**LETTER**

Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice

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Immunoglobulin A (IgA), the major class of antibody secreted by the gut mucosa, is an important contributor to gut barrier function1–3. The repertoire of IgA bound to gut bacteria reflects both T-cell-dependent and -independent pathways4,5, plus glycans present on the antibody’s secretory component6. Human gut bacterial taxa targeted by IgA in the setting of barrier dysfunction are capable of producing intestinal pathology when isolated and transferred to gnotobiotic mouse7,8. A complex reorientation of gut immunity occurs as infants transition from passively acquired IgA present in breast milk to host-derived IgA9–11. How IgA responses co-develop with assembly of the microbiota during this period remains poorly understood. Here, we (1) identify a set of age-discriminatory bacterial taxa whose representations define a program of microbiota assembly and maturation during the first 2 postnatal years that is shared across 40 healthy twin pairs in the USA; (2) describe a pattern of progression of gut mucosal IgA responses to bacterial members of the microbiota that is highly distinctive for family members (twin pairs) during the first several postnatal months then generalizes across pairs in the second year; and (3) assess the effects of zygosity, birth mode, and breast feeding. Age-associated differences in these IgA responses can be recapitulated in young germ-free mice, colonized with faecal microbiota obtained from two twin pairs at 6 and 18 months of age, and fed a sequence of human diets that simulate the transition from milk feeding to complementary foods. Most of these responses were robust to diet, suggesting that ‘intrinsic’ properties of community members play a dominant role in dictating IgA responses. The approach described can be used to define gut mucosal immune development in health and disease states and to help discover ways of repairing or preventing perturbations in this facet of host immunity.

To define the relationship between assembly of the gut community and gut mucosal IgA responses, we collected faecal samples monthly for the first 24–36 months of postnatal life from each member of a birth cohort of 40 twin pairs (21 monozygotic) who lived in the greater metropolitan area of a single city in the USA (St. Louis, Missouri). All twins had healthy growth phenotypes as judged by serial anthropometry; 13 pairs were delivered vaginally, 24 by Caesarean section, and three pairs were discordant for mode of birth; 96% received breast milk, infant formula, or a combination of the two as the predominant food source throughout the first 6 months of postnatal life (Supplementary Tables 1–4).

Gut microbiota assembly was defined following an approach based on our previous studies of healthy Bangladeshi and Malawian infants and children12,13. We generated a random forests (RF)-derived model of microbiota development from a bacterial V4-16S rRNA data set generated from 1,670 faecal samples collected from the 40 twin pairs (20.9 ± 6.2 (mean ± s.d.) samples per individual). The sparse RF-generated model, based on the relative abundances of the 25 most age-discriminatory operational taxonomic units (OTUs), could predict chronological age for members of twin pairs as well as for biologically unrelated individuals (OTUs defined by mapping sequenced reads to a reference database of 16S rRNA sequences; see Methods, Extended Data Figs 1 and 2 and Supplementary Tables 5–8). We then conducted a series of reciprocal tests with the data sets we generated from the three birth cohorts. We applied each sparse model to the population of healthy infants and children from which it was generated as well as to the other two populations. We found that the USA model performed consistently across the three populations (Spearman’s correlation coefficients of 0.73 and 0.78 for the Bangladeshi and Malawian data sets, respectively; see Methods and Supplementary Table 9).

Although previous studies have identified taxa that are shared more commonly between adult monozygotic compared with dizygotic twin pairs14,15, our analysis indicated that none of the 25 age-discriminatory OTUs showed significantly greater concordance in their relative abundances in monozygotic compared with dizygotic twin pairs (Supplementary Table 10). The impact of age, family, milk feeding history, and birth mode on the overall phylogenetic configuration of the microbiota was evaluated with a permutational multivariate analysis of variance (PERMANOVA) and the UniFrac metric. Family had the largest effect (Extended Data Fig. 3), followed by age, and milk feeding (that is, breast milk versus formula) (36%, 11%, and 1%, respectively, when considering only those samples with associated feeding data; P < 0.001 for all variables except birth mode, which did not have a significant effect). A previous study, conducted in the immediate postpartum period, reported that infants born by Caesarean section have a greater representation of skin-derived taxa than those that were vaginally delivered16. A caveat to our study is that we were not able to determine the very early effects of birth mode since the median time point for first faecal sampling was postpartum day 52.

