IMMUNOLOGICAL ANALYSIS OF THE INTRINSIC ALLERGENICITY OF GLUTEN EXTRACTS FROM FOUR WHEAT SPECIES IN AN ADJUVANT-FREE MOUSE MODEL OF FOOD ALLERGY

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ABSTRACT

Wheat allergy (also known as immediate hypersensitivity) is a growing global public health hazard of critical significance. There is extensive evidence that such hypersensitivity reactions to wheat pose a lethal risk due to high incidence of systemic anaphylaxis. Currently existing wheat species encompass four distinct genomes (AA, DD, AABB, and AABBDD) of which exist in numerous lines and varieties. The extent of natural variation in the intrinsic allergenic potential of glutens from various wheat species remains unknown. Our research was piloted by the central hypothesis that glutens obtained from three different currently consumed wheat species [*Triticum monococcum* (AA genome), *Triticum aestivum* (AABBDD genome), and *Triticum durum* (AABB genome)], as well as an ancient DD genome progenitor (*Aegilops tauschii*) would exhibit natural variation in their respective intrinsic allergenicities *in vivo* in an adjuvant-free transdermal sensitization/systemic elicitation of anaphylaxis (TS/SE) mouse model of food allergy. To test this hypothesis, two aims were established: i) to validate the TS/SE mouse model for intrinsic allergenic sensitization to both alcohol and acid-soluble gluten extracts from the four above mentioned wheat species; and ii) to validate the TS/SE model for elicitation of systemic anaphylaxis by both alcohol and acid-soluble gluten extracts from the four wheat species.

Our findings demonstrate that repeated skin exposures to both the alcohol and acid-soluble gluten extracts from the four different wheat species elicited significant gluten-specific IgE antibody responses. Furthermore, skin sensitized mice demonstrated clinical sensitization for life-threatening systemic anaphylaxis upon intraperitoneal challenge with the respective glutens as evidenced by significant hypothermic shock responses (HSR) and mucosal mast cell responses (MMCR). Comparative mapping analysis revealed differences in capacity to elicit sIgE among

the four wheat species with *T. aestivum* being the most potent sensitizer for both types of gluten extracts. HSR analysis revealed that the four wheat species elicited significant largely comparable systemic anaphylactic reactions. Nevertheless, among the four wheat species, both types of gluten extracts from *T. aestivum* emerged as the most potent elicitors of MMCR.

In summary, our study validates the TS/SE mouse model of food allergy for wheat gluten extracts from four wheat species. Furthermore, a comparative map of intrinsic allergenic potential of gluten extracts was created for the first time. This validated model serves as a cost-effective pre-clinical testing tool for assessing the intrinsic allergenic potential of glutens from novel wheats including genetically engineered wheats, various wheat lines developed by gene editing, cross-hybridization, as well as altered wheat glutens by food/industrial processing methods. This model may also be used to conduct basic research on the mechanisms underlying gluten allergy as well as developing novel methods to prevent and treat life-threatening systemic anaphylaxis elicited by glutens.

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TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION

1.1 Background and significance

Wheat is one of the world's most consumed foods due to its high yield, adaptability to various climates, and high nutritional value. However, despite these benefits, wheat proteins are among a rather short list of 9 to 14 foods that account for life-threatening disease burdens in individuals with food allergies (Sicherer & Sampson, 2018; Gangur & Acharya, 2021; Sicherer et al., 2020). Wheat proteins are an increasingly recognized trigger for both IgE and non-IgE mediated immune disorders (Cianferoni, 2016; Jin et al., 2019; Renz et al., 2018; Sicherer & Sampson, 2018; Jorgensen et al., 2023a; Jorgensen et al., 2023b). Wheat allergy is known to affect both children as well as adults, with an increased prevalence seen in children because most patients outgrow their allergy by the age of 16 (Cianferoni, 2016; Jin et al., 2019; Renz et al., 2018; Sicherer & Sampson, 2018; Sicherer et al., 2020). The prevalence of wheat allergy has recently been estimated to be around 0.22% to 1.93% globally (Liu et al., 2023). Although the economic impact of wheat allergies is unknown, the overall impact of food allergies (caused by 8 major allergenic foods including wheat) in the United States is around $24.8 billion annually (Gupta et al., 2013). The economic burden of food allergy in other countries including Canada, the European Union, and the United Kingdom have also been estimated to be enormous (Fong et al., 2022).

