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A Murine Oral-Exposure Model for Nano- and Micro-Particulates: Demonstrating Human Relevance with Food-Grade Titanium Dioxide

*Sebastian Riedle, John W. Wills, Michelle Miniter, Don E. Otter, Harjinder Singh, Andy P. Brown, Stuart Micklethwaite, Paul Rees, Ravin Jugdaohsingh, Nicole C. Roy, Rachel E. Hewitt, and Jonathan J. Powell**

Human exposure to persistent, nonbiological nanoparticles and microparticles via the oral route is continuous and large scale (10^{12}–10^{13} particles per day per adult in Europe). Whether this matters or not is unknown but confirmed health risks with airborne particle exposure warns against complacency. Murine models of oral exposure will help to identify risk but, to date, lack validation or relevance to humans. This work addresses that gap. It reports i) on a murine diet, modified with differing concentrations of the common dietary particle, food grade titanium dioxide ($fgTiO_2$), an additive of polydisperse form that contains micro- and nano-particles, ii) that these diets deliver particles to basal cells of intestinal lymphoid follicles, exactly as is reported as a "normal occurrence" in humans, iii) that confocal reflectance microscopy is the method of analytical choice to determine this, and iv) that food intake, weight gain, and Peyer's patch immune cell profiles, up to 18 weeks of feeding, do not differ between $fgTiO_2$-fed groups or controls. These findings afford a human-relevant and validated oral dosing protocol for $fgTiO_2$ risk assessment as well as provide a generalized platform for application to oral exposure studies with nano- and micro-particles.

Humans are exposed, orally, to vast numbers of nonbiological nanoparticles and microparticles, many of which are chemically persistent. Within this category, the ingestion of silicates and food grade titanium dioxide ($fgTiO_2$) is estimated at 37.5 mg (median), or 10^{12}–10^{13} particles, per adult per day in the UK.[1] For $fgTiO_2$, this equates to an intake of ≈0.04 mg kg^{-1} body weight per day for a 70 kg adult.[1] A further carefully conducted study reported broadly similar results in the Netherlands (0.06–0.17 mg kg^{-1} body weight per day at >7 years of age), with even higher $fgTiO_2$ intakes recorded for children of 2–6 years of age.[2]

What the effects of exposure are to these or other particles, via the oral route, is unknown but has become a key agenda item for the relevant regulatory bodies.[3–9]

With this in mind, this issue's focus on "rethinking nanosafety" is timely. This is especially so, given that accumulated data

Dr. S. Riedle,[+] Dr. D. E. Otter, Dr. N. C. Roy
Food Nutrition & Health Team
Food & Bio-based Products Group
AgResearch
Grasslands Research Centre
Tennent Drive
Private Bag 11008, Palmerston North 4442, New Zealand

Dr. S. Riedle, Prof. H. Singh, Dr. N. C. Roy
Riddet Institute
Massey University
Private Bag 11222, Palmerston North 4442, New Zealand

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/smll.202000486.

[+]Present address: Conreso GmbH, Neuhauser Str. 47, München, 80331, Germany

DOI: 10.1002/smll.202000486

Dr. J. W. Wills, M. Miniter, Dr. R. Jugdaohsingh, Dr. R. E. Hewitt, Dr. J. J. Powell
Biomineral Research Group
Department of Veterinary Medicine
University of Cambridge
Madingley Road, Cambridge CB3 0ES, UK
E-mail: jjp37@cam.ac.uk

Dr. A. P. Brown, Dr. S. Micklethwaite
School of Chemical and Process Engineering
University of Leeds
Leeds LS2 9JT, UK

Prof. P. Rees
College of Engineering
Swansea University
Fabian Way, Crymlyn Burrows, Swansea SA1 8EN, UK

Prof. P. Rees
Broad Institute of MIT and Harvard
Cambridge, MA 02142, USA

Dr. N. C. Roy
High-Value Nutrition National Science Challenge
Auckland 92019, New Zealand

