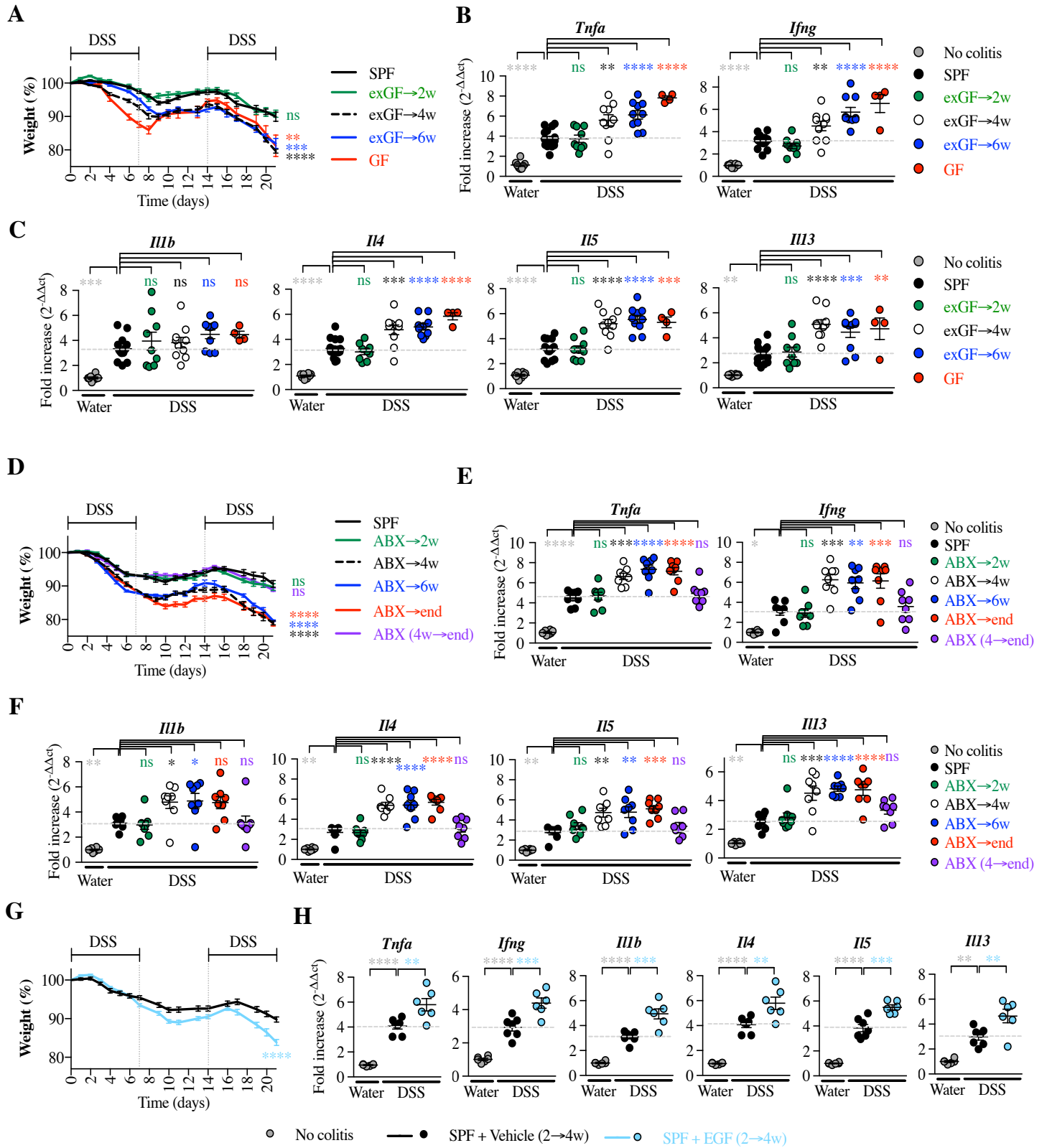


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