The genesis of wheat allergy, much like other food allergies, occurs in two steps: sensitization upon exposure to wheat allergens, and disease elicitation among sensitized subjects upon re-exposure to the wheat proteins (Gangur & Acharya, 2021, Jin et al., 2019; Renz et al., 2018; Sicherer & Sampson, 2018). Sensitization of genetically susceptible individuals to wheat can occur through various routes of exposure such as the eyes, nose, oral, and skin resulting in

the production of IgE antibodies agonist to wheat allergens (Jin et al., 2019; Renz et al., 2018; Sicherer & Sampson, 2018; Sicherer et al., 2020; Jorgensen et al., 2023a; Jorgensen et al., 2023b). These antibodies then load on to basophils and mast cells by specifically binding to their cell surface high affinity IgE receptor (FcER-I), resulting in the state of allergic sensitization (Jin et al., 2019; Renz et al., 2018; Sicherer & Sampson, 2018; Sicherer et al., 2020; Jorgensen et al., 2023a; Jorgensen et al., 2023b). Disease elicitation in sensitized individuals can occur upon re-exposure to wheat allergens via eyes, nose, oral, or skin routes. The wheat allergens then bind to the IgE-loaded mast cells and basophils, causing them to degranulate releasing inflammatory mediators such as histamine, thus resulting in clinical symptoms. Common symptoms include vomiting, diarrhea, rhino-conjunctivitis, dermatitis/eczema, and potentially deadly asthma and systemic anaphylaxis (Jin et al., 2019; Renz et al., 2018; Sicherer & Sampson, 2018; Sicherer et al., 2020).

Since both IgE production upon food allergen exposure causing sensitization, and later binding of food allergens to IgE antibodies upon re-exposure leading to disease elicitation are central to the genesis of food allergy, both steps are commonly used to estimate the allergenicity of various food proteins including wheat proteins (Gao et al., 2019; Kumar et al., 2017; Nakamura et al., 2005; Pastorello et al., 2007). Therefore, for the proposed studies both types of readouts of wheat allergenicity will be used to evaluate intrinsic allergenicity of wheat gluten proteins.

The earliest cultivated forms of wheat were diploid (AA genome, einkorn) and tetraploid (AABB genome, emmer) wheats, and later AABBDD wheat was developed (Shewry, 2009; Gao et al., 2021). These wheats contain 3 distinct genomes: A, B (extinct now), and D. The parental D genome contributor to the AABBDD wheat, known as *tauschii* wheat (*Aegilops tauschii*), is

not commercially available. Nearly 95% of the wheat grown (known commonly as bread wheat) belongs to the AABBDD genome (Shewry, 2009). The common durum wheat (or pasta wheat) belongs to the AABB genome. Although not very common, einkorn wheat of the AA genome is also commercially available (https://www.einkorn.com/where-to-buy-einkorn/).

Wheat proteins were first classified in 1924 by Osborne into glutens and non-glutens based on their solubility in different solvents which is often referred to as "Osborne fractionation" (Shewry & Halford, 2002). These proteins are non-glutens (water/salt-soluble albumins and globulins), and 2 types of glutens: alcohol-soluble wheat gliadins (ASWG), and acid-soluble wheat glutenins (ADSWG) (Shewry, 2009; de Sousa et al., 2021; Matsuoka et al., 2022). Both glutens and non-gluten proteins are linked to human wheat allergies (Jin et al., 2019, Cianferoni, 2016).

Dr. Gangur and his colleagues have developed and characterized a novel adjuvant-free mouse model to evaluate intrinsic allergenicity of food proteins (Navuluri et al., 2005; Birmingham et al., 2007; Parvataneni et al., 2009, 2016; Gonipeta et al., 2010, 2015; Jin et al., 2020, Gao et al., 2022, 2023). This method involves the use of transdermal exposure to food proteins to induce clinical sensitization for systemic anaphylaxis elicitation. Recently, this model was validated for non-gluten wheat proteins (Jin et al., 2020; Gao et al., 2022, 2023). This transdermal sensitization/systemic elicitation (TS/SE) model more closely simulates the human wheat allergenicity mechanisms from skin exposure to wheat proteins in occupational settings such as in the baking industry (Jin et al., 2020; Gao et al., 2022, 2023). Furthermore, it was shown that robust IgE as well as mucosal mast cell responses were elicited similarly in the both the classical alum adjuvant-based model, as well as in this novel adjuvant-free model,

showcasing the effectiveness of this TS/SE mouse model for evaluating intrinsic allergenicity of non-gluten wheat proteins without the need to use adjuvants.

The work presented in this dissertation validated the TS/SE mouse model for both types of wheat gluten extracts from 4 wheat species representing four distinct genomes: AA, DD, AABB, and AABBDD. Once validated, the TS/SE model can be used to monitor genetically modified wheats of these 4 genomes for changes in the allergenic potential of their modified gluten proteins. With wheat contributing up to 20-50% of total calories in wheat-producing countries, and even being found in cosmetic items such as facial soap, it is imperative to monitor changes made in wheat varieties that may cause allergies, and in a worst-case scenario, potentially deadly anaphylaxis (Gao et al., 2021, Jin et al., 2019; Shewry, 2018).

The TS/SE model will also be of significance in a more generalized sense: Are genetically altered foods more potent in eliciting allergic responses than unaltered foods? As no validated method exists to characterize the intrinsic allergenic potential of genetically modified foods versus natural food, this model will also advance science, and advance the 2 decades-long efforts by Gangur and colleagues, to address this concerning general question (Birmingham et al. 2003, 2005, 2007, Parvataneni et al., 2009; Gonipeta et al., 2010, 2015a, 2015b; Navuluri et al., 2006; Domingo, 2016; Hollingworth et al., 2003; Ladics et al., 2015; Selgrade et al., 2009; Gao et al., 2022, 2023; Jin et al., 2020; Jorgensen et al., 2023a, 2023b).