Dr. N. C. Roy
Liggins Institute
The University of Auckland
85 Park Road, Auckland 1142, New Zealand

now show real and definitive health risks as a result of our exposure to common environmental particulates.[10] Notably, the relationship between airborne particles and cardiopulmonary diseases has been consistently demonstrated. However, much of this research has relied upon epidemiological approaches whereby large populations under different exposure conditions are correlated against adverse outcomes (i.e., mortality or biomarkers of disease).[11,12] To understand the risk of oral exposures to common particles, however, such approaches are limited. This is because while exposures undoubtedly vary across populations, they do so in a diffuse fashion and not in geographical subpopulations. Data, therefore, are difficult to capture: dietary intake measures are reasonably crude and, unless specifically designed, do not include intakes of pharmaceuticals, nutraceuticals, or swallowed toothpastes, which are major sources of oral particle intakes.[1] Even when studies are specifically designed to track all sources of oral particle exposure to humans, the complexity is such that subject numbers are limited by practicable design.[1] Moreover, oral intake studies do not track long-term exposures, but provide information on a short window of a few days or a week.

Distinct methodology is therefore required for the oral route and, likely, an aggregate of approaches, including carefully conducted murine studies, will be necessary to inform human health risk assessment, effectively.

Recently, the pitfalls of murine oral exposure models for nanoparticle and microparticle research have been reviewed.[13] In particular, it was noted that real-world human exposures are poorly represented by gavage whereby concentrated, daily boluses are forced into the stomach of rodents via an oral tube. Aside from physiological stress and its effects on the intestine, particles have a tendency to agglomerate and aggregate when concentrated. This may occur in the dosing apparatus, or in the intestine itself but, importantly, it may be irreversible resulting in no or limited uptake from the intestine. In any case, bolus delivery does not reflect human exposure patterns to particles, which will trickle in throughout the day from the acts of brushing teeth, through to intakes of diet and pills, to brushing of the teeth again. Usefully, rodents tend to graze on their diet providing a model to mimic the nature of human exposure to particles. Any criticism that dietary intakes are variable in rodents, and cannot be precisely monitored, can be readily countered by the arguments above, which outline much greater problems with the alternatives. Moreover, Organisation for Economic Co-operation and Development (OECD) guidelines for repeat dose oral toxicity testing (e.g., TG 408)[14] do not mandate gavage but also allow for a test material to be incorporated in the diet or dissolved in drinking water, so any dietary approach could still be adopted within the OECD framework.

The next aspect for consideration is whether particle absorption from the small intestine via the murine diet "vehicle" is successfully achieved. Indeed, the importance of confirming particle uptake for proper in vitro hazard identification, and avoidance of false-negative outcomes, has been a topic of considerable focus in recent years.[15–17] In much the same way, this same logic must apply to in vivo experimentation and, especially so, when human precedence has been so clearly demonstrated. In humans, the known target tissues for orally ingested, persistent particulates are the large lymphoid follicles of the small intestine, typically referred to as Peyer's patches. Repeatedly, these have been shown to contain basally located "pigment cells" that accumulate, nano- and micro-particles including a marked proportion of $fgTiO_2$.[18–23] It is beyond reasonable doubt that these particles accumulate in such cells as a result of the "every day" oral exposures described above. By no means does this rule out other tissue sites for environmental particle accumulation in humans, and, indeed, this is known to occur for liver and spleen.[24] However, as of yet, no other sites have been so thoroughly studied, or the precise location of their particle accumulating cells so accurately pin-pointed, as for Peyer's patches. In turn, if, a diet with added persistent particles is to be considered a successful mimic of regular human exposures then basal Peyer's patch accumulation of the fed particles must be observed.

Here, we develop diets incorporating three differing levels of $fgTiO_2$ to permit a physiologically relevant exposure method. We then confirm that these diets act as suitable "delivery vehicles." We achieve this, for the first time in a murine model, by assessing the formation of particle-loaded cells at the base of gut lymphoid follicles (Peyer's patches) as occurs in humans. Finally, we also establish sex-specific, key baseline measures such as intakes of $fgTiO_2$ as a result of the dosing regimen, animal weight gain, and how immune cell fractions of the Peyer's patch change at three different time points under confirmed exposure; all referenced to a negative control group (i.e., same diet but no $fgTiO_2$ supplementation).

The diets were constructed through incorporation of $fgTiO_2$ into the basal diet, namely American Institute of Nutrition (AIN)-76A, by "Research Diets, Inc." (USA). The primary particle size distribution of the $fgTiO_2$ has been described previously.[25] Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) spectroscopy confirmed that the particulate nature of the $fgTiO_2$ was retained after diet incorporation (Figure S1, Supporting Information).

