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Early-life exposure to gut microbiota from disease protected mice does not impact disease outcome in type 1 diabetes susceptible NOD mice

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RUNNING TITLE

NOD mice are resistant to gut-microbiota transfer

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KEYWORDS

Co-housing, genetic susceptibility, gut microbiota, type 1 diabetes, autoimmunity, environmental determinants.

ABSTRACT

The microbial community making up the gut microbiota can profoundly influence intestinal homeostasis and immune system development, and is believed to influence the development of complex diseases including type 1 diabetes (T1D). T1D susceptible non-obese diabetic (NOD) mice have been shown to harbour a distinct microbiota to disease protected mice. We hypothesised that the T1D susceptible genetic background of NOD mice would be resistant to the introduction of a C57BL/6 derived microbiota. NOD and C57BL/6 mice were cohoused either continually from birth, from birth until weaning or from weaning onwards, allowing transfer of microbiota between the mice. Cohousing NOD with C57BL/6 mice from before birth, resulted in moderate changes to the gut microbiota, whereas initiating co-housing at weaning only led to minimal changes. Terminating cohousing at weaning reduced the changes in the microbiota composition. However, diabetes onset was not significantly delayed and there was no reduction in intestinal inflammation or the proportion of regulatory T cells in the co-housed NOD mice. However, insulin but not IGRP-specific CD8+ T cells were reduced by co-housing suggesting an epitope-specific modulation of the autoreactive response by the gut microbiota. These results suggest that the T1D susceptible genetic background of the NOD mouse was resistant to the introduction of a C57BL/6 derived microbiota.

INTRODUCTION

A number of studies have described alterations in the gut microbiota associated with type 1 diabetes (T1D) which is thought to be linked to disease pathogenesis [1-5]. Recently, we have shown that genetic susceptibility to T1D contributes to the structure of the intestinal microbiota in both T1D susceptible NOD mice and humans [6]. In NOD mice, manipulations that lead to major disturbances in the microbiota can both protect from and accelerate disease [7-9]. This suggests that a balance between host genetics and environment contributes to dysbiosis associated with T1D risk.

We previously reported low-level intestinal inflammation in NOD compared to disease protected mice [6]. It is unknown the extent to which genetically driven gut inflammation is a barrier to therapies designed to restore a healthy microbiota in T1D. In this study, we have investigated whether the genetic effect on the NOD microbiota composition could be overcome by the introduction of a highly dissimilar microbiota from diabetes-protected mice and whether this could reduce intestinal inflammation and disease progression.

METHODS

Mice and Cohousing Design:

Female mice were housed in specific pathogen free conditions within the University of Queensland Biological Resource Facility. All experiments were approved by the University of Queensland Animal Ethics committee. NOD/Lt (NOD) and C57BL/6 (B6) mice were from the Animal Resources Centre (ARC, Perth, WA, Australia). For experiment 1, NOD mice were randomly allocated into two groups; control or cohoused at weaning. The cohoused group were caged with age matched B6 mice at a 1:1 ratio. For experiment 2, an adult virgin B6 female was cohoused with a pregnant NOD dam until weaning. At weaning, pups were

either cohoused with B6 aged matched mice at a 1:1 ratio or housed in single strain cages. Diabetes was tested weekly using urine or blood glucose measurement (≥16 mmol/L considered diabetic) and confirmed on a second consecutive day.

16s rRNA gene sequencing:

Faecal pellets were collected from multiple cages to minimise cage effects. Bacterial DNA was extracted from faecal pellets and used for 16S rRNA gene sequencing as previously described [10]. Rarefaction was applied at 3150. Communities were visualized with R package mix0mics for sparse partial least squares discriminant analysis (sPLS-DA) and for visualizing plot loadings [11].

Histology:

Insulitis and intestinal inflammation was scored from sections stained with Hematoxylin and eosin or Periodic Acid-Schiff stain and scored as previously described [6] [12]. Lysozyme staining used antibodies listed in Supplementary Table 1 and 3,3′-diaminobenzidine (DAB). Goblet cell abundance was determined using region of interest area of goblet staining intensity of each crypt using NIS Elements software (Nikon Instruments, Melville, NY).