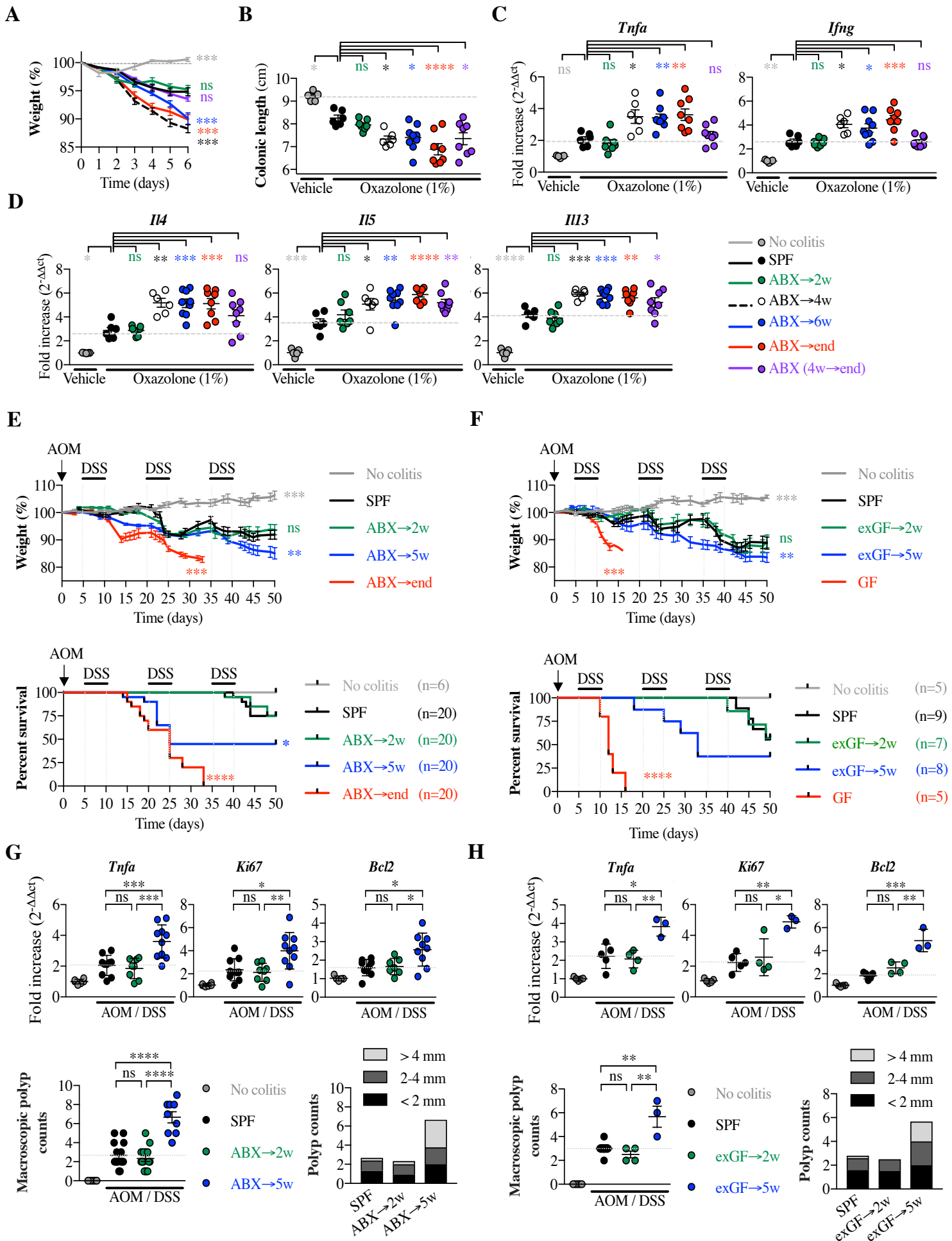