To validate whether the diets permitted uptake from the intestinal lumen, and thus cellular exposures to the $fgTiO_2$, we surveyed the basal regions (i.e., serosal aspect) of Peyer's patches for $fgTiO_2$-containing cells at the end of the feeding study (18 weeks). The tissue harvest and method used to prepare sections—being careful to avoid inadvertent contamination—are shown in Figure S2 (Supporting Information). This time point was chosen on the basis that the cellular accumulation of particles, deep in tissues, will require time and, pragmatically, because the goal was simply to assess whether the diet had achieved successful particle delivery or not. To achieve this, reflectance confocal microscopy, as previously used in human tissue analyses, was employed to detect the presence of $fgTiO_2$ (**Figure 1**).[21] A robust threshold for the detection of reflectant foci consistent with $fgTiO_2$ was defined by removing all signal from complete, tile scanned images of Peyer's patch tissue sections taken from three negative control animals (Figure 1A). Reflectant foci, indicating the presence of $fgTiO_2$, were found at the base of Peyer's patches for all three dose groups (Figure 1C–E) (additional "*Z*-stack" 3D images also shown in Figure S3 in the Supporting Information). As previously shown in humans, SEM/EDX analyses confirmed that the tissue contained subsurface particles rich in titanium (Figure 1F–H), further validating the reflectance findings.