1.2 Central hypothesis and aims

The extent of natural variation in the intrinsic allergenic potential of glutens from various wheat species remains unknown. Our research was piloted by the central hypothesis that glutens obtained from three different currently consumed wheat species [*Triticum monococcum* (AA

genome), *Triticum aestivum* (AABBDD genome), and *Triticum durum* (AABB genome)], as well as an ancient DD genome progenitor (*Aegilops tauschii*) would exhibit natural variation in their respective intrinsic allergenicities in vivo in an adjuvant-free transdermal sensitization/systemic elicitation of anaphylaxis (TS/SE) mouse model of food allergy.

Aim 1. Validate the TS/SE mouse model for transdermal sensitization to both types of glutens from four wheat species representing four distinct genomes: AA, AABB, AABBDD, and DD

Hypothesis: The 8 gluten extracts from four different wheat species will vary in their capacity to sensitize mice in TS/SE model. Specific-IgE antibodies were utilized to determine the degree of sensitization.

Aim 2. Validate the TS/SE mouse model for systemic allergic reaction to glutens from four wheat species representing four distinct genomes: AA, AABB, AABBDD, and DD

Hypothesis: The 8 gluten extracts from four different wheat species will vary in the capacity to elicit systemic anaphylaxis in TS/SE mice. Hypothermia shock response (HSR) and mucosal mast cell responses (MMCR) in skin-sensitized mice were used to quantitatively establish the potency differences among 8 wheat glutens to elicit systemic anaphylaxis.

Table 1.1 Operational definitions and quantification of sensitization and disease elicitation potencies of gluten extracts.

Allergenic Potencies	Definition and Experimental Quantification
Sensitization potency	***Definition***: The capacity of gluten to trigger the production of specific (s)IgE antibodies in naive mice through transdermal application without the use of adjuvants. ***Quantification***: Blood tests to quantify gluten-specific IgE antibodies before vs. after exposure.
Disease elicitation potency	***Definition***: The capability of gluten to provoke a systemic anaphylactic reaction in pre-sensitized mice following intraperitoneal challenge ***Quantification***: Blood tests to assess mucosal mast cell protease-1 levels subsequent to a systemic gluten challenge, serving to evaluate mucosal mast cell degranulation response (MMCR). Rectal thermometry to quantify the hypothermic shock response (HSR).

1.3 Findings from this work

Findings from this work are organized and presented in this dissertation as follows:

- Review of literature is covered in Chapter 2 (Partly published in Jin et al., 2019; Gao et al., 2021 where Jorgensen is a co-author) and Chapter 3 (under revision for publication as Jorgensen et al., 2024)
- Preliminary published research leading to the dissertation research is presented in Chapter 4 (Jorgensen et al., 2022)
- Published work on the development and validation of TS/SE mouse model for alcohol-soluble gluten extract from *T. durum* wheat is presented in Chapter 5 (Jorgensen et al., 2023a).

- Published work on the development and validation of TS/SE mouse model for acid-soluble gluten extract from *T. aestivum* wheat is presented in Chapter 6 (Jorgensen et al., 2023b).
- Comparative map of the intrinsic allergenicity of diploid, tetraploid, and hexaploid wheats is presented in Chapter 7 (Jorgensen et al., 2024 manuscript in preparation)
- Evaluation of intrinsic allergenicity of alcohol and acid-soluble gluten extracts from the ancient wheat progenitor *Ae. tauschii* is presented in Chapter 8 (Jorgensen et al., 2024 manuscript in preparation).
- Conclusions/Future directions are presented in Chapter 9

1.4 Scope of this work

This research used a Balb/c mouse model to study IgE antibody responses and systemic anaphylactic reactions to gluten extracts from four wheat species representing four distinct genomes. IgE responses were elicited upon skin exposure to gluten extracts without the use of adjuvants and without causing any skin wounds. Systemic anaphylaxis was elicited by intraperitoneal injections with gluten extracts without the use of adjuvants. The following wheat species were sued to obtain gluten extracts in this study: *T. monococcum* (einkorn, genome AA), *T. durum* (durum wheat, Carpio variety, genomes AABB), *T. aestivum* (common wheat, Ambassador variety, genomes AABBDD) wheats, and an ancient wheat progenitor *Ae. tauschii* (genome DD). *Ae. tauschii* was grown at Michigan State University (MSU) with the help of Dr. Eric Olson; the *T. aestivum* (Ambassador variety) and *T. durum* (Carpio variety) were obtained from the MSU Wheat Breeding Program and North Dakota State University respectively; and the *T. monococcum* (einkorn wheat) was purchased from a commercial source (einkorn.com).

Breeder pairs of Balb/c mice strain were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in the MSU animal facilities where mouse breeding was conducted to produce all animals used in this study. All animals were maintained on a plant-protein-free diet throughout this study (AIN-93G, Envigo). Adult female mice were used in the experiments described in this dissertation.