Faecal biospecimens were categorized as obtained from donors who were ‘predominantly formula fed’ or ‘predominantly breast fed’ at the time of sampling (‘predominant’ defined as comprising ≥50% of that individual’s total milk feedings; Extended Data Fig. 4a and Supplementary Table 2). Linear mixed-effects modelling disclosed that milk feeding practice had a significant effect on maturity (P < 0.001, ANOVA with predicted microbiota age as the dependent variable and individual/family/chronological age as nested effects). In a post-hoc analysis, infants receiving >50% of their milk from formula feedings had significantly accelerated development of their microbiota during the first 6–7 months of postnatal life compared with infants receiving most of their milk from breastfeeding (n = 619 and 127 samples, respectively; Mann–Whitney U-test). These differences were no longer statistically significant by 12 months (Extended Data Fig. 4b). This finding can be explained in part by the significantly lower aggregate relative abundance of members of the genus *Bifidobacterium* represented in the RF model in the faecal microbiota of formula-fed infants (Extended Data Fig. 4c, Supplementary Table 11 and ref. 17).
Faecal samples collected during the first postnatal month, and at 3-month intervals thereafter from each member of the 40 twin pairs, were subjected to fluorescence-activated cell sorting (FACS) to characterize the patterns of IgA targeting of bacterial taxa in their developing microbiota (Supplementary Table 12; see Methods for a description of ‘BugFACS’ with anti-human IgA). V4-16S rRNA gene sequencing was performed on three fractions generated from each sample (‘input’, IgA+, and IgA−). The differential representation of a given taxon between the IgA+ and IgA− fractions is expressed in the form of a log-normalized ‘IgA index’ that ranges, in theory, from −1 to 1, with positive and negative values indicating enrichment in the IgA+ and IgA− fraction, respectively (Fig. 1a). IgA indices are not a simple reflection of the relative abundances of organisms in the input fraction (Extended Data Fig. 5a).

We identified 30 OTUs that were significantly enriched in either the IgA+ or IgA− fraction in three or more age bins (Fig. 1a). Seven OTUs exhibited consistently positive IgA indices after the third month of life, including two age-discriminatory members of the sparse RF-generated model of gut microbiota development (Clostridium xiletile OTU 4436046, Bifidobacterium bifidum OTU 365385; Fig. 1a). Seventeen OTUs remained untargeted throughout the first 24 months, including six OTUs in the RF-based model (Fig. 1a). Two OTUs manifested significant differences in their IgA targeting during the first 2 postnatal years: B. longum (OTU C.1) and Escherichia coli (OTU C.3) (Extended Data Fig. 5b and Supplementary Table 13).

We performed an indicator species analysis across all age points to obtain a metric complementary to the IgA index that could describe the strength of partitioning of the 30 OTUs into the IgA+ or IgA− fractions. The results were largely concordant with those obtained from the IgA index-based analysis and provided an additional level of resolution of the temporal patterns and specificity of targeting (Supplementary Table 14 and Extended Data Fig. 6).

IgA indices were highly correlated within twin pairs during the first 21 months of life (Fig. 1b). Indices between unrelated infants were very weakly correlated during the first 6 postnatal months, became increasingly more correlated during the second year of life, and by 24 months co-twins no longer had an IgA response that was significantly more similar to one another than to other unrelated children (Wilcoxon signed-rank test; Fig. 1b). As the effects of family membership diminished, variation of the IgA index for a given taxon across the population of twins also diminished (Extended Data Fig. 5c). The similarity in the IgA profiles between mothers sampled during the first 12 postpartum months (39 mothers; 3.0 ± 1.0 (mean ± s.d.) samples per mother) and children at 24 months of life supports the notion that development of a child’s gut mucosal IgA responses reaches a state of maturation that resembles that of adults by this age (Supplementary Tables 15 and 16 and Fig. 1c).

On the basis of Pearson’s correlation distance, we determined that age and family membership explained the most variance in IgA indices (25% and 19%, respectively), while zygosity and mode of delivery had small but statistically significant effects (0.6% and 0.5%, respectively; PERMANOVA with 999 permutations). Breastfeeding explained 5% of the variance in the model (P < 0.001 for breast milk versus formula feeding as well as for age and family). Intriguingly, IgA targeting of two taxa, E. coli (OTU C.3) and Ruminococcus gravis (OTU C.4), varied between children who were predominantly breastfed and those who were predominantly formula-fed, with breastfed children exhibiting significantly higher IgA targeting of E. coli at 3 months of age and significantly lower IgA targeting of R. gravis during the latter half of the first year (Extended Data Fig. 7).