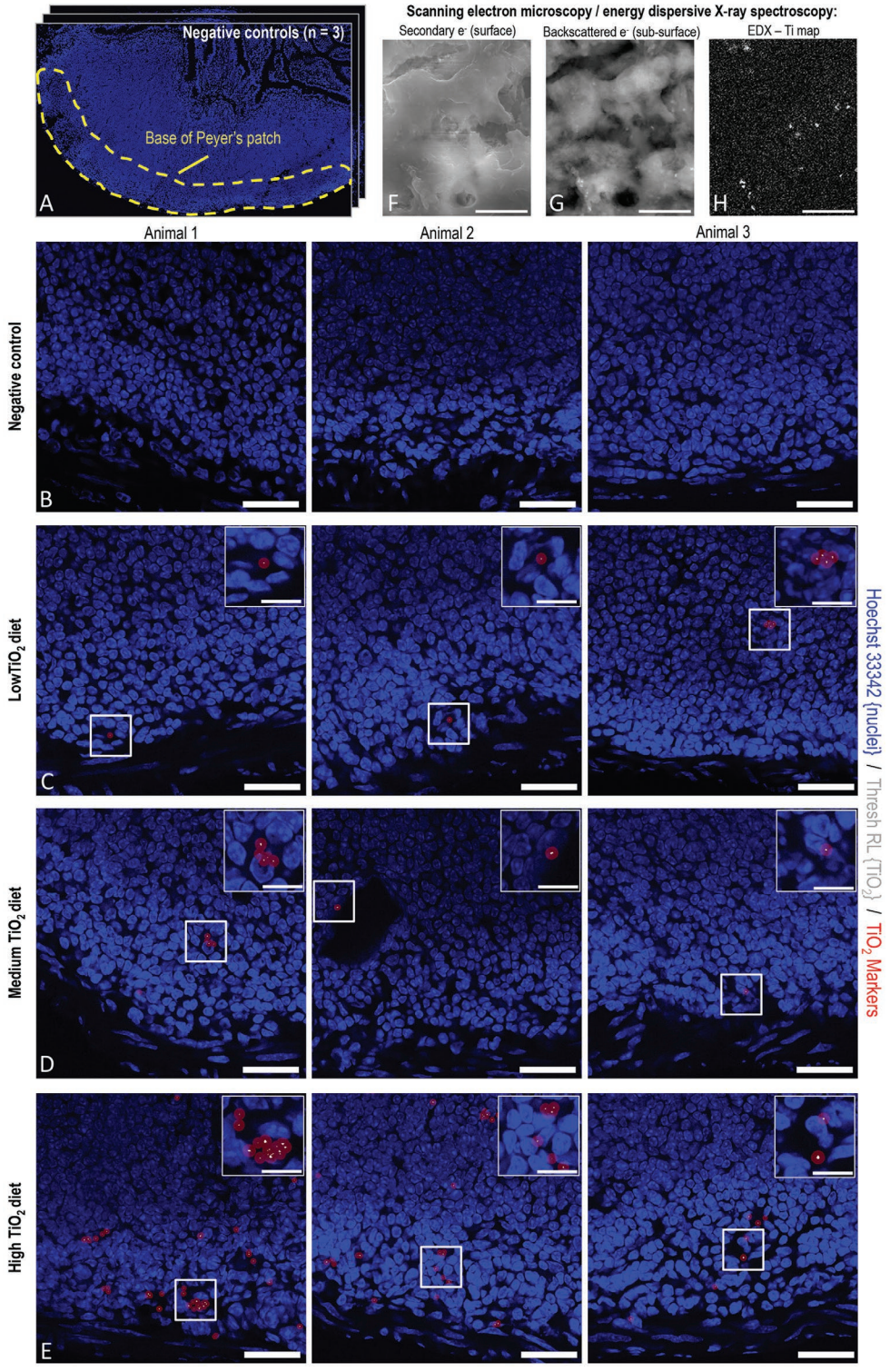

Figure 1. Accumulation of $fgTiO_2$ in basal cells of murine Peyer's patches following feeding. A) Tile scanned "overview" image of a typical murine ileal tissue section containing a Peyer's patch lymphoid follicle. Cell nuclei are labeled in blue by the fluorescent dye Hoechst 33342. The "base" region of the patch, which lies just above the muscularis layer, is indicated (yellow). The example shown is from an unexposed mouse. Tissues from B) negative control and C–E) $fgTiO_2$-exposed mice: Inset are boxed regions at higher magnification. Confocal reflectance microscopy (0.8 µm optical sections taken from the center of tissue sections) reveals the increasing formation of particle-loaded cells when mice are fed diets containing increasing amounts C–E) of $fgTiO_2$ (red circle markers placed on reflectance signal to aid visualisation). F–H) Imaging and physical analysis by scanning electron microscopy and energy dispersive X-ray analysis H) confirms the presence of titanium-rich particles inside of the tissue sections taken from the $fgTiO_2$-fed mice. B–E) Scale bars = 25 µm, with 10 µm in zoomed insets. F–H) Scale bars = 10 µm.

Interestingly, the low and medium diets (6.25 and 62.5 mg $fgTiO_2$ kg^{-1} diet) only generated very lightly impacted cells at the base of the Peyer's patch, whereas the high diet (625 mg $fgTiO_2$ kg^{-1} diet) generated something akin to "precursor pigment cells" (Figure 1B–E). These latter findings were much more reminiscent of the cellular $fgTiO_2$ loading previously observed in humans in this tissue region.[18–23]

Throughout the feeding, the mice appeared healthy and weight gains were, as expected, different between female and male mice within each diet group, but there was no difference between the diet groups (**Figure 2**). Overall, from these data we could estimate that mice were, on average, each exposed to ≈17 µg (low), 165 µg (medium), and 1672 µg (high) of $fgTiO_2$ per day (**Table 1**). Titanium dioxide was not present in the baseline diet of the control group.

In the flow cytometry-based analyses of digested Peyer's patch cell suspensions (Figures S4 and S5, Supporting Information), at different time points, repeated-measures analysis of variance (ANOVA) demonstrated an effect of time on the mean live phagocyte gate ($P < 0.0001$), with significant differences between week 6 and week 12 and between week 6 and week 18 (**Figure 3**). However, there was no influence of sex or diet ($P > 0.6$) on the percentages of live phagocytes. There was also no significant effect of time, sex, or diet on the live lymphocytes ($P > 0.2$). For the subpopulations of cells, i.e., CD4 or CD8 T cells, B cells, or different phagocytes (CD11b/CD11c/double negative), the cell marker percentages were reliably consistent between groups (Figures S6 and S7, Supporting Information). CD8α $CD11c^{high}$ dendritic cells are renowned for the cross-presentation of exogenous antigen and stimulation of $CD8^+$ T-cell responses.[26] Phenotypic analyses of the phagocytic population present in the Peyer's patches whole digests were extended to examine CD8α and CD11b expression on $CD11c^{high}$ subsets. This also failed to demonstrate any indication of the promotion of proinflammatory innate immune cell subsets resulting from the $fgTiO_2$ diet (Figure S8 and Tables S1 and S2, Supporting Information). The findings show, therefore, that long-term feeding of $fgTiO_2$ does not affect immune cell populations of the Peyer's patch, despite additionally confirming this to be a site of uptake and basal accumulation for the particles. More detailed analyses can now be undertaken in future work and outcomes in Peyer's patches linked to particle exposure, rather than some indirect effect of cell recruitment or migration.

