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Dietary whey protein increases brain leukocytes in mice regardless of their hypersensitivity status

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Abstract

Cow's milk allergy (CMA) often manifests as milder reactions and may be linked to neurological problems. Previously, we demonstrated that C57BL/6J mice sensitized to a bovine whey allergen, β-lactoglobulin (BLG, Bos d 5), moderately increased BLG-specific IgE levels and exhibited behavioral changes without severe allergic reactions. When these non-anaphylactic CMA mice were placed on a whey-protein (WP)-containing diet for 2 weeks to simulate continuous dairy consumption, we found neuropathology indicative of neuroinflammation and cortical demyelination. Since immune cells migrate to the central nervous system (CNS) and promote neuroinflammation in demyelinating conditions such as multiple sclerosis, we hypothesized that the number of leukocytes would increase in BLG-sensitized mouse brains to orchestrate neuropathology. To test this hypothesis, we used flow cytometry to determine the number and phenotypes of leukocytes in the brains of naïve, sham, and BLG-sensitized mice after the 2 weeks of the WP diet. The frequencies of cells expressing common leukocyte marker CD45, pan T cell marker CD3, cytotoxic T cell marker CD8, integrin CD11b, myeloid cell marker CD14, and co-stimulatory marker CD86 significantly increased, regardless of the sensitization status. The percentages of these cells were low in mice that never received WP. This result indicated that WP diet consumption alone increased CNS leukocyte populations. Additional immunophenotyping is needed to determine whether the identified cells can be differentiated among the experimental groups. Detailed characterization of CNS leukocyte phenotypes and dynamics will help elucidate the mechanism of CMA-induced neuroinflammation and cortical demyelination.

Background

Cow's milk allergy (CMA)

- Reactions range from mild and delayed to severe and immediate (1).
- CMA tends to manifest with milder allergic reactions (2).
- People with mild allergic reactions have increased re-exposure risks.
- CMA has been associated with neuropsychiatric disorders (3,4,5).
- How allergen-triggered inflammatory signals from the gut reach the brain is unclear.

Hypothesis

- Repeated allergen consumption by individuals with mild food hypersensitivities promotes leukocyte migration to the central nervous system (CNS), leading to neuroinflammatory pathologies and subsequent behavioral changes.

Objectives

- Compare the number of leukocytes in the brains of naïve, sham, and β-lactoglobulin (BLG)-sensitized mice by flow cytometry.
- Determine the immunophenotypes of the leukocytes in the brain.

Methods

Animals: Four-week-old male C57BL/6J mice were used. All procedures involving animals were approved by the University of North Dakota IACUC.

Sensitization phase: Mice were given either 200 µL vehicle (10 µg cholera toxin in $NaHCO_3$ buffer, pH 9.0) without (sham) or with 1 mg β-lactoglobulin (BLG) once a week orally for 5 weeks. Naïve mice were not treated. All mice were fed whey-protein-free control (CTL) diet (Envigo Teklad 2018) during the sensitization phase.

Exposure phase: Except for a designated group of naïve mice that stayed on the CTL diet, all other mice were placed on a whey-protein (WP) containing diet (Envigo Teklad 8640) for 2 weeks following the sensitization phase.

Sample collections: Mice were euthanized via CO_2 asphyxiation at the end of week 7 and brains were collected after intracardiac perfusion with PBS.

Flow cytometry: Single-cell suspensions were prepared from the collected brains (Miltenyi Biotec, cat #130-107-677), incubated with selected antibodies against cell surface markers, and flow cytometry was performed using BD FACSymphony.

Week 1-5	*Week 6-7*
Naïve: no treatment; CTL diet	CTL diet
Naïve: no treatment; CTL diet	WP diet
Sham: CT only x 5; CTL diet	WP diet
BLG: 1 mg BLG+CT x 5; CTL diet	WP diet

Fig 1: Experimental groups and timeline. CTL diet: whey-protein-free rodent chow (Envigo Teklad 2018); **WP diet:** whey-protein-containing rodent chow (Envigo Teklad 8640). **Week 1-5:** sensitization phase; **Week 6-7:** allergen exposure phase. **CT:** cholera toxin. **CTL:** control diet. **WP:** whey protein diet.

Adoptive transfer and cell tracking: After 2 weeks of the allergen exposure phase, single-cell preparations from the spleens of naïve, sham, or BLG-sensitized mice were labeled with a cell tracking dye (AB269446) and adoptively transferred to naïve recipient mice via tail vein injection. Recipient mice were subsequently placed on the WP diet for 2 weeks and the dura was collected at Week 7.

