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Transient inflammatory-like state and microbial dysbiosis are pivotal in establishment of mucosal homeostasis during colonization of germfree mice

S. El Aidy, M. Derrien, R. Aardema, G. Hooiveld, S.E. Richards, A. Dane, J. Dekker, R. Vreeken, F. Levenez, J. Doré, E.G. Zoetendal, P. Van Baarlen and M. Kleerebezem

Supplementary methods

Correlation between the microbial profiling of gut contents and the transcriptome data

To validate the association of microbial dysbiosis with the transient augmented pro-inflammatory response, correlation analysis of MITChip and transcriptome datasets was performed as previously described (Swann *et al*., 2011). In brief, a correlation matrix between the MITChip (genera-like) data and the transcriptome was computed using the absolute expression values. To reduce the probability of type I errors (false positives) for multiple testing, the Benjamini-Yekutieli method (Benjamini and Yekutieli, 2001) was employed and the data were corrected for multiple testing using (False discovery rate) (FDR) method. FDR was set at <0.05% for expected proportion of false positive correlations in the multiple comparison testing. Detailed explanation of the methods used to perform correlation analysis can be obtained from the authors upon request.

Plasma measurements of amine metabolites

Quantitative analysis of primary and secondary amines was performed with an aliquot of 5 µL plasma using LC-MS/MS after derivatisation using the AccQ·Tag Ultra Derivatisation Kit (Waters, UK) as described by Noga *et al.* (2012). Briefly, this method involves the reaction of primary and secondary amines present in protein precipitated mouse plasma with the AccQ-Tag Reagent, to form stable urea analogues. The samples were injected onto the AccQ-Tag column (Waters, UK) and 59 primary and secondary amines were detected using the XevoQqQ MS (Waters, UK) in MRM mode. A binary gradient of water – eluent A (10:1, v/v) (AccQ·Tag eluent A, Waters) and 100% eluent B (AccQ·Tag eluent B, Waters), was used. Elution of the amines was achieved by ramping the percentage of eluent B from 0.1 to 90% in approx. 9.5 minutes. The flow rate was 0.7 ml/min. An ACQUITY ultra pressure liquid chromatography (UPLC) system (Waters, UK) was hyphenated to a XevoQqQ mass spectrometer, which was operated in positive-ion mode. The desolvation gas flow was 1000 l h-1, and the desolvation temperature was set at 450 °C. The cone gas flow was set at 50 l/h and the source temperature was 140°C. The cone voltage was set at 52V. The capillary voltage was 3.20 kV. Collision energy and collision gas (Ar) pressure were 22eV and 2.5 mbar, respectively. The samples were analyzed in one batch. Nine samples were at random chosen to be analyzed in duplicate. All samples (including the nine replicas) were randomized and divided into blocks of five. Between each block a QC sample was analyzed. These QC samples were created by

pooling an aliquot of all the samples. Each sample was injected twice in a row. The acquired data was analyzed using Quanlynx (Waters, UK). The automated peak integration was checked manually and when necessary corrected by hand. All peak areas were corrected for system trends by using the QC samples as described by Van der Kloet *et al.* (2009). The data was normalized by dividing the intensity of a compound by the mean intensity of all compounds and globally checked on inconstancies. There were no unexpected differences between duplicate samples and the duplicate injections were within range of each other. The quality of the measurements for the individual metabolites was assessed by means of the relative standard deviation (RSD) on the QCs and the replicates. In general, we consider metabolites with RSD values on both below 15% as measured with enough precision. For data analysis: to correct for multiple testing a Benjamini-Hochberg correction was applied to generate corrected p values. When significant differences $P<0.05$ were detected, a Tukey Honestly Significant Difference test was performed to determine which groups were significantly different from each other. For data analysis: to correct for multiple testing a Benjamini-Hochberg correction was applied to generate corrected p values. When significant differences $P<0.05$ were detected, a Tukey Honestly Significant Difference test was performed to determine which groups were significantly different from each other.