In summary, in contrast to current mainstream work, this study demonstrates that incorporation of $fgTiO_2$ into the AIN-76A diet permits uptake and subsequent delivery of $fgTiO_2$ to target sites of known human relevance, which is a significant advancement for enabling oral particle risk assessment. Importantly, diets as high as ≈100 mg kg^{-1} body weight per day can be delivered via normal feeding without any evidence of gross perturbation of immune-cell physiology. We also demonstrate how simple reflectance microscopy of forming pigmented cells at the base of Peyer's patches can be used to validate successful exposure. In the view of the authors, this kind of validation should become an essential component for in vivo studies with $fgTiO_2$. For example, a recent study where growing rats were fed a pelleted diet containing ≈40, 400, or 5000 mg $fgTiO_2$ kg^{-1} diet, and then a large range of analyses undertaken, provided no information to confirm that tissues were actually exposed in the study.[27] This makes the suite of "no effect outcomes" that were reported difficult to interpret.

The reporting of persistent particles, including titanium dioxide, associating with Peyer's patches in murine experiments is not new. However, prior studies bear no relation to the advances reported here. For example, following oral gavage with large TiO_2 particles (475 ± 24 nm), Jani et al. report an implausible tissue uptake of 11.9% and, with electron microscopy, they show a particle "in the vicinity of a Peyer's patch" with some apparently also noted in the "granular areas."[28] Bettini et al. report analytical signals that are suggestive of titanium dioxide somewhere in the murine Peyer's patch after addition of the particles to drinking water.[29] Again, in the context of orally dosed titanium dioxide, Warheit et al. mention Peyer's patches in a table.[30] The work that we present is entirely different in that it tracks properly-fed $fgTiO_2$ into specific cell sites, namely the base of the Peyer's patch, exactly as is seen in humans. Furthermore, we demonstrate how this is measurable with a simple light microscopy technique, yielding a readily accessible, site-specific "biomarker" that can be used to confirm successful uptake and cell exposure after feeding.

In short, the diet and methodology presented here provide for a physiologically relevant, oral dosing approach in particle risk assessment with appropriate tissue exposure measures. It will allow much more detailed analyses of the potential effects of $fgTiO_2$ to now be undertaken, with proper human relevance, and should serve as a base for any such nanoparticle or microparticle research that is interested in the physiological and long-term dosing of persistent particles. Moreover, as noted above, this approach can be readily adopted within the OECD framework for next-generation risk assessments. For example, it is known that human cells are impacted by $fgTiO_2$, and accumulation increases with age. Further steps would be a careful assessment of toxicity potential in a tissue-relevant fashion. Indeed, Peyer's patches are key structures in intestinal immunosurveillance and in the generation of immune responses, notably involving immunoglobulin A. How human-relevant exposure to $fgTiO_2$ impacts such functionality can now be addressed, beyond our current knowledge that gross perturbation of the system (cell infiltration) does not occur. In addition, how mesenteric lymph nodes are impacted by oral $fgTiO_2$ can also now be investigated, alongside the implications for gastrointestinal immune responses.

Experimental Section

The study procedures described were approved by the Grasslands Animal Ethics Committee (Palmerston North, New Zealand) in accordance with the New Zealand Animal Welfare Act 1999. The $fgTiO_2$ was obtained from Sensient Colors (St. Louis, MO, USA). C57BL/6 mice aged 6 weeks were randomly assigned to one of four diets (0, 6.25, 62.5, or 625 mg $fgTiO_2$ kg^{-1} of diet) exposing the mice to, respectively, 0 and ≈1, 10, and 100 mg $fgTiO_2$ kg^{-1} body weight per day. Mice were housed conventionally with feed intake and body weight recorded biweekly. At 6, 12, and 18 weeks, mice were euthanized and the gastrointestinal tracts harvested. Ileal tissues containing the most distal Peyer's patch were excised and snap frozen. Those from 18 weeks feeding were examined via confocal microscopy and SEM with EDX analysis. Remaining Peyer's patches were collected and enzymatically digested to form single-cell suspensions for flow cytometry analysis after staining with either lymphocyte