Flow cytometry

Single cell suspensions were prepared from spleen and lymph nodes and red blood cells were lysed with ACK buffer. Dead cells were excluded using live-dead stain (Biolegend, San Diego, CA). Antibodies are listed in Supplementary Table 1. Foxp3 staining used the Foxp3 Staining Set (eBioscience). Lymphocytes were stained with PE-conjugated tetramers ($IGRP_{206-214}$-K^d, $Insulin_{15-23G9V}$-K^d and LLO_{91-99}-K^d , NIH tetramer core facility) for 15

minutes at room temperature. Samples were run on the FACS LSR Fortessa (BD Bioscience, San Jose, CA) and analysed using FlowJo (Tree Star, Ashland, OR).

Statistics

16s rRNA gene sequencing data were analysed through QIIME (MacQiime 1.9.1) and RStudio (Version 1.0.143). Permutation ANOVA, ANOSIM and ADONIS were carried out using R scripts with R vegan v2.4-3, limma v3.30.13 and RVAideMemoire v 0.9-64 (Version 6). Correction for multiple testing used the Benjamini and Hochberg method. Taxa with adjusted *P*-values <0.05 were further investigated with post-hoc Tukey tests performed in GraphPad Prism. Level of significance is denoted: *= *P*-value < 0.05, **= *P*-value < 0.01, ***= *P*-value < 0.001, ****= *P*-value < 0.0001.

RESULTS

Cohousing NOD and B6 mice from early-life results in modest alterations in the gut microbiota.

We aimed to test whether co-housing T1D susceptible NOD and disease-protected B6 mice would result in the NOD microbiota becoming more B6-like and protect from disease. The study was split into two experiments (Figure 1a). In the first experiment, NOD pups were cohoused with age-matched B6 pups from weaning (CoH@W). In the second experiment, NOD mice were cohoused with B6 mice from birth either continually (long-cohoused: LCoH) or until weaning (short co-housed: SCoH). Fecal samples were collected at weaning and 10-12 weeks of age and used to profile the microbial community composition.

By 10-12 weeks of age, sPLS-DA multivariate analysis showed that cohousing from weaning modestly altered the NOD microbiota (Figure 1b, NOD control vs CoH@W *P*=0.05). Non-cohoused NOD and B6 mice had distinct gut microbial communities (*P* < 0.001,

Supplementary Figure 1a). Only two genera (*Clostridiaceae* and *Clostridium*) had altered abundances in NOD CoH@W compared to control NOD mice (Supplementary Figure 1a), but not in the direction of the B6 mice. Consistent with this, the Shannon diversity index was not increased by cohousing from weaning (Supplementary Figure 2a). In contrast, both LCoH and SCoH NOD mice had a significantly altered microbiota to control NOD mice (Figure 1c, P=0.018 and P=0.003 respectively). Eight genera were significantly altered in LCoH compared with the control NOD mice (Figure1d), though many other genera were not altered (Supplementary Figure 1b). Some taxa that were significantly increased in abundance by long-term cohousing were not altered or had a partial increase following short-term cohousing (e.g. *Parabacteroides*, unclassified *YS2* and *Clostridium*). Consistent with this, the Shannon diversity index was not increased in SCoH NOD mice but was in the LCoH NOD mice (Supplementary Figure 2b). Together, these data suggest that the majority of changes to the microbiota were introduced prior to weaning, but the NOD intestinal environment may cause the microbiota to revert back to a steady state over time.

We also compared the microbiota of the cohoused mice at weaning when the LCoH and SCoH groups were separated from each other. As expected, while the microbiota profiles of cohoused and non-cohoused NOD mice were different at weaning (P = 0.02), the LCoH and SCoH groups were not significantly different at the time of separation (Supplementary Figure 3a). Likewise, the Shannon diversity index was increased at 3 weeks of age in both LCoH and SCoH groups compared with the control NOD mice (Supplementary Figure 3b). These data indicate that cohousing during the neonatal period is essential for effective transfer of bacterial species into the NOD gut, although maintaining co-housing past weaning was required to preserve these changes to a maximal effect.

Cohousing NOD mice with B6 did not significantly affect the incidence of diabetes.

We next investigated whether introduction of B6 derived taxa by cohousing impacted the onset of diabetes. Cohousing from weaning showed a trend to delaying the progression of diabetes (Figure 2a, P=0.056). LCoH NOD mice showed a trend to a reduced frequency of diabetes; however, this was not significant (Figure 2b, P =0.244). SCoH NOD mice had a similar disease onset to control NOD mice (Figure 2b, P = 0.24). There was also no difference in insulitis scores between LCoH and control NOD mice (Figure 2c-d). Thus, the changes in the NOD microbiota brought about by cohousing with B6 mice were not sufficient to significantly prevent or slow progression to diabetes.

