2023

Continuous consumption of whey protein maintains neuroinflammation in a mouse model of asymptomatic cow's milk allergy

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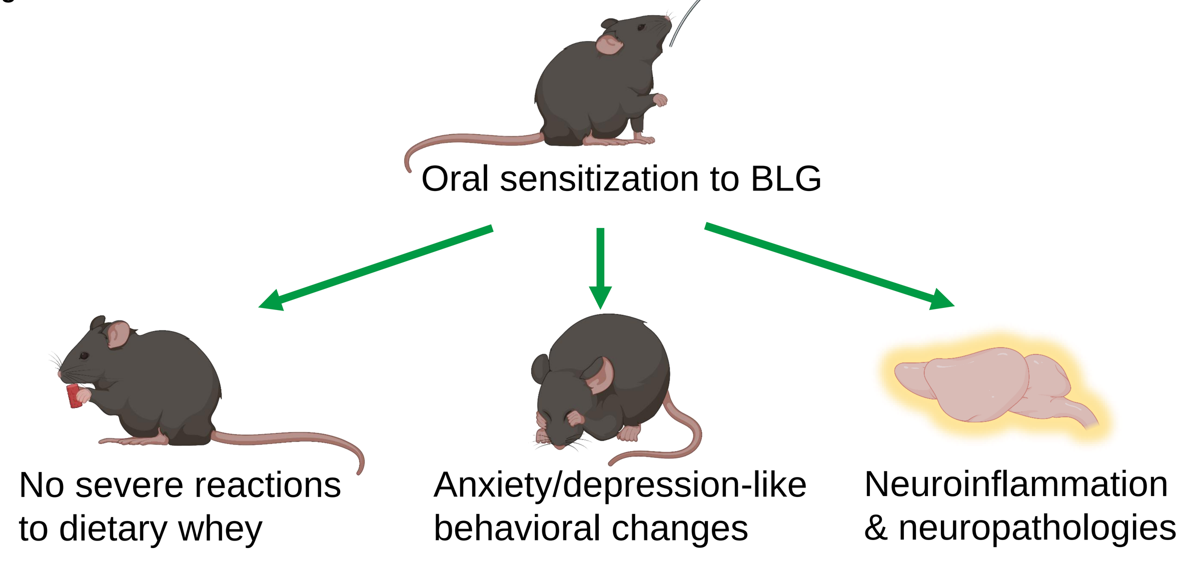
Introduction

Using a mouse model of cow's milk allergy (CMA), we previously showed that C57BL/6J mice sensitized to a bovine whey allergen, β-lactoglobulin (BLG), exhibited anxiety/depression-like behavior and neuroinflammation in the absence of overt anaphylaxis upon allergen exposure (1, 2).

Fig 1. Previous observations in our CMA mouse model.



Hypothesis

Inflammatory immune responses and behavior changes observed in BLG-sensitized mice after the initial allergen exposure would be resolved with allergen avoidance.