Results

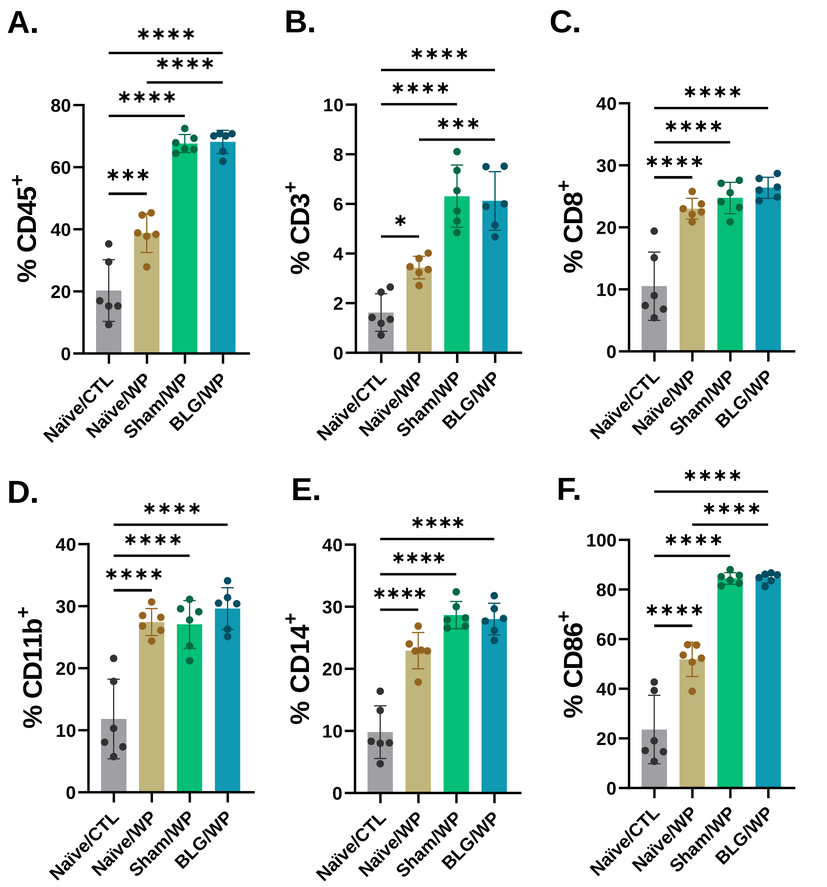

Fig 2: Allergen consumption increased leukocytes in the brain. Naïve, sham, and BLG-sensitized mice were placed on a WP diet during the exposure phase. A group of naïve mice that stayed on the CTL diet throughout the experiment served as a control. **(A)** CD45: pan leukocyte marker; **(B)** CD3: pan T-cell marker; **(C)** CD8: cytotoxic T-cell marker, (**D**) CD11b: a marker for macrophages, dendritic cells, neutrophils, and natural killer cells, **(E)** CD14: a marker for macrophages, dendritic cells, and neutrophils; **(F)** CD86: a surface marker for memory B cells, germinal center B cells, and macrophages. **CTL:** whey-free control diet, **WP:** whey-protein diet. Bars indicate group average ± SEM. One-way ANOVA. ***p<0.001 and ****p <0.0001. N=6 per group.

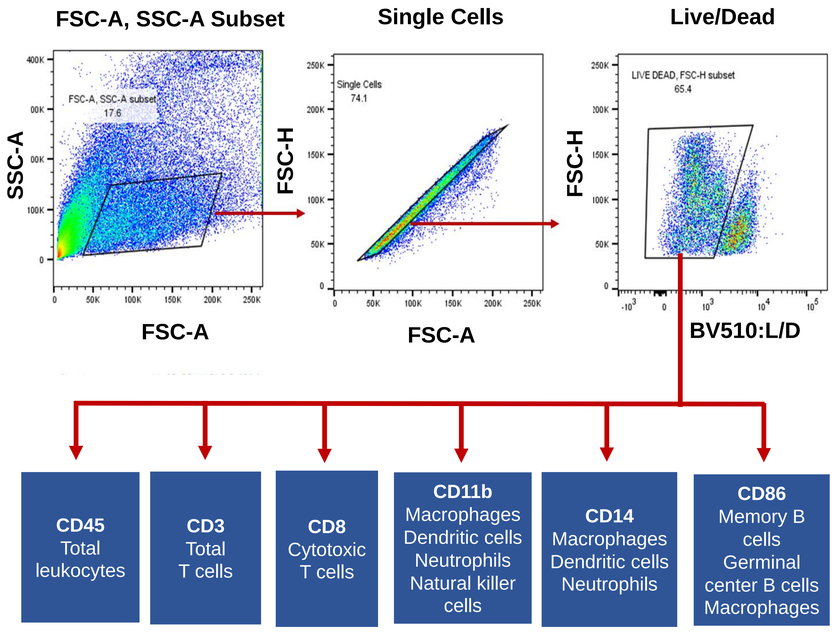

Fig 3: Gating strategy for flow cytometric analysis. SSC: side scatter, FSC: forward scatter. Live cell population further gated to the different surface cell markers to identify the immunophenotypes of cells (Blue boxes).