Cohousing did not reduce subclinical intestinal inflammation in NOD mice

We next questioned whether cohousing had any effect on the subclinical intestinal inflammation seen in NOD mice[6]. As expected, control NOD had significantly higher inflammation scores compared to B6 mice (Figure 3a). B6 mice had an increased goblet cell area (Figure 3b) and Paneth cell staining of lysozyme P in the ileum to NOD mice (Fig 3c). However, there were no statistically significant differences between LCoH and control NOD mice in inflammatory scoring, goblet cell area or lysozyme P staining. We concluded that the altered abundance of the taxa shown in Figure 1d did not impact intestinal inflammation present in NOD mice.

Cohousing did not have a significant impact on the frequency of regulatory T cells but reduced the frequency of insulin-specific CD8+ T cells

Regulatory immune T cells (Tregs) play an important role in suppressing autoimmunity and defects in Tregs activity, contribute to the development of T1D [13-15]. Specific members of the gut microbiota have been shown to impact peripheral induction of Tregs [16]. We examined

Treg frequencies in the mesenteric, pancreatic and inguinal lymph nodes and spleen. B6 mice had significantly higher proportions of CD4+Foxp3+ Tregs in their lymph nodes and relatively less in spleen compared with both NOD and LCoH NOD mice as previously described (Figure 4a) [17]. No difference was seen between the proportions of Tregs in LCoH and non-cohoused NODs. Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) is a major autoantigen in the NOD mouse [18]. Several species of the *Bacteroides* genera express an epitope that mimics IGRP and can promote recruitment of diabetogenic CD8+ T cells to the gut[19, 20]. We did not observe any differences in the frequency of IGRP-specific CD8+ T cells in lymph nodes of NOD and LCoH NOD mice using IGRP-specific tetramers (Figure 4b). Strikingly, insulin-specific CD8+ T cells were significantly decreased in the pancreatic and mesenteric lymph nodes of LCoH NOD mice (Figure 4c). We concluded that while cohousing NOD mice with B6 mice did not have a significant effect on peripheral Tregs, autoreactive CD8+ T cells were differentially impacted depending on their specificity.

DISCUSSION

We hypothesised that cohousing NOD and B6 mice would change the gut microbiota of the NOD mice to become more B6-like. However, the effects of cohousing were modest and terminating cohousing at weaning reduced the effect. Exposure during the pre-weaning period was essential for optimal establishment of a new microbiota. The changes induced in the gut bacterial communities of the NOD mice were not sufficient to alter either the gut inflammatory environment or to prevent diabetes development. Notably, insulin but not IGRP-specific CD8+ T cells were modulated by co-housing suggesting that the microbiota influence autoreactive T cells in an epitope-specific manner.

In our colony, the B6 mice had a microbiota dominated by *Allobaculum* while the NOD mice had a predominance of *Lactobacillus*. Surprisingly, neither cohousing from before birth or from weaning changed the abundance of these genera. The taxa that were altered were all obligate anaerobes, which might infer that cohousing leads to a less oxidative intestinal environment. An aerobic intestinal environment has been associated with dysbiosis and disease [21]. However, intestinal inflammation observed histologically in NOD mice was not reduced by cohousing.

Introduction of a new microbiota into NOD mice may be dependent on the specific strain and sex of the donor. For example, cross-fostering NOD pups to non-obese resistant (NOR) mothers permanently altered the microbiota of the offspring[22]. However, this only resulted in a significant reduction in diabetes in the male offspring. An adult male NOD microbiota transferred to females by oral gavage was able to protect from diabetes and alter the recipient's microbiota [23]. Others have reported that B6 foster parents did not transfer diabetes protection to NOD mice, whereas ICR foster parents did [24]. It is therefore likely that the microbiota composition of the donor strain is critical for determining protection.

The small effects on the NOD microbiota may be because of passive microbiota transfer through cohousing. While we introduced the B6 and their microbiota, we did not cross foster the NOD pups onto B6 dams. This would have allowed closer interaction between the NOD pups and the B6 females as well as exposing them to maternal antibodies from the B6 mothers. Maternal antibodies contribute to shaping the microbiota of the offspring [25].

The small changes in the microbiota we observed may have been due to an inherent incompatibility for one another’s microbiota due to genetic pressure. Constraints to colonisation may occur that allow only an inflammation tolerant microbiome in the NOD strain. An enterotype associated with low-grade inflammation has been described in NOD mice [26], suggesting that subclinical inflammation contributes to shaping the gut microbiota.