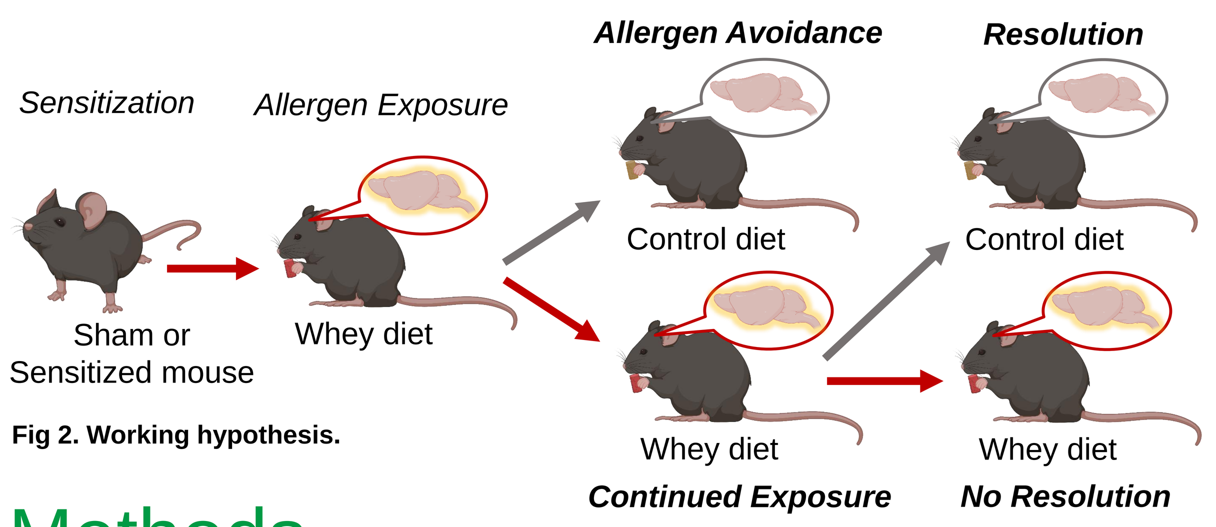


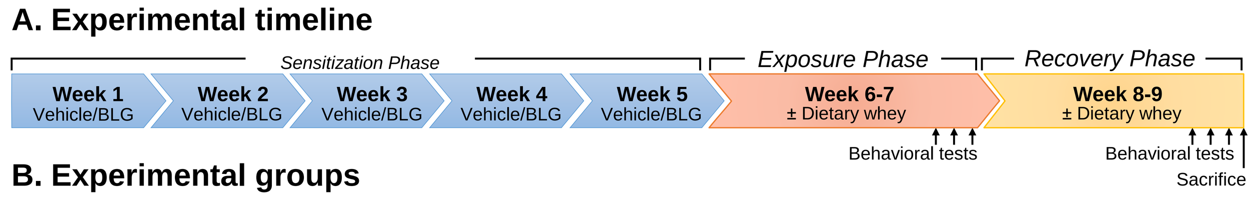
Fig 2. Working hypothesis.

Methods

Allergen sensitization, exposure, and avoidance: Four-week-old C57BL/6J male mice were subjected to five weekly oral gavage with either a carbonate buffer (pH 9.0) containing 10 µg cholera toxin (adjuvant) or 1 mg BLG in the vehicle to generate sham or BLG-sensitized mice, respectively. Following the 2-week allergen exposure phase when all mice were placed on a whey-protein (WP) diet, assigned groups of mice continued on the WP diet or placed on a WP-free control (CTL) diet. *All procedures were approved by UND IACUC prior to the experiments.*

Behavior assessments: The all-limb grip strength test and the cross-maze test were performed during the second week of the recovery phase (Week 9) to assess the animals' motor and cognitive functions (2). All mice were sacrificed one day after the completion of all behavior tests, and blood and brain samples were collected.

A. Experimental timeline



B. Experimental groups

Treatment Phases	C57BL/6J Male Mice			
Sensitization Phase (Week 1-5)	Sham (Vehicle Only)		BLG (1 mg BLG)	
Exposure Phase (Week 6-7)	WP	WP	WP	WP
Recovery Phase (Week 8-9)	CTL	WP	CTL	WP

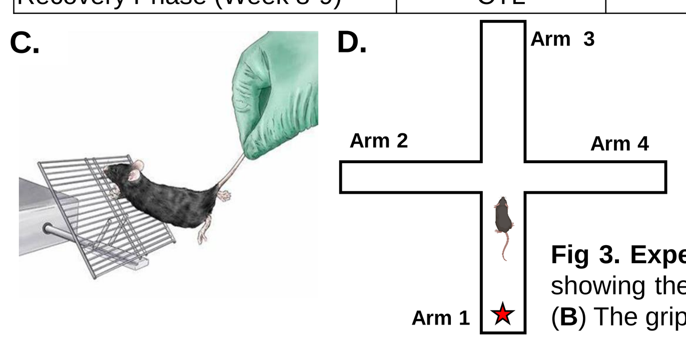


Fig 3. Experimental design. (**A**) The procedural timeline showing the sensitization, exposure and recovery phases. (**B**) The grip strength and (**C**) cross maze tests.

Cytokine/chemokine assay: The Q5 RayBiotech Quantibody® cytokine/chemokine array was used to determine inflammatory changes in the midbrain region.

Immunohistochemistry. Brain sections (40 µm) were stained with antibodies against GFAP (1:1000) or CD45 (1:500) to detect astrocytes and immune cells, respectively.