1.5 Impact

The TS/SE mouse model will allow for the assessment of the intrinsic allergenic potential of glutens from any genetically engineered strains of wheat, gene edited wheat lines, novel wheat lines developed by cross-hybridization, and processed gluten proteins without the use of artificial adjuvant. This will create a cost-effective method to monitor this important issue. The TS/SE mouse model will also advance animal-welfare issues because it will be the first study to test the allergenicity of glutens in a mouse model without the use of i) adjuvant, and ii) without causing painful skin-wounds for eliciting skin-sensitization (Adachi et al. 2012, Bodinier 2009, Jin et al. 2020). Lastly, the TS/SE mouse model may also be beneficial in advancing the treatment and prevention of wheat gluten allergies (e.g., immunotherapies).

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CHAPTER 2 REVIEW OF LITERATURE

(Partly published in Jin Y, Acharya HG, Acharya D, **Jorgensen R**, Gao H, Secord J, Ng PKW, Gangur V. (2019). Advances in Molecular Mechanisms of Wheat Allergenicity in Animal Models: A Comprehensive Review. Molecules. Mar 22;24(6). pii: E1142. doi: 10.3390/molecules24061142; and Gao, H., **Jorgensen, R**., Raghunath, R., Nagisetty, S., Ng, P. K. W., & Gangur, V. (2021). Creating hypo-/nonallergenic wheat products using processing methods: Fact or fiction? Comprehensive Reviews in Food Science and Food Safety. (https://doi.org/10.1111/1541-4337.12830)

2.1 Significance of wheat allergy

Food allergies, including wheat allergy, are increasing in prevalence globally for unknown reasons. Recent estimates of the prevalence of food allergy within the United States is 10.8% among adults and 8% among children, with other countries reporting similar trends (Allen & Koplin, 2015; Berin & Sampson, 2013; Gao et al., 2021; Gupta et al., 2013; Gupta et al., 2019; Lee & Shek, 2014; Nwaru et al., 2014; Platts-Mills, 2015). The economic impact of food allergies was estimated to be $24.8 billion annually, with $4.3 billion resulting from direct medical costs such as doctor's visits, emergency department visits due to clinical symptoms, and hospitalizations (Gupta et al., 2013).

Food allergens are regulated in the US because they are potentially deadly for sensitive consumers. In 2004, Congress passed the Food Allergen Labeling and Consumer Protection Act (FALCPA) (US FDA, 2021). This law identified eight foods are major "red flag" food allergens: milk, eggs, fish, shellfish, peanuts, tree nuts, soybeans, and wheat. Recently, the Food Allergy Safety, Treatment, Education, and Research (FASTER) act was signed into law, thus declaring

sesame as the ninth major food allergen recognized by the United States, effective January 1st, 2023 (US FDA, 2021).

The prevalence of IgE-mediated confirmed wheat allergy is between 0.33% to 1.93%, (Liu et al., 2023). As of 2021, wheat is the second most grown cereal crop behind corn, and ahead of rice (FAO, 2021). Despite being so widely utilized and consumed, wheat allergens remain under studied when compared to other major allergens, such as peanuts.

Currently, the only management of wheat allergy is a life-time dietary avoidance to wheat-containing products (Cabanillas, 2020; Ricci et al., 2019). However, oral immunotherapy has been proposed to promote tolerance of wheat allergens in wheat-allergic children (Ricci et al., 2019). If wheat is consumed by a wheat-allergic patient, symptoms include vomiting, abdominal pain, urticaria, angioedema, diarrhea, bronchial obstruction, cramps, hives, nausea, rhinitis, or even life-threatening anaphylaxis (Cabanillas, 2020; Renz et al., 2018; Ricci et al., 2019; Sicherer & Sampson, 2018; Cox et al., 2021). Stress and anxiety associated with both the need for continuous allergen avoidance and the looming threat of life-threatening anaphylaxis upon consumption of allergens, significantly impairs the quality of life for individuals with food allergies (Warren et al., 2016). Alarmingly, in 2020, there were 187 food recalls, with 52% of these recalls being due to undisclosed food allergens being present in these foods (FDA, 2020). Thus, wheat allergy affects a significant portion of the global human population as well as negatively impacting the global economy.

2.2 Wheat processing vs. wheat gluten allergenicity: how a validated mouse model of wheat gluten allergenicity would benefit science

In general, the inherent factors affecting a food protein's allergenic potential include the protein's structure, glycosylation patterns, and stability to the gut digestion process (Gao et al., 2021). Typically, wheat foods are consumed only after processing due to preservation of the food product, and to make the food edible (Mills & Mackie, 2008; Gao et al., 2021). Most food processing procedures modify wheat protein structure affecting how the protein is broken down during digestion, or how the proteins are presented to the body's immune system (Chizoba Ekezie et al., 2018; Gao et al., 2021; Huby et al., 2000; Jin et al., 2019; Lepski & Brockmeyer, 2013). Common types of wheat processing are fermentation followed by thermal treatment, thermal treatment with or without pressure, and enzyme or acid hydrolysis (Gao et al., 2021). Common thermal treatments include baking, extrusion, frying, pasteurization, boiling, drying, and roasting (Gao et al., 2021; Table 2.1).