Plasma measurements of cytokines

All samples were stored at -80 °C until tested. The samples were thawed at room temperature (RT), vortexed, spun at 13,000×g for 5 minutes for clarification and 35 µL was removed for Multi-Analyte Profile (MAP) analysis into a master microtiter plate. Using automated pipetting, an aliquot of each sample was introduced into one of the capture microsphere multiplexes of the Mouse CytokineMAP A v1.0, Mouse CytokineMAP B v1.0, Mouse CytokineMAP C v1.0, and Rodent CustomMAP: RAMB1, RAMB2 and RAMB5. The mixture of sample and capture microspheres were thoroughly mixed and incubated at RT for 1 h. Multiplexed cocktails of biotinylated reporter antibodies for each multiplex were then added robotically and after thorough mixing, were incubated for an additional hour at RT. Multiplexes were developed using an excess of streptavidin-phycoerythrin solution which was thoroughly mixed into each multiplex and incubated for 1 h at RT. The volume of each multiplexed reaction was reduced by vacuum filtration and the volume increased by dilution into matrix buffer for analysis. Analysis was performed in a Luminex 100/200 instrument and the resulting data stream was interpreted using proprietary data analysis software developed at Rules-Based Medicine (www.rulesbasedmedicine.com). For each multiplex, both calibrators and controls were included on each microtiter plate. Eight-point calibrators were run in the first and last column of each plate and three-level controls were included in duplicate. Standard curve, control, and sample QC were performed to ensure proper assay performance. Unknown values for each of the analytes localized in a specific multiplex were determined using four and five parameter, weighted and non-weighted curve fitting algorithms included in the data analysis package.

References

Benjamini, Y. and Yekutieli, D., 2001. The control of the false discovery rate in multiple testing under dependency. The Annals of Statistics 29: 1165-1188.

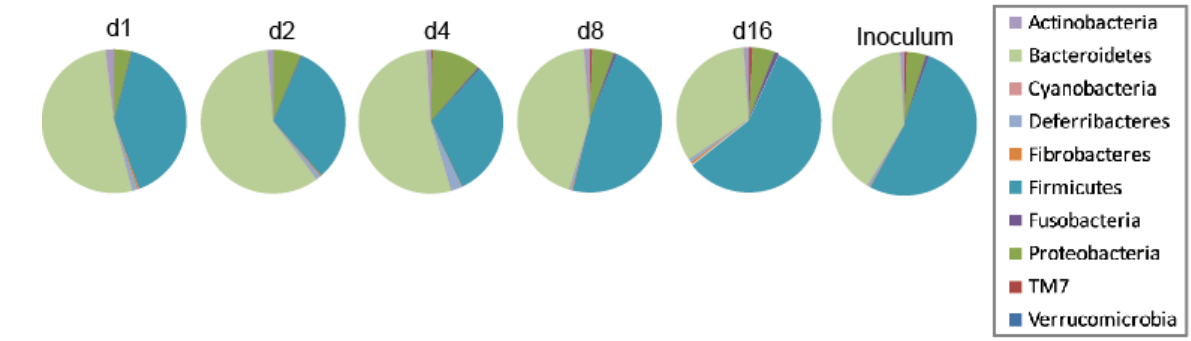
Bogomoletz, W.V., Williams, G.T. and Potet, F., 1987. HID-blue alcian (high iron diamine-alcian blue) and the histochemistry of mucins in colonic pathology: 20 years later. Gastroenterology and Clinical Biology 11: 865-868.

Noga, M.J., Dane, A., Shi, S., Attali, A., Aken, H., Suidgeest, E., Tuinstra, T., Muilwijk, B., Coulier, L., Luider, T., Reijmers, T.H., Vreeken, R.J. and Hankemeier, T., 2012. Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis. Metabolomics 8: 253-263.

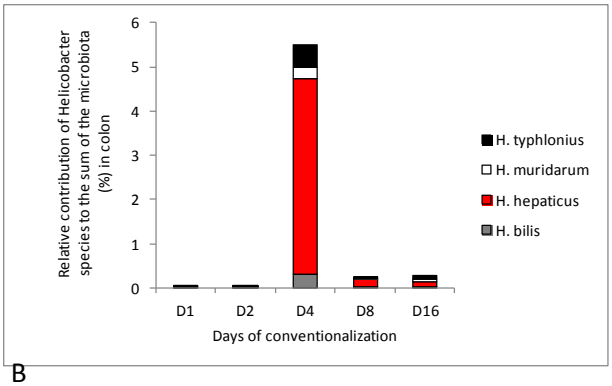
Swann, J., Richards, S.E., Shen, Q., Holmes, E., Marchesi, J.R. and Tuohy, K., 2011. Culture-independent analysis of the human gut microbiota and their activities. In: De Bruijn, F.J. (ed.) Handbook of Molecular Microbial Ecology, Volume II: Metagenomics in different habitats, First edition. Wiley-Blackwell, John Wiley & Sons, Inc., pp. 207-218.

