Authors' Response to Reply: Ketogenic diet activates protective $\gamma\delta$ T cell responses against influenza virus infection

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We are glad for the broad interest and readership our recent publication “Ketogenic diet activates protective $\gamma\delta$ T cell responses against influenza virus infection” has received (1, 2). The goal of our study was to understand how the immune system might be involved in mediating the surprising protection against lethal influenza A virus infection we observed when we fed mice a ketogenic diet (KD) for one week. What made our findings particularly intriguing is that robust protective changes were occurring in the lungs, the site of influenza infection and, importantly, not a tissue traditionally considered in manipulations of systemic metabolism, and that these protective changes were $\gamma\delta$ T cell-dependent. Amidst this enthusiasm, Bass et al. have raised concern about the control diets used in our experiments, specifically with regards to the protein, fiber, and micronutrient content (3). We welcome critical feedback and appreciate the opportunity to respond to these criticisms.

Bass et al. recently wrote a reply to our study stating “the design of the dietary interventions reported in Goldberg et al., 2019 is confounded by the use of inappropriately matched comparator diets” and that this “obviate[s] our ability to make causal inferences about the dietary factors impacting the observed phenotypes.” The authors express particular concern

for mismatches in protein, fiber, and micronutrient contents. In addition, they question why we did not use the commercial control chow that matches the percent of calories from protein contained in the KD. Bass et al. rightfully point out that many previously-published rodent studies use KD formulations with low protein content. However, what Bass et al. have neglected to discuss is the actual amount of protein in each of these diets (18.6% in standard chow, 17.6% in KD, 10.2% in KD control chow, as % w/w), instead focusing solely on the percent of calories from protein (24% in standard chow, 10.4% in KD, 10.4% in KD control chow, as % of calories derived from protein). When designing our experiments, we considered the actual amount of protein in each diet (Table 1) and decided that, given clinical recommendations for protein intake can be based on body weight rather than percent of calories, we opted for control diets with similar *amounts* of protein. This aspect is especially important when considering total protein intake because the control chow sold to match percent calories from protein with KD actually contains 42% less protein compared to KD and 45% less protein compared to the 2018S standard chow we used in our experiments. Thus, contrary to Bass et al.'s comments, mice fed this alternative recommended control are likely to be more protein-restricted than any of the mice in our experiments. Moreover, in opposition to an initial increase of $\gamma\delta$ T cells as observed in influenza-infected mice, long-term KD feeding (which, according to Bass et al.'s prediction, should further exacerbate restriction-driven effects) caused the depletion of $\gamma\delta$ T cells (4). Indeed, low protein content in KD (5% of calories, 9.5% w/w) promotes sustained weight loss (5) whereas normal protein content actually permits *ad libitum* KD-induced weight gain (6, 7). These results underscore our choice of using a control diet with protein content matched by “amount” by weight.

Bass et al. correctly point out the importance of adequate protein and micronutrient intake for immunology studies. The authors recommend using the “prudent approach” of designing a customized high-fat diet in which Primex (the source of excess fat in KD) be added in place of dextrose to control for other dietary background differences. Had our scientific question been focused on the dose-responsiveness of Primex or isolating effects of other specific dietary factors, we would have been obliged to use this approach. Instead, we used a standard high-fat diet simply to ask if providing excess calories could rescue mice from lethal influenza A virus infection. This was a reasonable approach given that weight loss is a gross indicator of disease severity and that energy restriction increases murine susceptibility to influenza virus infection (8-10).

We acknowledge that fiber and micronutrient mismatches in diets deserves further attention. We would argue that this is also a clinically-relevant problem that people on KD face, and thus is an important aspect of the model. It should also be noted that previous studies describing the impact of nutrient restriction on immune system function indicate that restrictions induce lymphopenia (11-15), which is opposite to our findings that 7 days of KD increased $\gamma\delta$ T cell numbers in the lungs and adipose tissue (1, 4). Furthermore, as described above in relation to protein, if our phenotype were driven by differences in relative amounts of various macronutrients and/or micronutrients (based on Kcal composition), one might expect long-term feeding to potentiate our $\gamma\delta$ T cell phenotype. Instead, we observed the opposite in adipose tissue: 7 days of KD feeding expanded $\gamma\delta$ T cells, like we reported in the lung, but 2+ months of KD feeding led to loss of $\gamma\delta$ T cells. If the reduction of fiber, etc was driving the $\gamma\delta$ T cell expansion, this effect would be sustained after 2 months of KD feeding, which was not borne out by our experiments (4). The sticky texture of KD prevented us from obtaining reliable food

intake measurements, but it is known that mice over-consume high-fat-containing diets which is why they've been a longstanding popular diet-induced obesity model. Therefore, we also contend that food amount, rather than calories, is a more appropriate denominator for comparing dietary exposures when caloric density is variable. Moreover, this is especially important for macronutrients like protein and fiber which are not the preferred calorie-producing substrates like fat and carbohydrates.

Taken together, our data support a model that mitochondrial substrate switch favored by ketogenesis drives the immunological effects observed in our experiments. We thank Bass et al. for the opportunity to address their concerns. We share their enthusiasm for rigorous nutritional immunology studies and interest in harnessing metabolic interactions that hopefully improve health outcomes in people. Indeed, additional studies are needed for better understanding of how nutrition and metabolism intersect with immune function for development of therapeutic diets against infections and chronic diseases.

Table 1. Comparison of macronutrient contents of selected diets.

	% of Calories				% w/w			
	2018S chow	KD	HFD	KD control recommended by authors	2018S chow	KD	HFD	KD control recommended by authors
Protein	24	10.4	20	10.4	18.6	17.6	26.23	10.2
Fat	18	89.5	60	11.5	6.2	67.2	34.89	5
Carb	58	0.1	20	78.1	44.2	0.2	25.56	76.2
Density (Kcal/g)	3.1	6.76	5.21	3.9				

The amounts of protein, fat, and carbohydrates are shown both as percentage of total calories (% of Calories) and percentage of food based on weight (% w/w).

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