To quantify the stage of development of gut mucosal IgA responses, we randomly selected 20 unrelated children from the healthy twin cohort and generated an RF model based on the IgA indices to the 30 taxa identified in Fig. 1a. The model was then applied to unrelated children represented in the remaining 20 twin pairs (‘test set’, n = 40). Even though the data set was smaller than the one used to generate the RF model of gut microbiota development, the effort produced a model of development of IgA responses that correlated with donor chronological age (Spearman’s correlation for training set and test set, 0.97, and 0.72, respectively; Supplementary Tables 13 and 17).

Faecal samples from two twin pairs whose pattern of gut microbiota development was well described by the RF-derived model and whose IgA responses exemplified those of the larger population were selected for transplantation into germ-free mice (pairs 4 and 40 in Supplementary Table 13). Both twin pairs were predominantly formula-fed throughout their first postnatal year (Supplementary Table 2). Faecal samples, collected from each of these four individuals when they were 6 and 18 months old, were introduced into separate groups of male 5-week-old C57BL/6J germ-free mice (n = 4 or 5 mice per donor sample; eight treatment groups). Two days before gavage, all mice were switched from a standard chow diet low in fat and rich in plant polysaccharides to a sterilized human infant formula diet (see Methods). After gavage, animals were maintained on this diet for 14 days and then switched to a diet constructed on the basis of a survey of fruits and vegetables most commonly consumed by infants transitioning to complementary food. This diet consisted of isocaloric amounts of the powdered infant formula diet and a mixture of sweet potatoes, green beans, bananas, and apples. After 10 days, animals were returned to the infant formula diet for another 10 days. Faecal samples were obtained from recipient mice at frequent intervals throughout all diet phases and subjected to 16S rRNA sequencing and/or to BugFACS (Fig. 1d, e).

Indicator species analysis revealed that the abundances of 15 of the top 60 taxa in the RF-derived model of gut microbiota maturation varied significantly in the context of one or the other diets (false discovery rate (FDR)-corrected P < 0.05 and indicator value > 0.5; Supplementary Tables 18 and 19). Most of these taxa responded in the same direction (increased or decreased in abundance) to the different diets, independent of the microbiota donor or donor age (Extended Data Fig. 8). For example, the age-discriminatory OTU C.1 (B. longum) and OTU 4439469 (a member of the Ruminococcaceae) have highest mean relative abundances during the first 6 months of postnatal life in members of the twin cohort (Extended Data Fig. 2c); these taxa also exhibited significantly greater relative abundance in the faecal microbiota of recipient gnotobiotic mice when they were consuming the infant formula diet. In contrast, Anaerostipes caccae (OTU 259772), which peaks in abundance during the latter half of the first postnatal year (the period corresponding to introduction of complementary foods in our twin study; Extended Data Fig. 2c and Extended Data Fig. 4a), was significantly higher in its abundance during the ‘formula plus fruits and vegetables’ diet phase (Extended Data Fig. 8).

To determine whether age-associated differences in IgA responses to components of the donors’ microbiota could be recapitulated in gnotobiotic mice, we subjected their faecal samples collected at 7, 14, 24, and 34 days after gavage to BugFACS (Supplementary Table 20). IgA responses in mice broadly mirrored those of the human donor population; taxa that were consistently not targeted across members of the twin cohort during the first 2 years of postnatal life (for example, Clostridium clostridioforme OTU C.26 and Clostridium bolteae OTU 4469576) were generally not targeted in mice colonized with the 6- and 18-month microbiota samples from the two twin pairs, while bacteria targeted in mice belonged to the set of taxa that were consistently IgA targeted in infants/children from postnatal months 6–24 (for example, Ruminococcus torques (OTU C.6) and Akkermansia muciniphila (OTU 4306262)) (Fig. 1d and Supplementary Table 21).

IgA-targeting of five OTUs varied significantly with the diet oscillation, whether judged by a comparison of the first and second or second and third diet phases (FDR-corrected repeated-measures ANOVA): they included OTUs whose IgA targeting increased during the fruits and vegetables phase (4306262 (A. muciniphila) and C.39 (Ruminococcus sp. ce2)), and those whose targeting decreased (OTUs C.4 (R. gravis), 4469576 (C. bolteae), 4453304 (other Clostridiales)) (Supplementary Table 22). IgA responses were most similar in mice.
harbouring a given donor microbiota, and were more similar within members of a twin pair than between unrelated children (Extended Data Fig. 9).