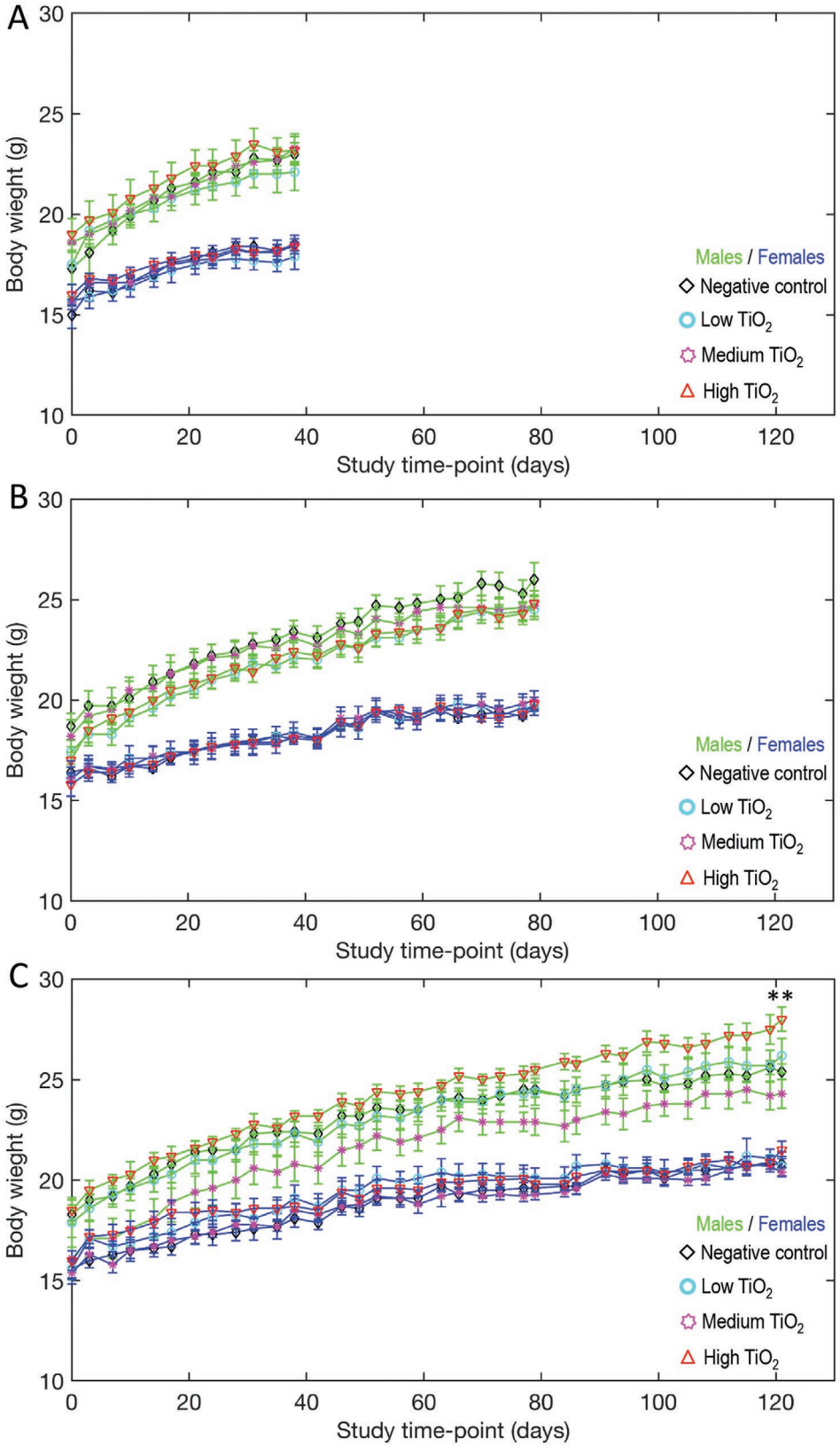

Figure 2. Body weights of wild-type C57BL/6 mice fed with AIN-76A diet ± fgTiO_2 at three levels. Body weights were recorded twice weekly for male (M: green) and female (F: blue) mice over A) 6 weeks, B) 12 weeks, or C) 18 weeks. Means ± standard error of the mean (SEM) are shown ($n = 6$ M and 6 F). ** $P \leqq 0.01$ male versus female body weights (one-way ANOVA with initial body weight used as a covariate).