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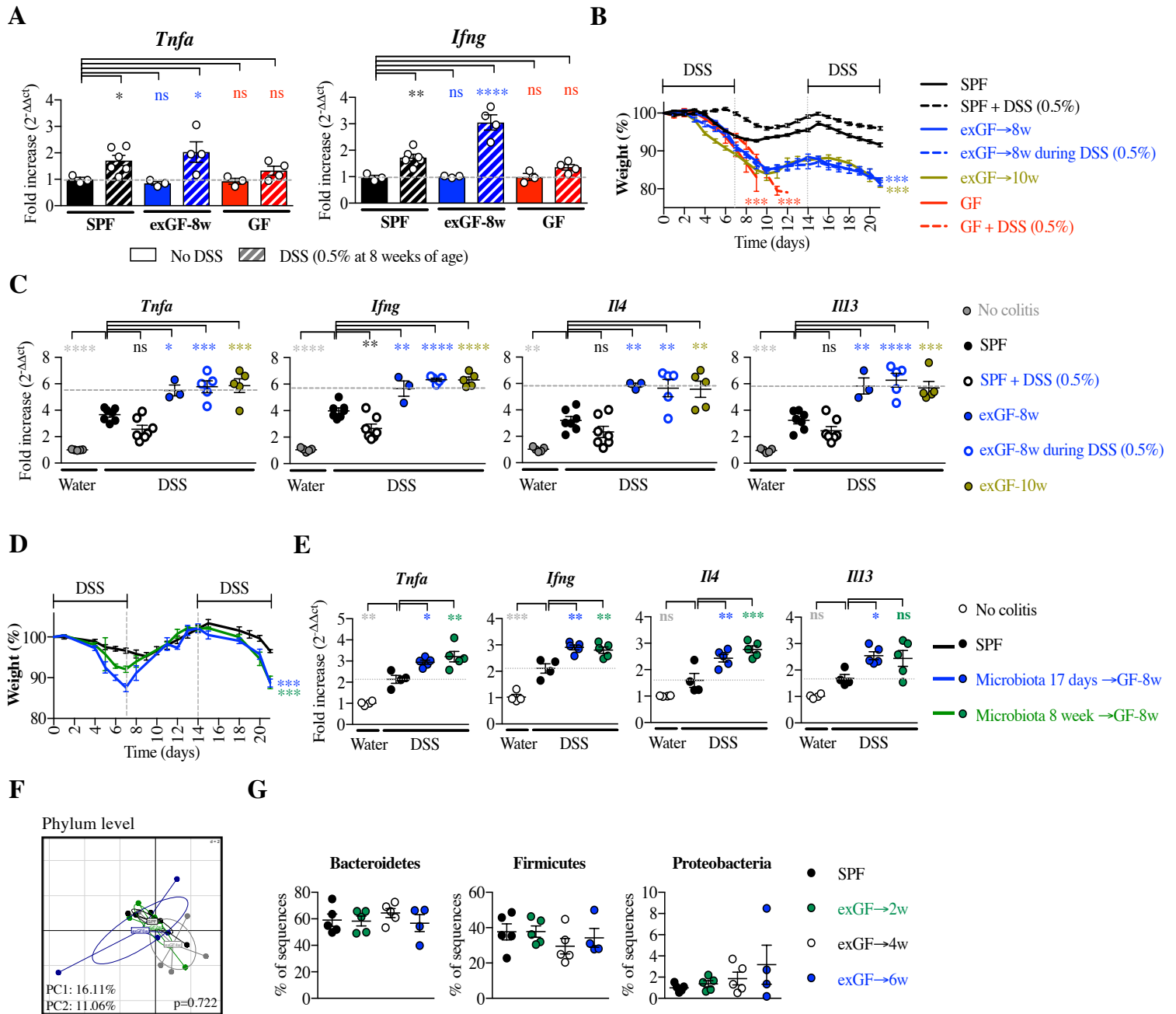