Applying our RF-derived model of maturation of human gut mucosal IgA responses to the mouse BugFACS data set showed that animals recapitulated distinct age-associated differences in mucosal IgA responses to the (transplanted) human microbiota; that is, for both twin pairs, the state of maturation of the IgA response in mice was significantly greater when animals were colonized with the 18-month compared with the 6-month donors’ communities. Remarkably, this significant difference in age-associated responses for a given co-twin or twin pair microbiota was evident in both diet contexts (Fig. 1F and Supplementary Table 21). We concluded that IgA responses to members of the 6-month-old gut microbiota were shared across the two twin pairs and robust to the transition to complementary foods. The fact that distinctive responses to the 18-month compared with the 6-month microbiota were identified in recipient mice, even in the context of a milk (formula) diet, supports the notion that ‘intrinsic’ properties of community members (for example, properties not clearly related to taxonomy or obviously affected by community composition) play a dominant role in dictating the gut mucosal IgA targeting response.

Our findings point to several directions for future investigation. The stability of the IgA molecule, the ease and safety of obtaining faecal samples, and the ability to sort members of a faecal microbiota sample into IgA-enriched versus non-enriched fractions provide a way to non-invasively quantify states of development of the gut mucosal immune system as a function of different host and environmental factors. BugFACS offers an opportunity to identify previously unappreciated IgA deficiencies presenting not as a lack of, or reduction in, the amount of total IgA in the gut lumen, but rather as aberrant patterns of IgA targeting. The effects of such deficiencies would need to be examined with the understanding that barrier function can be affected...
by multiple factors besides IgA, including for example mucin25, and less well understood elements21. BugFACS also provides a way to explore how IgA targeting of bacterial taxa varies as a function of their proximity to the intestinal epithelium and their location along the length of the gut. The importance of the small intestine as a source of the T-cell-independent IgA response to members of the microbiota was highlighted in a recent study4.

In principle, deviations from the pattern of convergence of IgA responses observed in the present study could occur in scenarios where colonization is abnormal, resulting in pathological immune responses in anatomically distant locations, such as that observed in asthma22 or various autoimmune/immunoinflammatory disorders. One obvious next step is to assess the generalizability of the shared pattern observed in this study by characterizing healthy members of birth cohorts representing different geographical areas, distinctive cultural and dietary traditions, and living environments with varying degrees of sanitation. Gut microbiota development is impaired in children with undernutrition12. Given that undernutrition is associated with impaired gut barrier function and responses to particular vaccines23, a comparison of the development of gut mucosal IgA responses to members of the microbiota in healthy and undernourished members of birth cohorts could provide a metric for disease classification, assessment of the impact of enteropathogen infection/burden, and a means of assessing the efficacy of current or new therapeutic interventions, including approaches for oral vaccination.

The ability to re-enact and recapitulate features of the development of gut mucosal IgA responses to human donor gut microbial communities in wild-type or genetically manipulated gnotobiotic mice should help delineate the mechanisms that control the temporal evolution and specificity of IgA responses to members of the gut community, the effects of the IgA response on targeted microbes and other members of the microbiota, as well as the impact on host biology. As such, these models could be used to identify new strategies for deliberately manipulating mucosal barrier/immune function, including food-based and/or microbial interventions.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.I.G. (jgordon@wustl.edu).

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Author Information 16S rRNA sequences in raw format before post-processing and data analysis have been deposited at the European Nucleotide Archive under project PRJEB11697. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.I.G. (jgordon@wustl.edu).
METHODS

Human studies. Protocols used for recruitment of participants, obtaining informed consent, collecting and de-identifying faecal samples, and acquiring and de-identifying clinical metadata were all approved by the Human Research Protection Office of Washington University School of Medicine. A total of 40 twin pairs were included in this study; this number was not determined by a power calculation.

All breast milk provided was from the mothers of the twins themselves and was not pasteurized. Breast milk was given from the breast directly or from bottles after being expressed by the mother. Expressed milk was given immediately or stored temporarily in home freezers; in the latter case, mothers were instructed to thaw their milk in warm water.