Van der Kloet, F.M., Bobeldijk, I. Verheij, E.R. and Jellema, R.H., 2009. Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. Journal of Proteome Research 8: 5132-5141.

Supplementary Figures



A



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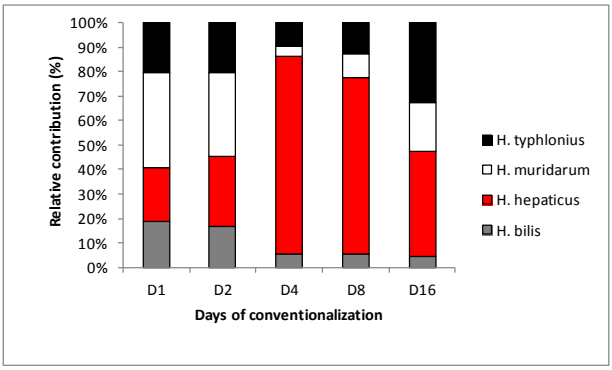

C

Figure S1. (A) Dynamics of the establishment of the gut microbiota in conventionalized mice and the inoculum in the colon, represented by the relative contribution of phylum-like levels (levels 0). (B) Dynamics of the relative contribution of level 3 (species-like) Helicobacter species to the total microbiota at day 4 after conventionalization in the colon as detected by MITChip. (C) Dynamics of the relative contribution of level 3 (species-like) Helicobacter groups to the level 2 (genera-like) Helicobacter genus at day 4 after conventionalization in the colon as detected by MITChip.

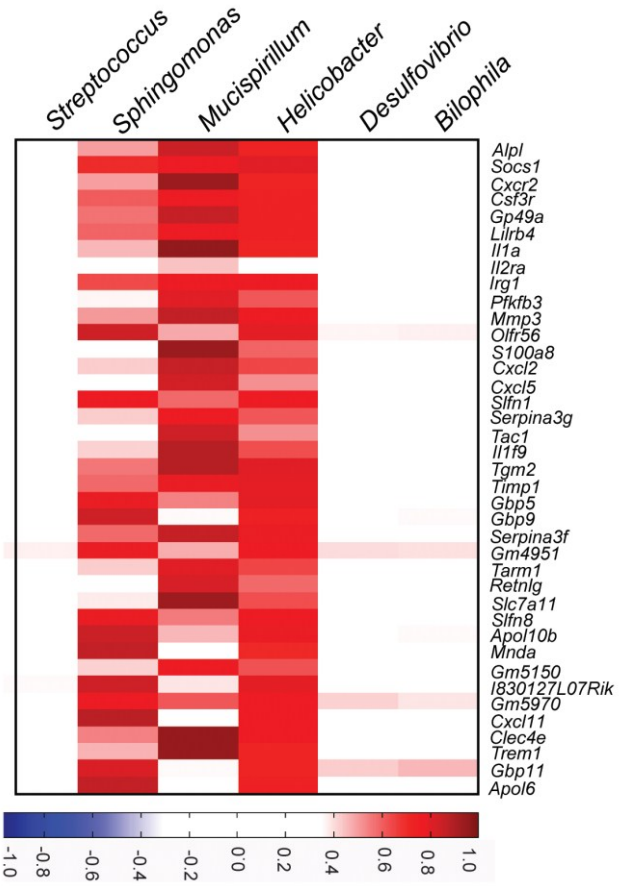

Figure S2. Association of specific Proteobacteria groups with proinflammatory genes. Correlation heat map between level 2 (genus-like) microbiota as identified by MITChip and the genes identified by STEM analysis to peak at day 4 post-conventionalization.