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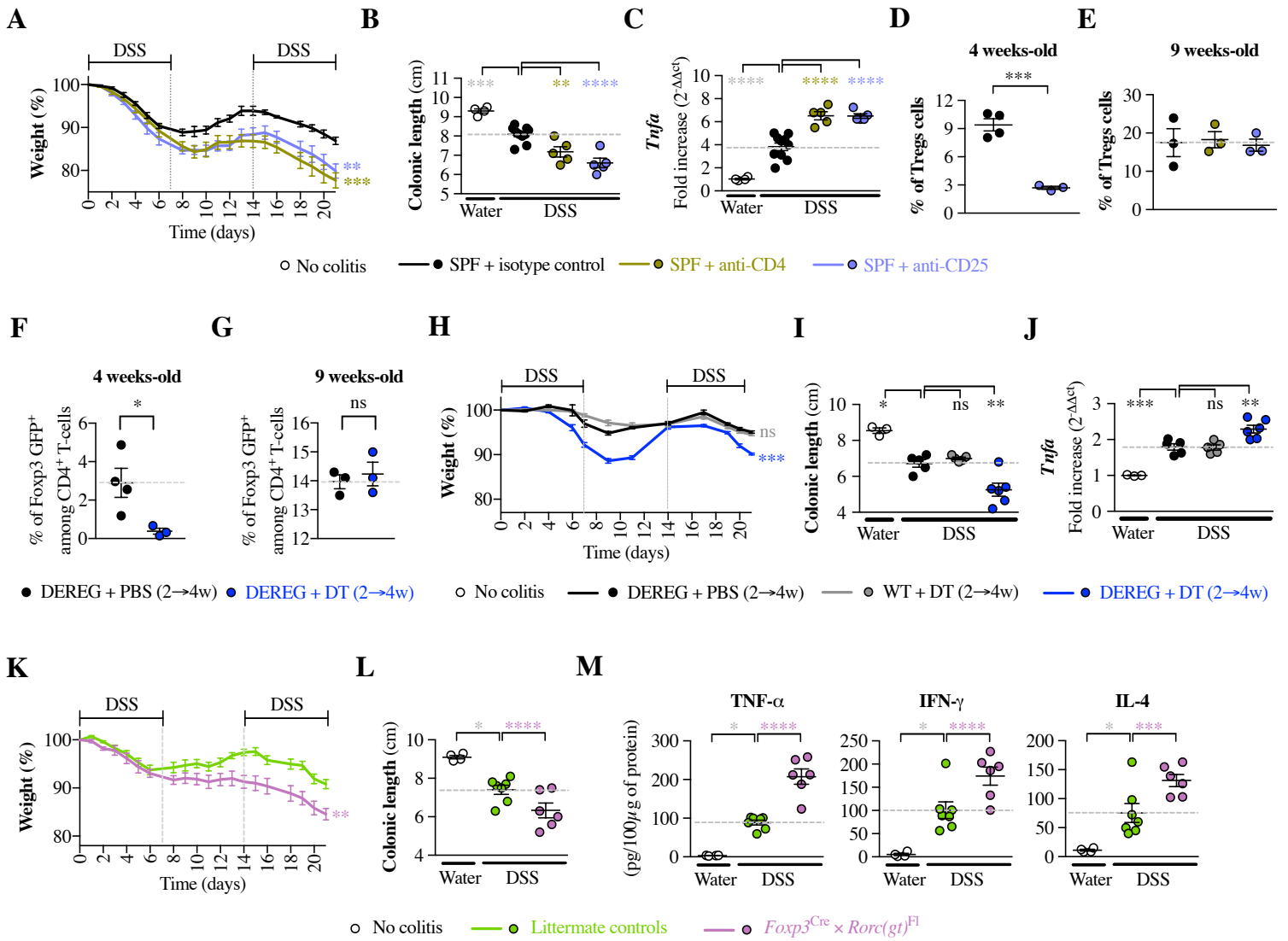