Determination of zygosity. Zygosity testing of same-gender twins was performed on residual blood samples obtained for clinical care, or samples obtained at the time of mandatory Missouri-state metabolic testing. Short tandem repeat polymorphic DNA markers were amplified from blood DNA by PCR, labelled with fluorescent markers, and separated by capillary electrophoresis to distinguish different alleles at each of ten different loci (D3S1358, vWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820). For each sample, 50,000 cytometer ‘events’ were recovered from the ‘Input’, and ‘IgA’ and ‘IgA’− gates (for details of the sorting protocol and gating strategies, see ref. 8). Additionally, samples of shear fluid were collected immediately before and after sorting to allow assessment of any potential contaminants in fluid lines. Sorted fractions and control shear fluid samples were frozen and stored at −20 °C. Each BugFACS-sorted fraction was subjected to V4-16S rRNA gene PCR in triplicate 20 μl reactions. Each reaction contained 2 μl of 10X HiFi PCR Buffer (Invitrogen), 0.8 μl of 50 mM magnesium sulfate (Invitrogen), 0.4 μl of dNTP mix (Invitrogen), 0.16 μl of Platinum Taq (Invitrogen), 1 μl of a 5 μM stock of forward PCR primer, 1 μl of a 5 μM barcoded reverse PCR primer, 2.5 μl of BugFACS sorted cells, and 1.2 μl of water. A negative control reaction with no sorted cells was included for each reaction. The following PCR conditions were used: 95 °C for 10 min followed by 31 cycles of 95 °C for 30 s, 53 °C for 30 s, and 68 °C for 45 s, followed by 68 °C for 2 min. Triplicate reactions were pooled and subjected to 1% agarose gel electrophoresis to verify the presence of a PCR product (these gels also contained negative control reactions). If any of the three sorted fractions from a given sample failed to amplify successfully, PCRs were repeated for 34 cycles for all three fractions under the same temperature cycling conditions. PCR-amplified fractions were pooled in equal proportion. Although ampiclon bands were not visible for shear fluid controls, a set volume of these reactions was also included in the sequencing pool. Pooled amplicons were purified with magnetic beads (AMPure XP, Agencourt) and subjected to multiplex sequencing (paired-end 250 nucleotide reads) on a MiSeq instrument as above.

After OTU picking, but before abundance filtering, shear fluid-containing OTUs were identified as sequences that constituted >1% of the reads in both the pre- and post-sort shear fluid samples for a given day. Contaminants that were identified on more than 2 days were removed from the OTU table. If multiple genera within a family-level taxon were identified as shear contaminants, the entire family was removed from the OTU table. This list included the following families: Burkholderiaceae, Xanthomonadaceae, Comamonadaceae, Brucellaceae, Pseudomonadaceae, Xanthobacteraceae, and Alcaligenaceae. Additionally, OTUs belonging to these families accounted for less than 0.05% of all sequences in the twin pair, maternal, and mouse faecal samples, and for less than 2% of all sequences in samples subjected to BugFACS. IgA indices were subsequently calculated for a given taxon in a given sample if that taxon comprised >0.5% of the 16S rRNA reads in either the IgA+ or IgA− fraction.

RF modelling. RF modelling of gut microbiota development was performed with the ‘randomForest’ package23 in R. Input data consisted of OTU data rarefied to a depth of 5,000 V4-16S rRNA reads per faecal sample. Feature importance scores for each OTU in the data set were calculated by randomly selecting one co-twin from half of the twin pairs (n = 20 individuals). An RF model was generated from this subset of data. Randomization and this process of model construction were performed 100 times (100 trees per model). Feature importance scores were extracted from each model, averaged across the 100 models, and used to rank the OTUs from highest to lowest feature importance.

To estimate the number of OTUs needed to build a sparse model, a new set of RF models was generated by selecting one co-twin from half of the twin pairs in the cohort as above, and evaluating the performance of the model (Spearman’s ρ and the adjusted r² of a linear model as metrics) when applied to (1) the individuals used to generate the model, (2) their co-twins, and (3) all unrelated faecal samples (training−‘co-twin’ and ‘test’ sets, respectively). A series of models was built with increasing numbers of OTUs, starting with the OTU assigned the highest feature importance score, and sequentially adding OTUs in decreasing order of feature importance. For each model of a different size, ten randomizations were performed; performance of the model was averaged across the independent replicates to generate standard error measurements. The subset of 25 OTUs with highest rank order of feature importance scores was used to create a sparse model. This sparse model, generated from samples collected during the first 36 months of postnatal life, was applied to 16S rRNA data sets generated from faecal samples collected between 1 and 24 months of age to predict chronological age in members of the study cohort. The Spearman’s ρ correlation to age was derived from IgA index data for the 30 OTUs shown in Fig. 1a. If a taxon was not detected in either the IgA+ or IgA− fraction, it was given a value of 0 before model construction. This model was applied to the ‘training’, ‘co-twin’, and ‘test’ sets.