Marker	IA/IE	Viability	CD14	CD45	CD19	CD83	CD86	CD8	CD25	CD11b	CD4	CD27	CD80	CD3
Fluoro-phore	BV 421	BV 510	BV 605	BV 711	BV7 86	BV 650	FITC	PerCP Cy5.5	PE	PE Dazzle	PE Cy7	AF 700	APC	APC Cy7

Table 1: Antibody panel used

Results (cont'd)

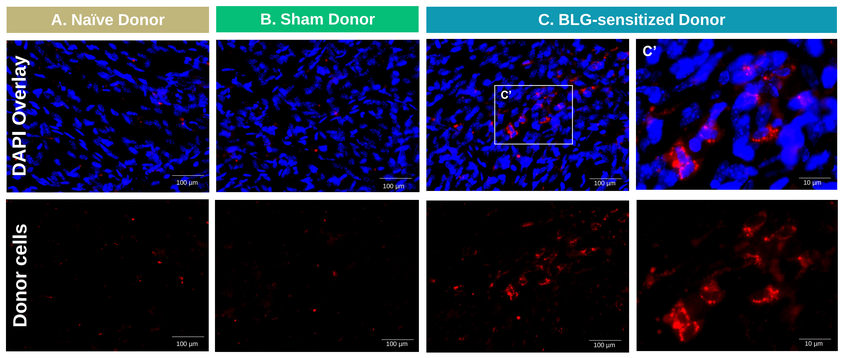

Fig 4: Splenocytes from BLG-sensitized donor mice trafficked to and stayed in the dura of naïve recipient mice. The air-dried, whole-mounted dural tissues were briefly rehydrated, cover slipped with VETASHIELD® mounting media with DAPI, and imaged using a Keyence microscope. Cy5-labeled donor splenocytes from naïve (**A**), sham (**B**), and BLG-sensitized (**C**) mice are shown in red, and DAPI nuclear stain is shown in blue. Magnification: 40x (A-C), 100x (C'). Imaged area: right upper area closed to the superior sagittal sinus. N=4 per group.

Discussions

- The consumption of the allergen alone increased the number of total leukocytes in the brain, regardless of the sensitization status of the animals. This result suggested that the introduction of an allergenic protein is communicated to the brain at least in part by leukocytes.
- Flow cytometric immunophenotyping suggested that the leukocytes found in the brain after WP consumption were likely T and B cells, macrophages, dendritic cells, neutrophils, and/or natural killer cells.
- The increases in the CD8+ and CD86+ cell populations indicated that the leukocytes trafficked to the brain included cytotoxic T cells and memory/germinal center B cells, respectively.
- Adoptively transferred splenocytes from BLG-sensitized donors, but not naïve or sham donors, were found in the dura of naïve recipient mice. These results suggested that BLG sensitization might influence peripheral leukocytes to reside longer in the central nervous system.

Conclusions

- Consumption of an allergenic protein promotes leukocyte trafficking to the brain, triggering gut-brain communication.
- The dura may serve as a "hub" for peripheral leukocytes that can elicit allergy-associated immune responses in the central nervous system. Whether they infiltrate the brain parenchyma and/or contribute to the development of neuropathology is yet to be investigated.

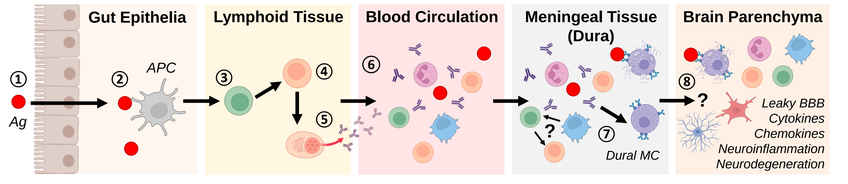

Fig 5: Working hypothesis. A diagram depicting a potential sequence of events following allergen (Ag) consumption. **1**) transepithelial Ag entry; **2**) Ag recognition by antigen-presenting cells (APC); **3**) T cell activation; **4**) B cell differentiation; **5**) antibody production; **6**) leukocyte & antibody intravasation from the circulation into the dura; **7**) antibody coupling to dural mast cells (MC) and additional interactions among aggregated leukocytes; **8**) possible infiltration of dural leukocytes to the brain parenchyma and the effects on the brain. BBB: blood-brain barrier.

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