The implication of our findings is that a therapeutic strategy aimed at inducing permanent changes in the gut microbiota of individuals at risk of T1D would be more effective if initiated in early life and continued long-term. A T1D-susceptible host genetic background imposes a barrier to fundamentally changing the microbiota composition. Major community remodelling may require therapy to reverse underlying inflammation to induce long-lasting effects.

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CONFLICT OF INTEREST STATEMENT

Authors disclose that they have no conflict of interest that may bias their work.

CONTRIBUTION STATEMENT

Conceptualization, E.H-W., J.M.; Methodology, J.M.; Resources, E.H-W.; Investigation, J.M., J.S., B.G. and E.H-W.; Formal Analysis JM.; Visualization, J.M.; Writing - Original Draft, JM., E.H-W.; Writing – Reviewing & Editing, J.M., E.H-W., Supervision, E.H-W, J.M.

REFERENCES

1. Alkanani AK, Hara N, Gottlieb PA *et al.* Alterations in Intestinal Microbiota Correlate With Susceptibility to Type 1 Diabetes. *Diabetes* 2015; **64:** 3510-3520.

2. Kostic Aleksandar D, Gevers D, Siljander H *et al.* The Dynamics of the Human Infant Gut Microbiome in Development and in Progression toward Type 1 Diabetes. *Cell host microbe* 2015; **17:** 260-273.

3. Endesfelder D, Hagen M, Winkler C *et al.* A novel approach for the analysis of longitudinal profiles reveals delayed progression to type 1 diabetes in a subgroup of multiple-islet-autoantibody-positive children. *Diabetologia* 2016; **59:** 2172-2180.

4. de Goffau MC, Fuentes S, van den Bogert B *et al.* Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. *Diabetologia* 2014; **57:** 1569-1577.

5. Giongo A, Gano KA, Crabb DB *et al.* Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J* 2010; **5:** 82.

6. Mullaney JA, Stephens JE, Costello M-E *et al.* Type 1 diabetes susceptibility alleles are associated with distinct alterations in the gut microbiota. *Microbiome* 2018; **6:** 35.

7. Mariño E, Richards JL, McLeod KH *et al.* Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. *Nat Immunol* 2017; **18:** 552.

8. Marietta EV, Gomez AM, Yeoman C *et al.* Low Incidence of Spontaneous Type 1 Diabetes in Non-Obese Diabetic Mice Raised on Gluten-Free Diets Is Associated with Changes in the Intestinal Microbiome. *PLOS ONE* 2013; **8:** e78687.

9. Livanos AE, Greiner TU, Vangay P *et al.* Antibiotic-mediated gut microbiome perturbation accelerates development of type 1 diabetes in mice. *Nat Microbiol* 2016; **1:** 16140.

10. Mullaney JA, Stephens JE, Costello ME *et al.* Type 1 diabetes susceptibility alleles are associated with distinct alterations in the gut microbiota. *Microbiome* 2018; **6:** 35.

11. Lê Cao K-A, Boitard S, Besse P. Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. *BMC Bioinformatics* 2011; **12:** 253.

12. Hamilton-Williams EE, Serreze DV, Charlton B *et al.* Transgenic rescue implicates β2-microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice. *Proc Natl Acad Sci* 2001; **98:** 11533-11538.

13. Jaeckel E, Mpofu N, Saal N *et al.* Role of Regulatory T Cells for the Treatment of Type 1 Diabetes Mellitus. *Horm Metab Res* 2008; **40:** 126-136.

14. Lindley S, Dayan CM, Bishop A *et al.* Defective Suppressor Function in $CD4^+CD25^+$ T-Cells From Patients With Type 1 Diabetes. *Diabetes* 2005; **54:** 92-99.

15. Brusko TM, Wasserfall CH, Clare-Salzler MJ *et al.* Functional Defects and the Influence of Age on the Frequency of $CD4^+CD25^+$ T-Cells in Type 1 Diabetes. *Diabetes* 2005; **54:** 1407-1414.

16. Kamada N, Núñez G. Role of the Gut Microbiota in the Development and Function of Lymphoid Cells. *J Immunol* 2013; **190:** 1389-1395.

17. James CR, Buckle I, Muscate F *et al.* Reduced interleukin-2 responsiveness impairs the ability of Treg cells to compete for IL-2 in nonobese diabetic mice. *Immunol Cell Biol* 2016; **94:** 509.