OTUs were reassigned to incorporate data sets from all three countries (USA, Bangladesh, and Malawi), resulting in a second set of consolidated OTUs (see Supplementary Table 9). Feature importance scores were calculated by iteratively regressing each country’s training set of samples 100 times against chronological age (100 trees per model). OTUs were ranked by the median values of their feature importance scores across the 100 models. The 25 most age-discriminatory OTUs were used to generate each respective country’s sparse RF model. Each model was used to predict the microbiota ages of members of that country’s corresponding test set, as well as the microbiota ages of all members of the healthy cohorts from each of the other two countries. Spearman’s correlation coefficients were generated
by building each sparse RF model ten times, correlating predicted microbiota ages with chronological ages, and averaging the coefficients.

**Animal studies.** All experiments involving mice were performed according to protocols that were in compliance with ethical regulations and approved by the Washington University Animal Studies Committee. No inclusion or exclusion criteria were established; all animals studied were included in our analyses.

**Gnotobiotic mouse husbandry.** Germ-free 5-week-old male C57BL/6J mice (*Mus musculus*) were maintained on a strict 12 h light cycle (lights on at 6:00) in flexible plastic gnotobiotic isolators (Class Biologically Clean). Mice were weaned onto an autoclaved, standard mouse chow diet low in fat and rich in plant polysaccharides (B&K Universal; diet 7378000). Two days before introduction of human donor faecal samples by gavage, 5-week-old animals were switched to the human infant formula diet.

**Diets.** The infant formula diet consisted of a mixture of Similac 'Sensitive with Iron' infant formula and unflavoured whey protein powder (GNC) mixed at a ratio of 1:1 (w/w). This powdered diet was reconstituted in the gnotobiotic isolator on a daily basis with sterile water. The infant formula plus fruit and vegetable diet was based on a survey of the fruits and vegetables most commonly consumed by infants transitioning to complementary foods39, and consisted of isocaloric amounts of the powdered infant formula diet and a mixture of 1:1:1:1 ratio (by mass) of sweet potatoes, green beans, bananas, and apples (Gerber 1st Foods). Formula was irradiated as a powder. Fruits and vegetables were irradiated in their original plastic containers before the start of the experiment (25–30 Gy; Steris Isomedix) and mixed with the irradiated formula powder. When mice were consuming infant formula diet alone, fresh food was prepared daily within the gnotobiotic isolator and presented to animals in sterile plastic trays that were changed daily. When animals were given the mixture of formula and fruits/vegetables, food was prepared every other day, and new aliquots given to animals in fresh trays daily. Bedding was changed with each phase of the diet oscillation; within a given diet phase, bedding was changed every 2–3 days.

**Microbiota transplants.** A given pulverized frozen human faecal sample (353 ± 184 mg; mean ± s.d.) was transferred to an anaerobic Coy chamber (atmosphere 75% N₂, 20% CO₂, 5% H₂) in a 2 ml Axygen screw topped tube. The tube was then opened and its contents were transferred to a 50 ml conical shaped polypropylene tube (Falcon). The faecal material was suspended in 10 ml of sterile PBS supplemented with 0.1% l-cysteine (Sigma) by vortexing with sterile glass beads (2 mm in diameter). The suspension was passed through a nylon 100 μm mesh filter (BD) and the filtrate was mixed with an equal volume of 30% glycerol in PBS/0.1% cysteine. Aliquots (1.2 ml) of this suspension were placed amber glass vials, each of which was sealed with a crimp top, and frozen at ~80 °C. Tubes were thawed, and transferred into gnotobiotic isolators (with surface sterilization achieved by treatment with Cldix). Aliquots (200 μl) were then introduced into each germ-free mouse in a given experimental group by oral gavage. A total of 38 animals were used for this study (n = 4 or 5 mice per donor microbiota). This size of each treatment group was not based on a formal power calculation but was informed by our previous work described in ref. 8. There was no randomization of mice for this study; male C57BL/6J animals in each group were age- and weight-matched before gavage. Investigators were not blinded to the donor microbiota.