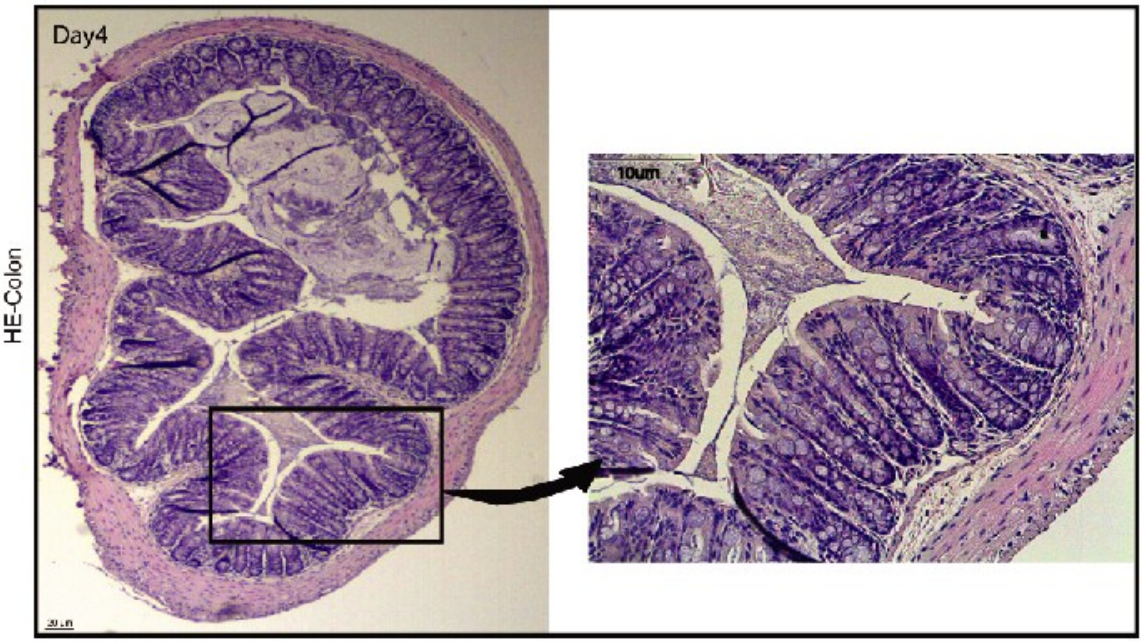

Figure S3. Absence of any sign of tissue damage in the colonic mucosa. Representative micrograph of HE-stained cross sections of colonic tissue at day 4 post-conventionalisation (the inset shows a higher magnification), illustrating the absence of overt tissue inflammation.