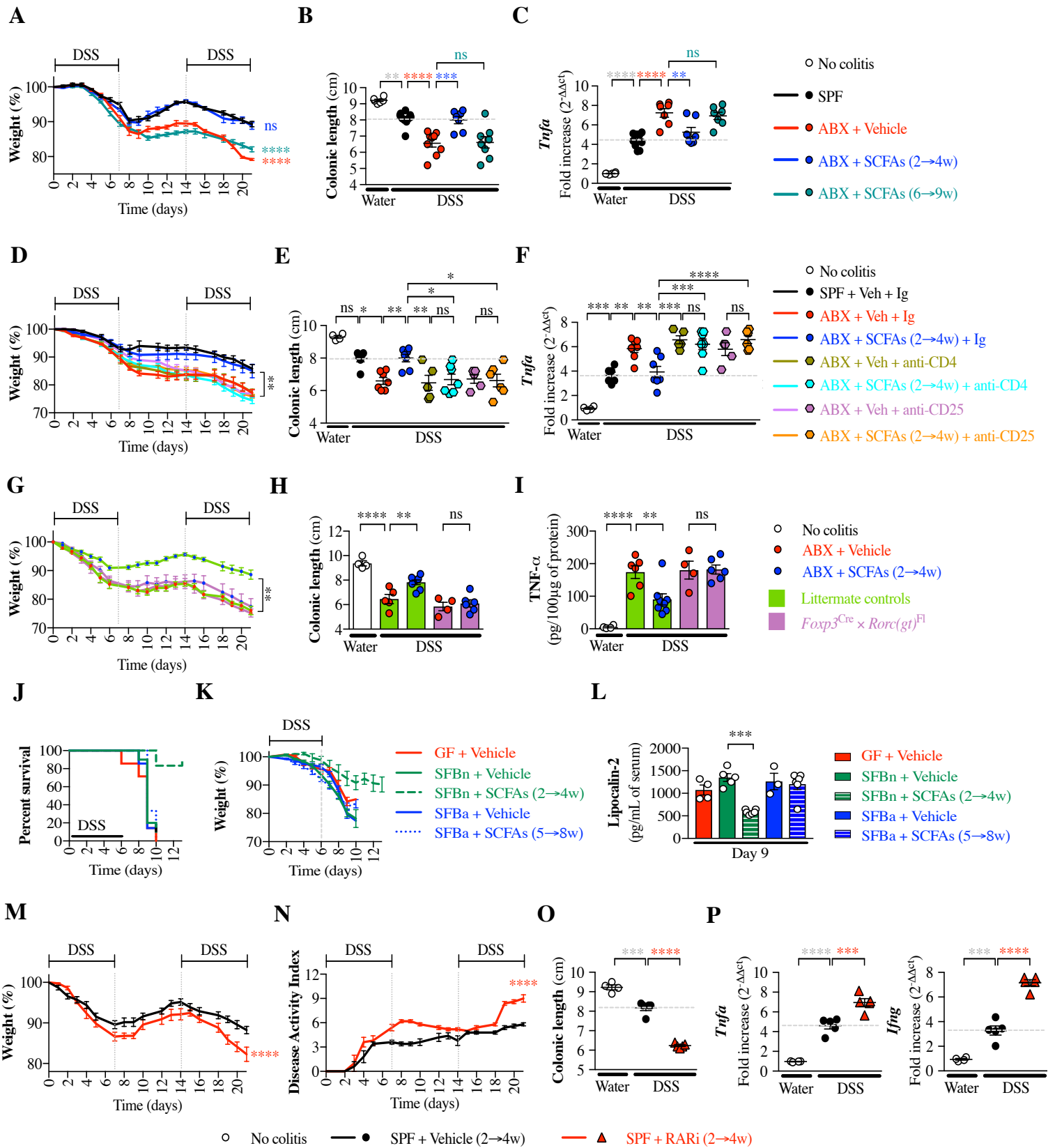


Figure 7:



Time window of opportunity