**BugFACS of mouse faecal samples.** The protocol used was similar to that described above for human faecal samples with several modifications. Faecal pellets were resuspended in PBS, vortexed, and a volume equivalent to 5 mg of faecal material was passed through a nylon 70 μm mesh filter. After washing with PBS, cells were incubated for 30 min on ice in the dark with a polyclonal goat antibody directed against mouse IgA conjugated to DyLight 650 (Abcam; catalogue number ab97014; diluted 1/50 in PBS/0.5% (w/v) bovine serum albumin). On each day that BugFACS was performed, a positive control of pooled material from all mouse faecal samples analysed on that day and stained with anti-mouse IgA antibody was used to verify staining, while a negative control of the same pooled faecal material stained with the anti-human IgA antibody (conjugated to DyLight 650, see above) was used as an isotype control.

**Statistics.** Statistical analyses, RF modelling, generation of plots, OTU consolidation, and OTU table rarefaction were performed in the R programming environment (R version 3.1.1) or Prism 6.0. For presentations of data in which group means are compared, confidence in mean values is displayed as the s.e.m.

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Extended Data Figure 1 | Method used for OTU consolidation. a, OTU consolidation was performed to limit pseudo-duplication of taxa. ‘Counts’ on the y axis refer to the number of OTU–OTU correlations falling within a given range of Spearman’s correlation values shown on the x axis (n = 341,640 OTU–OTU comparisons; see Methods for details). b, A subset of the matrix used to derive the distribution shown in a illustrates how OTUs within a single family-level taxon are consolidated. In this example, three clusters composed of OTUs with Spearman’s correlation coefficients of >0.7 are identified and the abundances of their constituent OTUs are summed. Each OTU cluster is assigned an identifier number with the prefix ‘C.’ and given a consensus taxonomic assignment (see Supplementary Table 5). Note that the OTUs used to generate a given ‘consolidated OTU’ shared 99.3 ± 0.4% (mean ± s.d.) nucleotide sequence identity in their V4-16S rRNA nucleotide sequences.
Extended Data Figure 2 | Modelling development of the gut microbiota during the first 24 months of life in healthy twins. a, To estimate the number of OTUs needed to maximize predictive accuracy, OTUs were iteratively added to a series of RF models, starting with the OTU with the highest feature importance score and adding additional OTUs in order of decreasing feature importance. To evaluate performance of the model, members of the 40 twin cohort were randomly assigned to ‘training’, ‘co-twin of training’, and ‘test’ sets (red, green, and blue, respectively) ten times, and the Spearman’s correlation coefficient and adjusted $r^2$ of a linear model were calculated for a given model size ($n = 10$ models for each data point, mean ± s.e.m. values are plotted). The dashed vertical line indicates performance of a 25 OTU model across the three different sets. b, Predicted age was calculated for all faecal microbiota samples with a sparse 25 OTU RF-generated model. Chronological versus model-predicted age is plotted for each of the three data subsets ($n = 1,477$ faecal samples). The inset shows mean ± s.d. values for predicted microbiota age of samples in each monthly age bin. c, Heatmap of mean abundances over the first 24 months of life for the 25 OTUs used to generate the sparse model. Taxa are normalized by row, with hierarchical clustering (complete linkage; $n = 1,477$ faecal samples).
Extended Data Figure 3 | Similarity of faecal microbiota composition within and between twin pairs. Similarity in composition of the faecal microbiota within and between twin pairs was analysed with unweighted UniFrac distance calculated before OTU consolidation. Statistical significance was evaluated with the paired Wilcoxon test for twin–twin versus twin–unrelated comparisons. Mean values + s.e.m. are plotted (n = 205 paired comparisons). ***P < 0.001. The results indicate that the overall phylogenetic composition of the faecal microbiota is more similar in infants/children sharing a common living environment and genetic background than between unrelated individuals; this is apparent as early as the first month of life and does not change significantly over the ensuing 23 months.
Extended Data Figure 4 | Feeding status and microbiota composition during the first year of life. a, The proportion of all feedings on the day of faecal sampling that consisted of either formula or breast milk (n = 746 observations). b, Microbiota age, defined by the sparse RF-derived model, compared for participants that were predominantly breast fed (≥50% of milk feeding) or predominantly formula fed across different age bins. Mean values ± s.e.m. are plotted (n = 681 faecal samples). *P < 0.05; **P < 0.01; ***P < 0.001 (Mann–Whitney U-test). c, Aggregate percentage relative abundance of age-discriminatory bifidobacteria included in the sparse RF-derived model; differences in their representation in the faecal microbiota as a function of breast or formula feeding evaluated in each age bin are shown. Horizontal lines within each column represent the median values; the horizontal dashed line represents the lower limit of detection. *P < 0.05; **P < 0.01; ***P < 0.001 (Mann–Whitney U-test comparing samples obtained from breast versus formula fed individuals; n = 681 faecal samples).