Table 1. Mean daily TiO_2 intake and daily TiO_2 dose of wild-type mice fed with the AIN-76A TiO_2 containing diet.

		Daily TiO_2 intake [mg day^{-1}]			
Sex	Diet	6 weeks	12 weeks	18 weeks	*P* value[a)]
Female	6.25	0.016 ± 0.002	0.016 ± 0.001	0.016 ± 0.001	0.89
	62.5	0.162 ± 0.008	0.154 ± 0.012	0.161 ± 0.012	0.41
	625	1.621 ± 0.126	1.569 ± 0.087	1.543 ± 0.081	0.32
		Daily TiO_2 dose [mg kg^{-1} body weight]			
	6.25	0.897 ± 0.071**	0.825 ± 0.084**	0.759 ± 0.029**	<0.05[b)]
	62.5	8.737 ± 0.365*	7.730 ± 0.552*	7.931 ± 0.620*	<0.05[b)]
	625	88.027 ± 6.886**	79.451 ± 6.708**	71.827 ± 4.640**	<0.01[b)]
		Daily TiO_2 intake [mg day^{-1}]			
Male	6.25	0.017 ± 0.001	0.018 ± 0.002	0.018 ± 0.002	0.34
	62.5	0.176 ± 0.018	0.172 ± 0.005	0.166 ± 0.007	0.38
	625	1.783 ± 0.133	1.750 ± 0.102	1.768 ± 0.145	0.98
		Daily TiO_2 dose [mg kg^{-1} body weight]			
	6.25	0.763 ± 0.049	0.737 ± 0.065	0.682 ± 0.076	0.11
	62.5	7.586 ± 0.990	6.965 ± 0.402	6.876 ± 0.516	0.24
	625	76.827 ± 4.700***	70.853 ± 5.564***	63.335 ± 4.933***	<0.05[b)]

[a)]Statistical comparison with one way ANOVA; [b)]Pairwise comparison of group means with Tukey's honestly significant difference test; significant differences between groups with superscript letters $^{*}P < 0.05$, $^{**}P \leq 0.01$, and $^{***}P < 0.001$.

(CD4, CD45R, and CD8a) or myeloid (CD11b, CD11c, and CD8a) antibody staining cocktails with propidium iodide (PI) for dead cell exclusion.

For confocal microscopy and SEM–EDX, frozen ileal tissue was embedded and 25 µm thick cryostat sections cut and collected on slides. Nuclei were labeled using Hoechst 33 342 and slides were mounted with Prolong Diamond mountant. Reflectance confocal microscopy images were obtained using a Leica SP8 confocal microscope equipped with a 40×/1.3 oil immersion objective. A 405 nm laser was used for Hoechst