Results

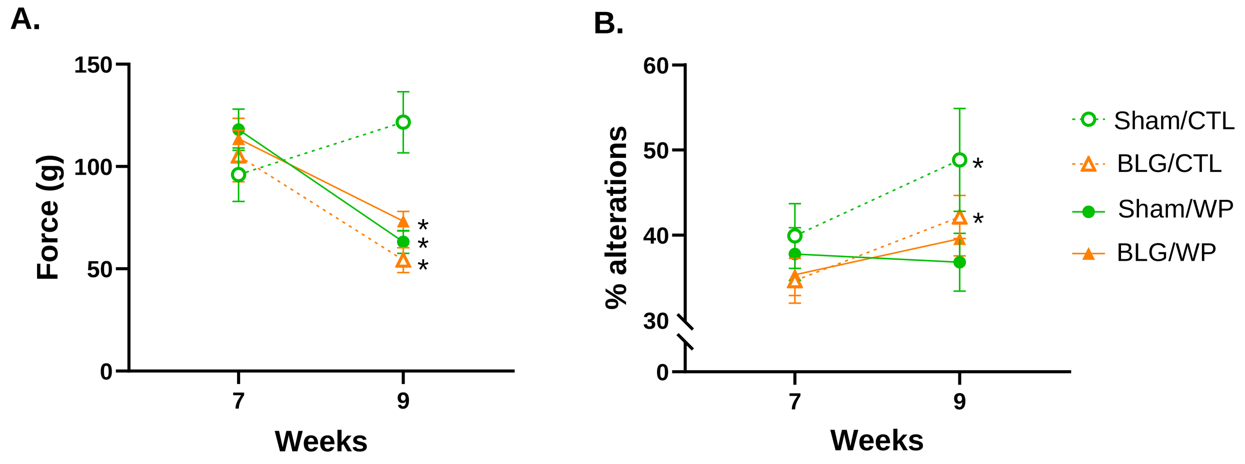
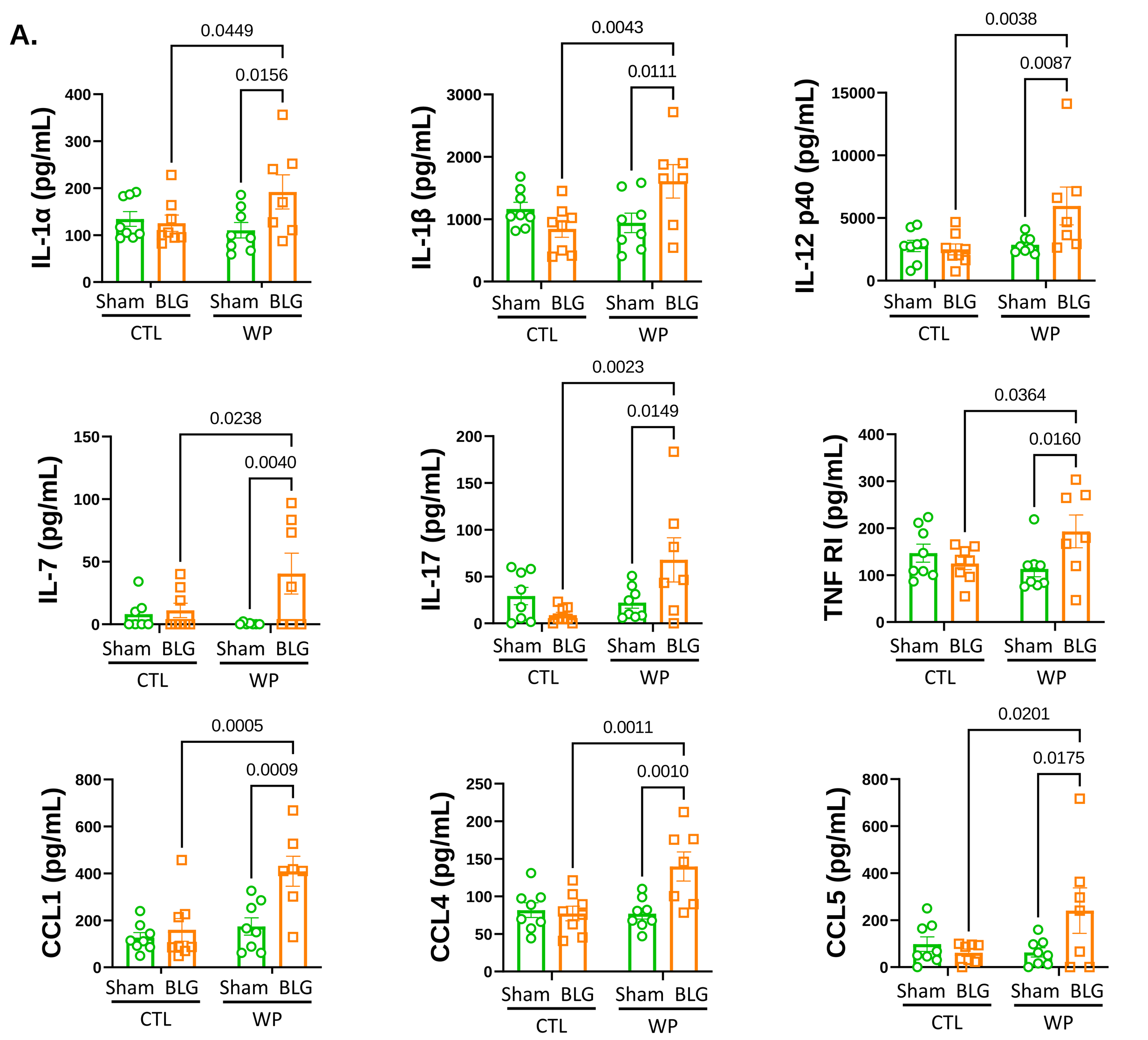