Extended Data Figure 5 | Further characterization of IgA responses to members of the microbiota in the USA twin cohort. a. Evidence that IgA indices are independent of relative abundance. IgA indices for all OTUs are plotted against their relative abundance in the 'input fraction' for all infant/child, maternal, and gnotobiotic mouse faecal samples analysed by BugFACS (n = 22,713 comparisons). b, Mean IgA indices ± s.e.m. for two OTUs whose IgA targeting varied significantly with age across all 80 individuals in the twin birth cohort (FDR-corrected Kruskal–Wallis test, P < 0.05). c, Variance of IgA indices as a function of age. A total of 26 OTUs were detected in at least two individuals in all of the age bins surveyed. The variance in their IgA indices was then calculated and the non-parametric repeated-measures Friedman test was used to test for statistical significance (P < 0.0001). Mean values + s.e.m. are plotted.
Extended Data Figure 6 | Specificity of targeting and temporal variation in the prevalence of IgA-targeted or non-targeted taxa. Specificity values from the indicator species analysis were calculated across all time points for the 30 OTUs identified as consistently IgA-targeted or non-targeted in Fig. 1a. Prevalence of the taxa, defined as detection in either the IgA+ or IgA− fraction, was plotted against the percentage of samples in which a given taxon had a positive or negative IgA index (n = 4,186 IgA index values analysed). The results reveal a group of OTUs that increased in prevalence over the course of the first 2 years of postnatal life and had very high ‘specificity’ for either the IgA+ or IgA− fraction (that is, across the population of faecal samples, most 16S rRNA reads for a given OTU were detected in one of the two fractions). This group included R. torques OTU C.6 and C. nexile OTU 4436046 that were IgA targeted in the majority of twins (when they are detectable in their microbiota), as well as Ruminococcus sp. ce2 OTU C.39 which was IgA− in most of the children. A second group of OTUs became more prevalent with age but members had a much weaker, albeit statistically significant, association with one or the other sorted fraction (for example, R. gnavus OTU C.4 and B. vulgatus OTU C.15). A third group of OTUs were highly specific for a given sorted fraction but were only detected in a minority (≤20%) of children. This last group included two strongly IgA-targeted OTUs assigned to A. muciniphila (OTU 588471 and OTU 4306262). Intriguingly, these two OTUs co-occurred just once among the 176 BugFACS samples in which A. muciniphila was detected (P < 0.0001, χ² test).
Extended Data Figure 7 | Effects of diet on gut mucosal IgA responses to members of the microbiota. The analysis was constrained to those faecal samples where a diet history had been collected within 10 days of procuring the specimens (n = 276). After FDR correction with the Benjamini–Hochberg procedure, IgA targeting of 2 of the 30 taxa identified in Fig. 1a varied significantly as a function of breast versus formula feeding. Each circle represents results from a given faecal sample. Samples are colour-coded on the basis of the type of milk diet being consumed by the donor at the time of faecal sampling. Horizontal lines in each column represent mean values. *P < 0.05 (Mann–Whitney U-test of the differences between breast and formula fed).
Extended Data Figure 8 | Diet-dependent changes in composition of the faecal microbiota of gnotobiotic mice. Indicator species analysis was used to identify taxa from the RF-derived model of gut microbiota maturation whose abundances varied consistently by diet treatments (n = 9,999 permutations with ‘mouse’ as the grouping variable). The top 60 ranked OTUs in the model (on the basis of their feature importance scores) were included in the analysis; those OTUs with statistically significant diet-dependent partitioning (P < 0.05) after FDR correction and with an indicator value > 0.5 are shown, ranked from highest to lowest indicator value for infant formula-discriminatory (upper portion of figure) and ‘infant formula plus fruits and vegetables’-discriminatory (lower portion of figure) (see Supplementary Table 19 for results of the indicator species analysis). Mean values for relative abundances in the faecal microbiota at each time point are plotted ± s.d.
Extended Data Figure 9 | Similarity in IgA responses as a function of microbiota donor, twin pair, and time after transplantation. Pearson’s correlation coefficients were calculated with IgA index data from all faecal samples collected from gnotobiotic mice that had been analysed by BugFACS. Mean values ± s.d. are shown for the indicated comparisons. ****P < 0.0001; **P < 0.01; *P < 0.05 (Kruskal–Wallis test with Dunn’s correction for multiple comparisons; n = 5,029 total comparisons).