18. Yang J, Danke NA, Berger D *et al.* Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein-Reactive $CD4^+$ T Cells in Human Subjects. *J Immunol* 2006; **176:** 2781-2789.

19. Tai N, Peng J, Liu F *et al.* Microbial antigen mimics activate diabetogenic CD8 T cells in NOD mice. *J Exp Med* 2016.

20. Hebbandi Nanjundappa R, Ronchi F, Wang J *et al.* A Gut Microbial Mimic that Hijacks Diabetogenic Autoreactivity to Suppress Colitis. *Cell* 2017; **171:** 655-667.e617.

21. Byndloss MX, Olsan EE, Rivera-Chavez F *et al.* Microbiota-activated PPAR-gamma signaling inhibits dysbiotic Enterobacteriaceae expansion. *Science* 2017; **357:** 570-575.

22. Daft JG, Ptacek T, Kumar R *et al.* Cross-fostering immediately after birth induces a permanent microbiota shift that is shaped by the nursing mother. *Microbiome* 2015; **3:** 17.

23. Markle JGM, Frank DN, Mortin-Toth S *et al.* Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity. *Science* 2013; **339:** 1084-1088.

24. Washburn LR, Dang H, Tian J *et al.* The postnatal maternal environment influences diabetes development in nonobese diabetic mice. *J Autoimmun* 2007; **28:** 19-23.

25. Rogier EW, Frantz AL, Bruno MEC *et al.* Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. *Proc Natl Acad Sci* 2014; **111:** 3074-3079.

26. Hildebrand F, Nguyen TLA, Brinkman B *et al.* Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biology* 2013; **14:** R4-R4.

FIGURE LEGENDS

Figure 1. Long-term cohousing of NOD and B6 mice from birth results in an altered gut microbiota. (a): Schematic of cohousing design. (**b)**: sPLS-DA multivariate analysis of mice cohoused from weaning and control non-cohoused B6 and NOD mice (n=6-7 per group). (**c)**: sPLS-DA analysis of mice cohoused from birth (n=10-11 per group). Ellipses show 95% confidence intervals. (**d**): Genera with differential abundance in cohoused NOD vs control NOD mice from (**c**) (adj $P < 0.05$). CoH@W NOD: Cohoused at weaning. NCoH NOD Not cohoused (control). SCoH NOD: short-term cohoused. LCoH NOD: long-term cohoused. Mean and SD are shown. Each experiment was performed once.

Figure 2. The effect of cohousing on diabetes frequency and insulitis. Diabetes frequency in (**a**) NOD mice not cohoused (NCoH, n=23) or cohoused from weaning (CoH@W NOD n=22) and (**b**) in control NOD mice (NCoH, n=20), NOD mice cohoused from birth short-term (SCoH n=20) or long-term cohoused (LCoH n=34). *P*-values from a log-rank test are shown. Insulitis scoring from individual mice (**c**) and mean insulitis scores (**d**) from mice cohoused continually from birth at 12-weeks of age. Mean and SEM are shown. Each experiment was performed once.

Figure 3. Intestinal architecture, inflammation, goblet cell mucous production and lysozyme production were not altered by cohousing NOD mice. Histological sections from NOD mice cohoused long-term from birth (LCoH), not-cohoused NOD (NCoH) and B6 mice culled at 12-weeks of age were scored (maximum possible score 29) (**a**): Examples of H&E stained colonic tissue and ileum Paneth structures with summary of gut scoring from n=6-8 mice per group. (**b**): Goblet PAS staining of goblet cells from colonic tissue and quantification of goblet cell area from n=6-8 mice per group. (**c**): IHC staining of lysozyme P

production by Paneth cells from the ileum and quantification of staining intensity in n=6-7 mice per group. Experiment was performed once.

Figure 4. Immune regulation was not restored by cohousing NOD and B6 mice. NOD mice cohoused long-term from birth (LCoH), not-cohoused NOD (NCoH) and B6 mice culled at 12-weeks of age and lymph node and spleen tissue analysed by FACS. (**a**): Foxp3+CD4+ Treg frequency within CD4+ T cells. (**b**): IGRP-specific and (**d**) insulin-specific tetramer+ cell proportion within CD8+ T cells. Dotted line: mean frequency of control LLO-specific tetramer+ cells. Mean and SD are shown. LCOH: long-cohoused. Experiment was performed once.