Fig 4. Behavior assessments during the recovery phase. (**A**) The grip-strength test showed declines in limb strengths of BLG-sensitized mice on either diet and sham mice on the WP diet. (**B**) The cross-maze test indicated that spatial working memory of sham and BLG-sensitized mice significantly improved with allergen avoidance. (n=7-8), *$p<0.05$; 2-way ANOVA.



B.

Class	Markers	Function(s)
Pro-inflammatory cytokines (inflammatory conditions)	IL-1α	Produced by macrophage, endothelial cells, neutrophils
	IL-1β	Monocyte activation
	IL-12 p40	Macrophage recruitment
	IL-17	Neutrophil recruitment
	TNF RI	TNFα effects
Cytokines involved in allergy	IL-4	Produced by mast cells, basophils and eosinophils, pleiotropic cytokine
	IL-13	IgE mediated allergic responses
	IL-21	B cell differentiation and Ig production
Chemokines	CCL1	Leucocyte recruitment
	CCL4	Chemoattractant for NK cells, monocytes
	CCL5	Recruits T cells
	CCL11	Chemoattractant for eosinophils, Th2 cells
	CCL12	Leucocyte recruitment
	CCL17	Chemoattractant for Treg cells
	CXCL1	Chemoattractant for neutrophils
	CXCL4	Promotes IL-6, TNFα
Adipokine	Leptin	Activation of effector T cells

Fig 5. Cytokine/chemokine changes in the brain. (A) Proinflammatory cytokines/chemokines were significantly increased in the midbrain region of BLG-sensitized mice with continued allergen consumption. Mean ± SEM (n=7-8), 2-way ANOVA. Significant p-values are shown. (B) Table showing the list of markers which significantly increased ($p<0.05$) in midbrain of BLG-sensitized mice with continuous allergen consumption compared to other groups.

Results (Cont'd)