Table 2.1 Commonly consumed wheat food products are either thermally processed or fermented plus thermally processed (Source: This table is reproduced from this publication where Jorgensen is a co-author: Gao et al., 2021).

	Processing method	
Wheat food product	**Fermentation**	**Thermal processing**
Cookies	*Saccharomyces cerevisiae*	Baking (177°C, 8–12 min)
Crackers	*Saccharomyces cerevisiae*	Baking (232°C, 12–15 min)
Pizza	*Saccharomyces cerevisiae*; *Lactobacillus sanfranciscensis*	Baking (232°C, 15–20 min)
Bread	*Saccharomyces cerevisiae*	Baking (232°C, 20–25 min)
Sourdough bread	*Saccharomyces cerevisiae*; *Saccharomyces delbrueckii*	Baking (218°C, 60 min)
	Lactobacillus brevis; *Lactobacillus amylophilus*	Baking (218°C, 60 min)
Cornbread	No	Baking (203°C, 20–34 min)
Breakfast cereal	No	Baking and extrusion
Naan	*Saccharomyces cerevisiae*	Baking (260°C, 4–5 min)
Pretzel	*Saccharomyces cerevisiae*	Baking (218°C, 10 min)
Graham cracker	No	Baking (180°C, 10–12 min)
Pie crust	No	Baking (177°C, 45–50 min)
Waffle	No	Baking (360°C, 2–3 min)
Cake	No	Baking (175°C, 30–40 min)
Cake doughnut	No	Baking (218°C, 12–14 min)
	No	Frying (190°C, 3 min)
Yeast doughnut	*Saccharomyces cerevisiae*	Baking (180°C, 13 min)
Saccharomyces cerevisiae	Frying (191° C, 2 min)	
Roti/chapati	No	Frying (8-9 min)
French toast	No	Frying (4-6 min)
Flour tortilla	No	Frying (1-1.5 min)
Beer	*Saccharomyces cerevisiae*	Pasteurization (98°C)
Pasta	No	Extrusion (51°C)
	No	Boiling (100°C, 10 min)
	No	Drying (12%–13% moisture)
Soy sauce	*Aspergillus oryzae/sojae*; *Pediococcus halophilus*; *Zygosaccharomyces rouxii*	Roasted (150°C, 30–45 s)

Notedly, wheat processing is not restricted only to food produces, as hydrolyzed wheat proteins (HWPs) are commonly used in cosmetics such as skin and hair care products. (Denery et al., 2012; Shinoda et al., 2012; Chinuki & Morita, 2012; Burnett et al., 2018). Thus, there is widespread exposure to processed wheat proteins both via mucosal routes (oral, airways, eyes) and via the skin.

There is significant evidence in the literature to suggest that wheat allergenicity is manipulated, either increased, decreased, or destroyed, using common food and industrial processing methods (Gao et al., 2021). However, the effect these processing methods have on the

sensitization capacity of the wheat allergens is not well understood (Gao et al., 2021). Thus, more research is needed to understand what effects processing has on sensitization to wheat allergens (i.e., de novo IgE production).

Therefore, it is apparent that models for monitoring changes in a protein's structure due to processing and the subsequent effects this has on both sensitization to wheat allergens, and the disease elicitation is necessary. The proposed TS/SE adjuvant-free mouse model will create such a validated pre-clinical method that can then be used to develop potentially hypo/non-allergenic wheat products, as well as prevent the possible induction of super-allergic processed wheat products.

2.3 With GE wheat varieties on the horizon, it is critical that super-allergenic varieties be avoided

Currently, genetically modified/genetically engineered (GM/GE) crops, mainly corn, cotton, and soybean, contain transgenes for herbicide tolerance and insect resistance resulting in increased profit margins for farmers and greater food stability (Wulff et al., 2018). Today, more than 184 million hectares were planted globally with crops containing genetically modified traits (Brookes & Barfoot, 2020). However, despite being a worldwide staple food, wheat has been abandoned regarding genetic modification. Today's wheat varieties were developed through careful selection and crossbreeding. Interestingly, field trails in the United States and Europe have begun to study new wheat varieties for future use (Lupi et al. 2013; Shewry et al., 2006; Beale et al., 2009; Bruce et al., 2015; Yadav et al., 2015; Meyer et al., 2013).

GM/GE technology is providing significant economic and environmental benefits such as reductions in chemical use by 37%, increased yield of crops by 22%, and improved farm profits

by 68% (Klümper & Qaim, 2014; Smyth, 2020). However, the benefits of GM technology extend to human health as well. Decreasing cancer incidences due to the adoption of crops such as Bacillus thuringiensis (Bt) maize has led to lower concentrations of mycotoxins, which are both toxic and carcinogenic to humans (Pellegrino et al., 2018; Smyth, 2020). Biofortified GM crops have been adopted, increasing the micronutrient availability (Hefferon, 2015; Smyth, 2020).