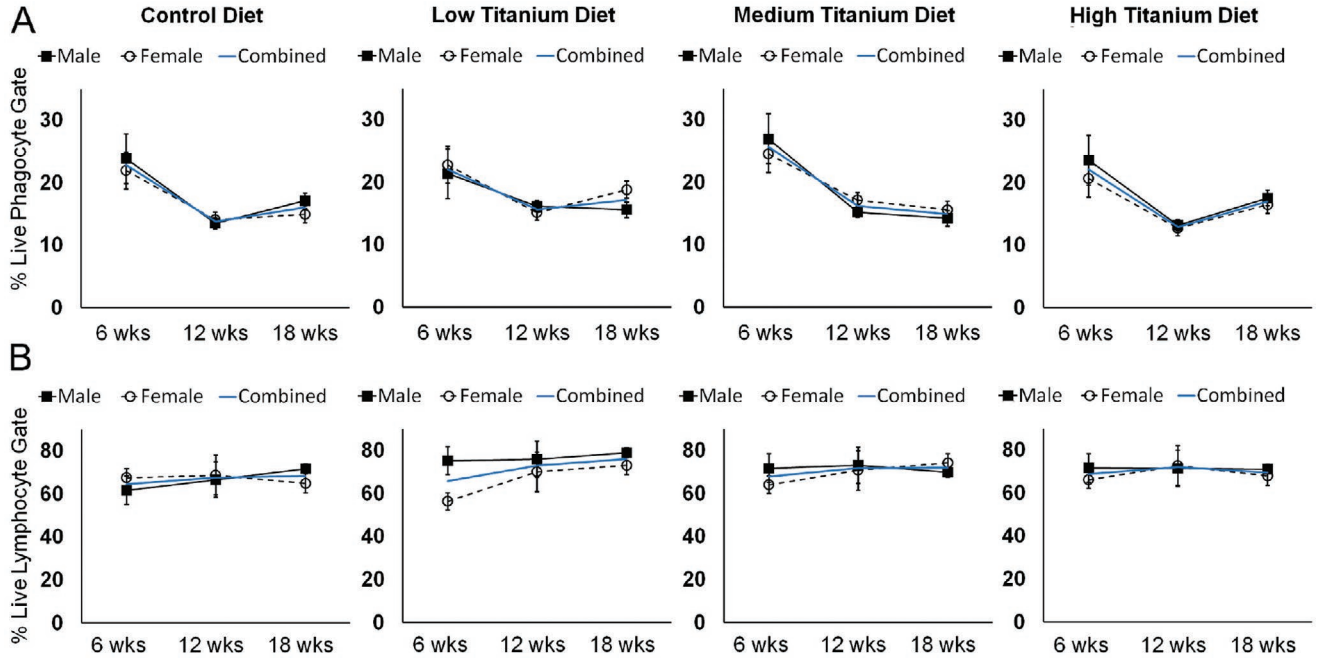

Figure 3. Frequencies of live cells residing within phagocyte and lymphocyte flow cytometric cell gates. Flow cytometric analysis of filtered, propidium iodide (PI)-stained single-cell suspensions derived from Peyer's patch tissues of mice fed the AIN-76A diet, with or without added $fgTiO_2$, for 6, 12, or 18 weeks. Control, low, medium, and high titanium dioxide diets refer to the amount of $fgTiO_2$ added to the basal diet as defined in the text. The mean ± SEM percentages of live (PI negative) cells residing within the A) phagocyte or B) lymphocyte gates are shown; (n = 6–8). Full flow cytometry gating and analysis strategy and a further breakdown of cell phenotype are shown in the Supporting Information. No significant differences were found between any of the diets for the combined (n = 6–8), male (n = 3–4), or female (n = 3–4) groups.

33 342 excitation. On a second sequence, reflectance from a 488 nm laser was used to detect the fgTiO_2. As noted in the text for results and discussion, a robust threshold for the detection of fgTiO_2 was made by removing all signals from tile scanned images collected from negative control mice (i.e., exposed to the base diet but not fgTiO_2). A red circle marker was placed on each reflectance pixel above the threshold using the inbuilt "insertShape" function in MATLAB R2019b (MathWorks). After confocal microscopy, cover slips were removed and the slides dried, cut down, mounted on stubs using colloidal graphite, and lightly sputter-coated with carbon for SEM/EDX of Peyer's patch regions. A description of the statistical analyses and further details of methods can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

S.R. and J.W.W. contributed equally to this work as first authors, N.C.R., R.E.H. and J.J.P. equally contributed as senior authors. The authors would like to thank Doug Hopcroft and Dr. Jianyu Chen (both Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North) for help with electron microscopy of titanium containing diets; Ric Broadhurst and Genevieve Baildon (Campus Services, AgResearch, Hamilton) for providing the mice; Leigh Ryan and Jason Peters (both Food Nutrition & Health Team, AgResearch, Palmerston North) for help with animal study and tissue sampling; and Dr. John Koolaard and Catherine Lloyd-West (Campus Services, AgResearch, Palmerston North) for help with statistical analyses. The research was mainly supported by the Riddet Institute through its Centre of Research Excellence funding which has been awarded to the Riddet Institute by the New Zealand government. Additional funding was provided by AgResearch, MRC Elsie Widdowson Laboratory (formerly MRC Human Nutrition Research, Grant No. U105960399) and Nutrigenomics New Zealand, a collaboration between AgResearch, Plant and Food Research, and The University of Auckland (primarily supported by funding from the Ministry for Science and Innovation contract C11 × 1009). S.R. was supported by doctoral scholarships from Massey University and AgResearch. All of the work in this study that was carried out at the University of Cambridge was supported by the UK Medical Research Council (R.J., R.E.H., and J.J.P.; Grant No. MR/R005699/1). J.W. would like to acknowledge Girton College and the University of Cambridge Herchel-Smith fund for providing him with Fellowships.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

diet, nanoparticles, Peyer's patches, titanium dioxide, validated exposure

Received: January 24, 2020
Revised: March 9, 2020
Published online:

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