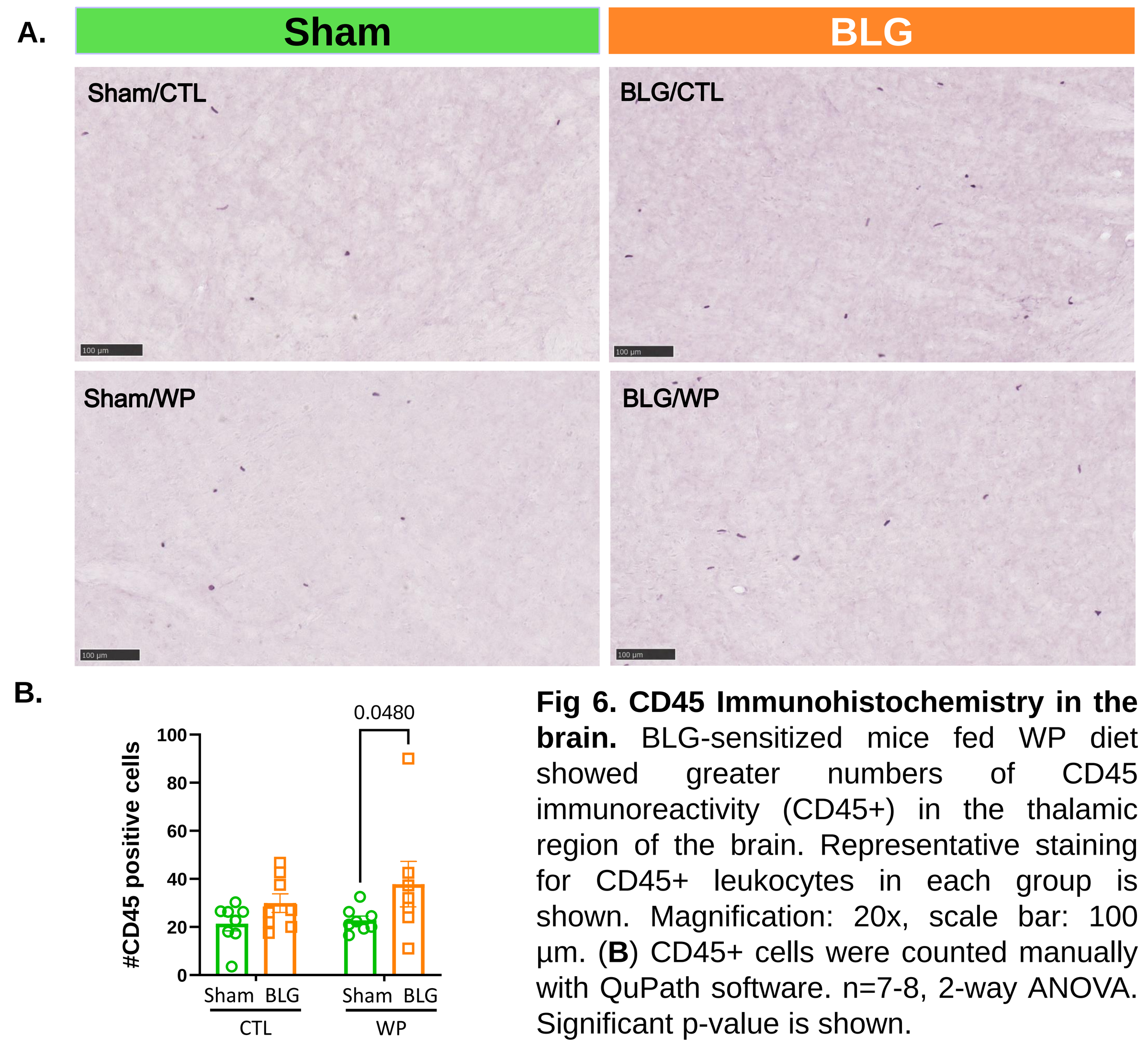


Fig 6. CD45 Immunohistochemistry in the brain. BLG-sensitized mice fed WP diet showed greater numbers of CD45 immunoreactivity (CD45+) in the thalamic region of the brain. Representative staining for CD45+ leukocytes in each group is shown. Magnification: 20x, scale bar: 100 µm. (**B**) CD45+ cells were counted manually with QuPath software. n=7-8, 2-way ANOVA. Significant p-value is shown.