However, despite the benefits of GM crops, we do not know whether GM wheat varieties will differ regarding their allergenicity when compared to conventional wheat. Therefore, it is imperative that this question must be addressed before the implementation of these new GM wheat lines. Currently, there is no validated pre-clinical method to access the allergenic potential of GM foods, including wheat (Domingo, 2016; Hollingworth et al., 2003; Ladics et al., 2015; Selgrade et al., 2009; Jorgensen et al., 2023a, 2023b). The proposed TS/SE model will look to solve this issue by validating a method for screening potentially hyper-allergenic wheat lines from entering the market.

2.4 Food triggers allergies in two phases: sensitization and systemic elicitation

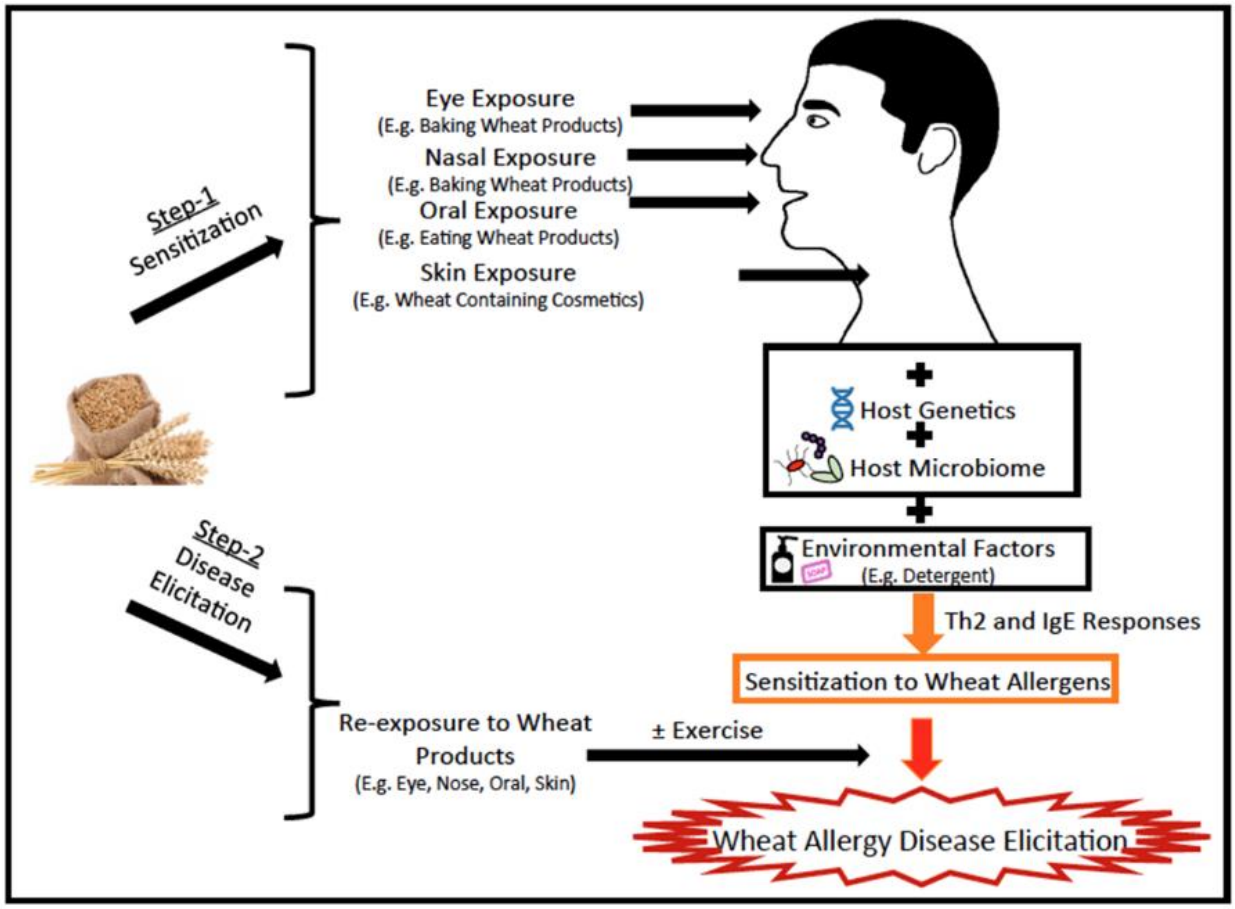

Figure 2.1 The genesis of wheat allergy occurs in two steps: sensitization and elicitation of disease (Source: This figure is reproduced from this publication where Jorgensen is a co-author and contributor of this figure in the paper: Jin et al., 2019).