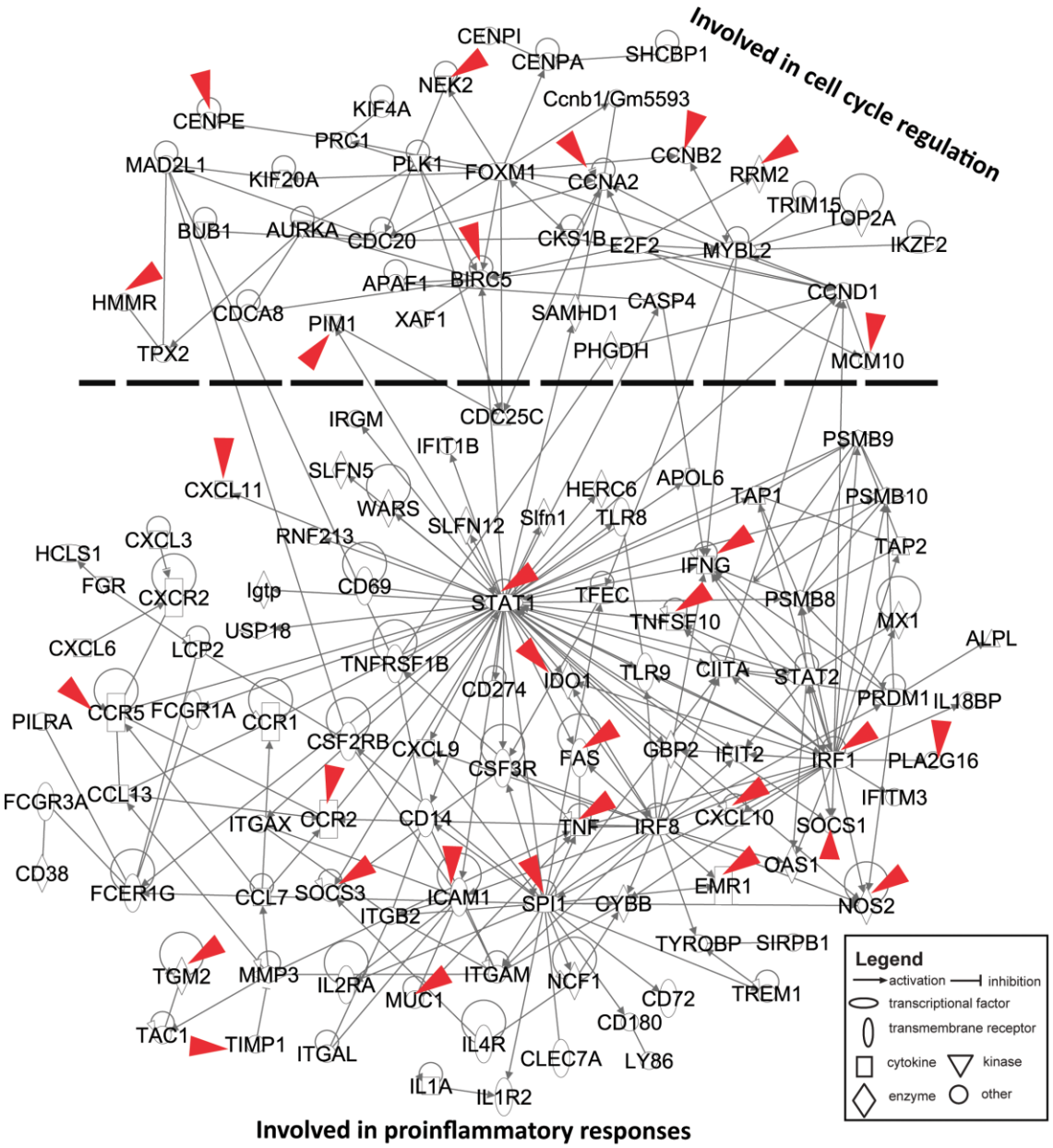

Figure S4. Transcriptome signature for priming the immune system during microbial colonization. The ingenuity protein-protein interaction network derived by plotting STEM output genes and exemplifies the transition towards a microbiota accommodating homeostasis during the process of conventionalization, (n=11 mice/day). Red arrow heads indicate genes associated with human inflammatory bowel disease and colon cancer.

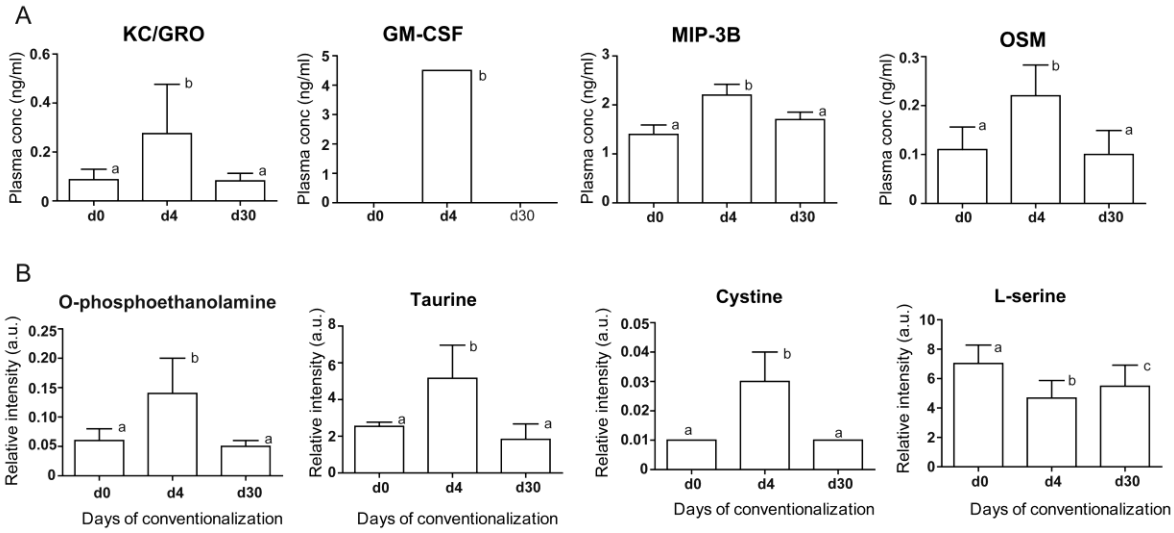

Figure S5. Changes in the plasma levels of proinflammatory cytokines and amines at day 4 post-conventionalization. Additional plasma analytes including (A) growth-regulated alpha protein (KC/GRO), (granulocyte-macrophage- colony stimulating factor 2) (GM-CSF or Csf2), and oncostatin M (OSM) and (B) o-phosphoethanolamine, Taurine, and Cystine were significantly increased at day 4 when compared to germfree and day 30 post-conventionalisation. Significant differences between time-points are indicated by distinctive characters above the measurement groups (P<0.05).