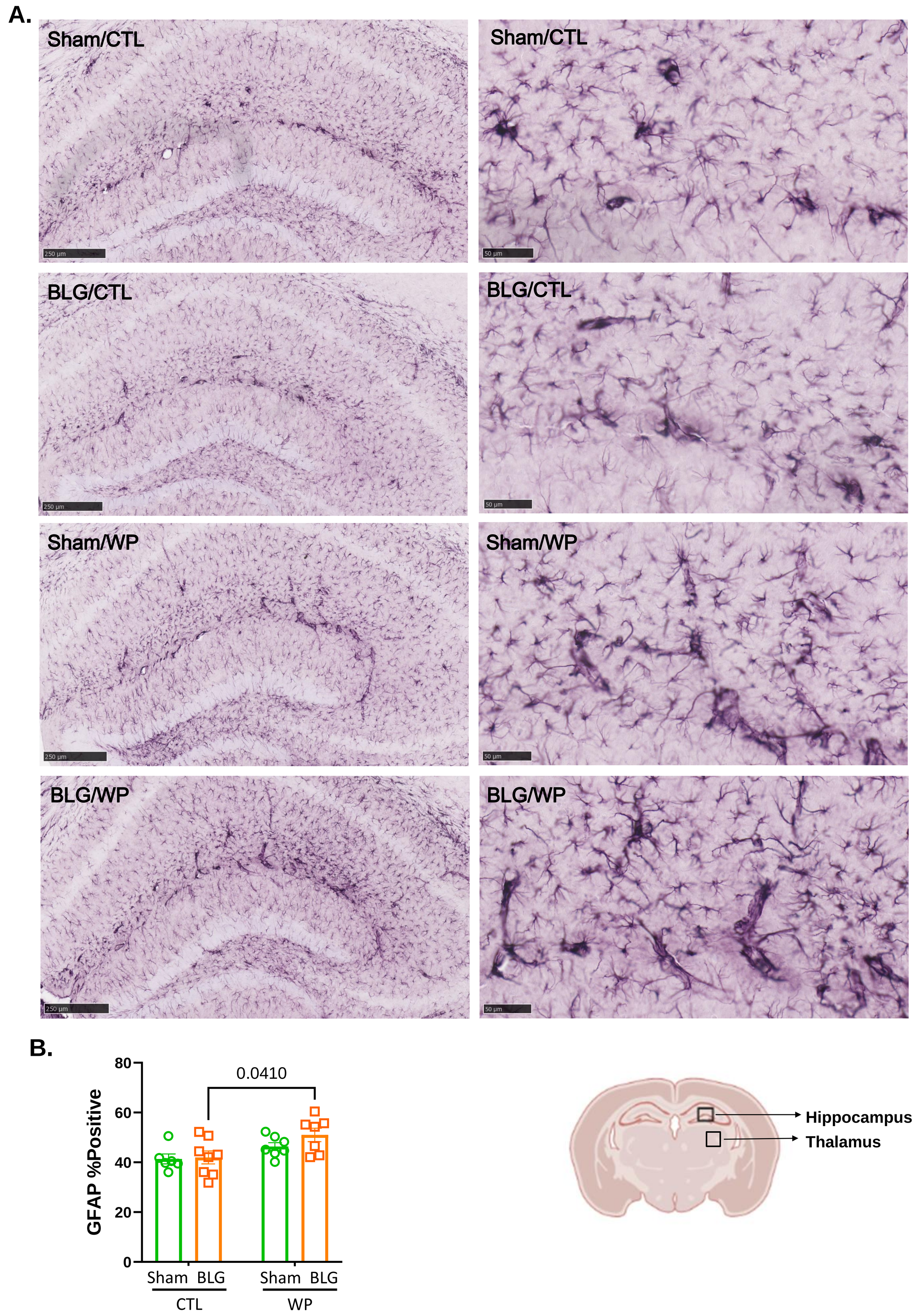


Fig 7. GFAP Immunohistochemistry in the brain. BLG-sensitized mice showed increased GFAP-immunoreactive (GFAP+) astrocytes in the hippocampal region of the brain. (**A**) Representative GFAP+ astrocyte staining for each experimental group. Magnification: 20x (left) and 40x (right), scale bars: 250 µm (left) and 50 µm (right). (**B**) GFAP immunoreactivity was quantified with the QuPath image analysis software. n=6-8, 2-way ANOVA. Significant p value is shown.

Conclusions & Discussions

- Continuous allergen exposure influenced motor function in both sham and BLG-sensitized mice.
- Dietary allergen removal improved spatial working memory of sham and BLG-sensitized mice.
- The increases in pro-inflammatory cytokines and chemokines in the BLG/WP group suggested that continued allergen exposure maintains neuroinflammatory conditions in sensitized individuals.
- Activation of astrocytes, as well as microglia and endothelial cells may be responsible for the chemotactic factors.
- Chemokines are involved in leukocyte trafficking.
- The CD45+ staining suggested that leucocyte trafficking was increased in BLG-sensitized mice, indicating ongoing immune responses and immune system-mediated pathology in brain.