The host immune system has evolved tolerances to food proteins and allergens; however, a growing number of individuals are losing this immune tolerance to several foods, including wheat (Platts-Mills, 2015; Renz et al., 2018; Sicherer & Sampson, 2018; Medina Sanchez et al., 2023). While the mechanisms of wheat allergy are not fully understood, the genesis of wheat allergy is thought to occur in two steps: sensitization to wheat allergens, and wheat allergy disease elicitation among wheat-sensitized subjects (Platts-Mills, 2015; Renz et al., 2018; Sicherer & Sampson, 2018).

There are multiple ways to induce sensitization with several mouse models opting for sensitization via intraperitoneal injection (IP) (Abe et al., 2014; Adachi et al., 2012; Bodinier et al., 2009; Denery-Papini et al., 2011a; Gourbeyre et al., 2012; Jin et al., 2017, 2019; Kozai et al., 2006; Tanaka et al., 2011). However, several studies suggest that a novel adjuvant-free mouse model that uses transdermal sensitization may be beneficial in the assessment of the intrinsic allergenic potential of food proteins such as wheat (Birmingham et al., 2007; Jin et al., 2020; Parvataneni et al., 2009). The proposed TS/SE (transdermal sensitization/systemic elicitation) mouse model will utilize transdermal sensitization to produce IgE antibodies agonist wheat gluten proteins.

Like sensitization routes, there are multiple ways to elicit an allergic reaction. In the case of animal studies using mice, intraperitoneal injection of allergenic proteins is common, with oral feeding, and nasal administration also established (Abe et al., 2014; Adachi et al., 2012; Bodinier et al., 2009; Denery-Papini et al., 2011a; Gourbeyre et al., 2012; Jin et al., 2017, 2019; Kozai et al., 2006; Tanaka et al., 2011; Kumar et al., 2019; Li et al., 2020). For eliciting systemic anaphylaxis, ingested food allergens must survive the gut digestion and then enter the blood. While oral elicitation of systemic anaphylaxis represents a typical route in humans, oral administration of gluten proteins to mice is technically problematic for 2 reasons: i) toxicity from alcohol/acid/alkali which are required to extract glutens; and ii) gluten proteins are also incredibly viscous making the administration of adequate amounts through an oral gavage needle almost impossible. Thus, in the proposed model we will elicit systemic anaphylaxis via systemic injection of gluten proteins into the intraperitoneal cavity as we have shown for non-gluten proteins recently (Jin et al. 2020).

Thus, for GE wheat to be determined to be safe for use, the allergenicity should be equivalent to traditional wheat varieties in eliciting similar levels of sensitization as well as the degree of systemic elicitation upon challenge with protein (Ladics et al., 2015; Selgrade et al., 2009). Therefore, validating the TS/SE model for predicting allergenicity of GE wheat varieties will take into account both the level of sensitization, as well as the systemic elicitation capacity.

2.5 Do the world's wheat genotypes naturally vary in intrinsic allergenicity of their glutens?

Currently, there are four wheat genomes utilized today: AA, DD, AABB, and AABBDD (Shewry, 2009). The fifth genome, BB, is now extinct (Poehlman, 1987; Jia et al., 2013). However, the natural variation in the intrinsic allergenicity among various wheat genome and varieties is largely unknown. There has been some success in breeding potentially coeliac-safe wheat utilizing various genomes that differ in immunotoxicity, suggesting that wheat genotypes may also elicit varying levels of intrinsic allergenicity (Shewry & Tatham, 2016). As wheat proteins are classified into two fractions based on their solubility in salt (salt-soluble albumins/globulins and salt-insoluble gliadins/glutenins) it is important to determine how these proteins differ in allergenicity from various wheat genotypes (Cianferoni, 2016).

The salt-soluble fraction of wheat proteins (albumins/globulins) represent roughly 15%-20% of total protein content with water-soluble albumins ranging from 10-12% and salt-soluble globulins from 5-8% of total protein content (Cianferoni, 2016; Jin et al., 2019). A study published in 2005 screened 324 varieties of wheat from various parts of the world to potentially identify less allergenic wheat varieties (Nakamura et al., 2005). This study identified several less allergenic wheat grains by utilizing direct ELISA to screen for IgE-binding in patient serum to

major wheat allergens: glutenin, gliadin, and α-amylase inhibitor (a salt-soluble wheat protein) (Nakamura et al., 2005). Recently, Dr. Gangur and his colleagues conducted a study examining the differences in in vitro allergenicity of salt-soluble wheat proteins from three wheat genotypes (DD, AABB, and AABBDD) (Gao et al., 2019). The results were that AABBDD and DD wheat genomes are significantly less allergenic than the AABB (carpio durum) wheat in this in vitro method. However, whether this finding translates to differences in *in vivo* allergenicity remains to be determined.

The salt-insoluble fraction of wheat proteins, gliadins and glutenins, account for 30-40% and 45-50% total protein content respectively (Jin et al., 2019; de Sousa et al., 2021). In a guinea pig model of WDEIA (wheat-dependent exercise-induced anaphylaxis), an AABBDD wheat line deficient in ω-5 gliadin was determined to have a lower sensitization capacity compared with regular wheat (Kohno et al., 2016). Another study found variations in the allergenic potential of wheat proteins from three different Ukrainian wheat cultivars: Sotnytsia, Panna, and Ukrainka (Lakhneko et al., 2020). Detected gliadins were the most life-threatening due to the highest number/density of epitopes and most grain proteins varied among genotypes (Lakhneko et al., 2020). Thus, it appears that wheat allergenicity of gluten proteins might differ among wheat genotypes, but more evidence is needed to make conclusions.