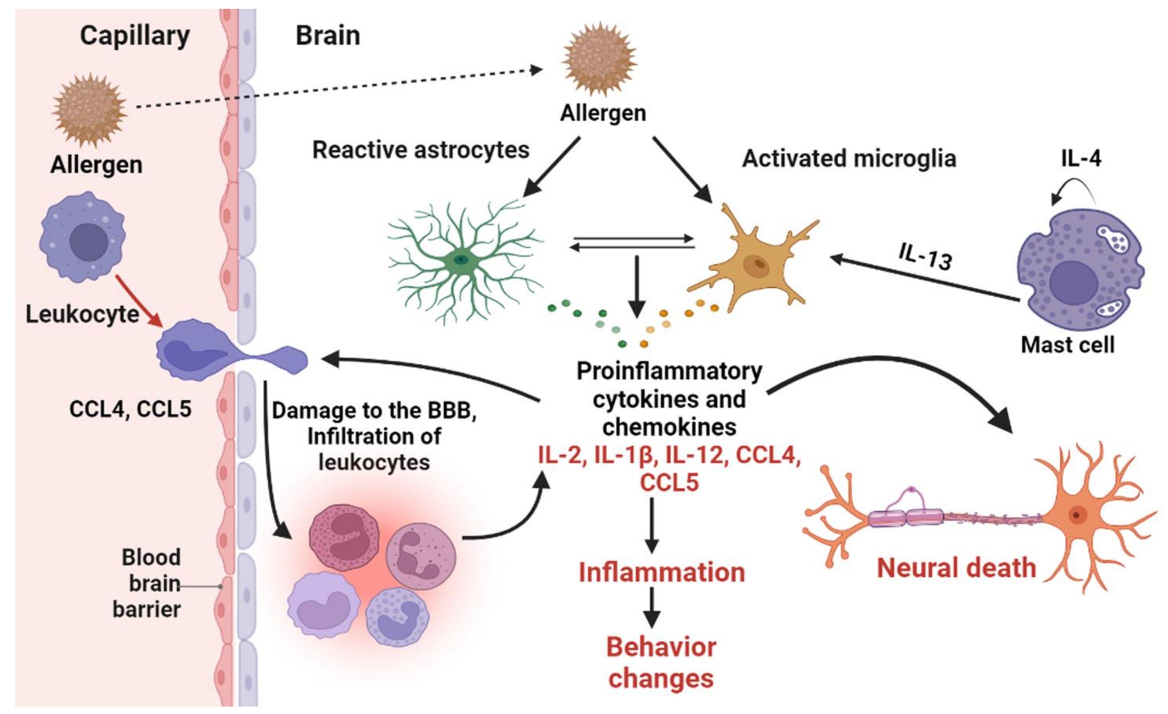


Fig 8. Proposed cellular events that lead to neuroinflammation in BLG-sensitized mice. The involvement of the astrocytes, microglia, and leukocytes in the production of inflammatory cytokines/chemokines is depicted. Infiltration of circulating allergens and recruited inflammatory cells across the leaky blood-brain barrier likely maintain neuroinflammation in the presence of allergen in the diet. These inflammatory markers causes neuropathologies which along with inflammation contribute to behavior changes.

Future Directions

- Validate cytokine/chemokine profiling in other brain regions as well as blood and peripheral tissues.
- Assess the BLG-mediated changes in intestinal permeability.
- Evaluate demyelination, microgliosis, and BBB integrity in more detail to determine regional differences within the brain.
- Repeat the study with female mice.

References

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2. Brishti A, Germundson-Hermanson DL, Smith NA, Kearney AE, Warda Y and Nagamoto-Combs K (2022) Asymptomatic sensitization to a cow's milk protein induces sustained neuroinflammation and behavioral changes with chronic allergen exposure. Front. Allergy 3:870628. doi: 10.3389/falgy.2022.870628

Acknowledgements

Research reported here was supported by the NIH/NIAID grant 1R01AI168563 to KNC. We thank the Behavioral Research Core and Histology Core Facilities at the University of North Dakota. The figures were made using BioRender.com.