It is currently not possible to evaluate the allergenicity of gluten from GE wheat without knowing the natural variation in allergenicity of gluten from traditional, non-GE wheat genotypes. Therefore, the proposed TS/SE mouse model will serve as the method for determining the variation in allergenicity of gluten among the four wheat genotypes: AA, DD, AABB, and AABBDD. This will serve as the baseline for non-GE wheat varieties for juxtaposition against the newly introduced GE wheat lines.

The TS/SE mouse model will fill a critical gap in the FAO/WHO decision tree for assessing allergenic potential of GM/GE foods.

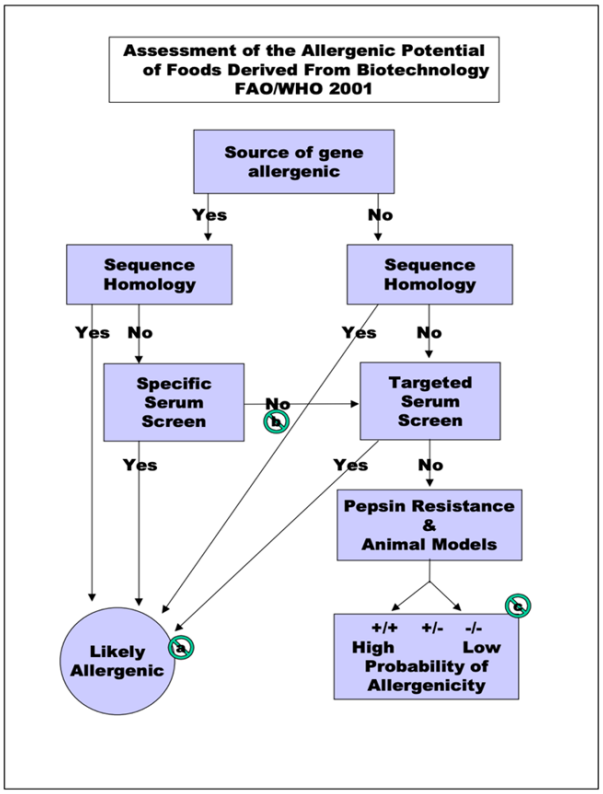

Figure 2.2 Animal models should be used to assess the allergenic potential of GM/GE foods according to the FAO/WHO decision tree (www.fao.org/docrep/007/y0820e/y0820e0e.htm).

Genetically modified/engineered foods must be as safe as the predicate, as this is the concept of "substantial equivalence" implemented by various regulatory agencies (Domingo, 2016; Hollingworth et al., 2003; Selgrade et al., 2009; Beale et al., 2009). In the FAO/WHO decision tree for assessing the allergenic potential of GM foods, the toxicological, allergenic, and nutritional properties of the genetically modified/engineered foods is compared to the traditional, non-GM food. If the GM food is not found to meet the criteria set forth by substantial equivalence, then the food would be deemed unfit for human use. Therefore, in 2001 the FAO/WHO developed the decision tree depicted above to assess the allergenic potential of GM foods.

Shown in the decision tree, the FAO/WHO recommends animal models to determine if the GM/GE food has a high or low probability of allergenicity. However, in the case of assessing wheat allergenicity for GM/GE foods, there are no validated animal models. From 2000 to 2005 there was a GM corn (StarLink™) that contaminated the United States food supply with the threat of allergenic potential, thus costing the U.S. several hundred million dollars resulting from clean-up costs (Buchini & Goldman, 2002; Siruguri et al., 2004). Such problems could have been prevented by validated animal models. The proposed TS/SE model could be implemented for not only assessing the allergenic potential of GM/GE wheat lines but could also prove instrumental in protecting the health of individuals by screening other GM foods as well.

Currently, safety assessments for GM/GE wheat products rely entirely on *in vitro* methods (Gao et al., 2019; Jin et al., 2019; Selgrade et al., 2009). However, as *in vitro* analysis is not predictive of *in vivo* allergenic potential, this is potentially hazardous when extrapolating to the use of GM/GE wheat crops (Gao et al., 2019; Ladics et al., 2015; Lupi et al., 2013; Selgrade et al., 2009). Thus, it stands to reason that an in vivo method for monitoring the allergenic potential of GM/GE wheat varieties is necessary in protecting public health.

2.6 Animal models of wheat allergenicity: a comprehensive review

A detailed evaluation of wheat allergenicity using animal models that have been generated thus far, was recently published by us (Jin et al., 2019). Below is a compiled summary of the most pertinent points from this publication.

The first animal model for studying wheat allergy used inbred high IgE-responding dogs (spaniel/basenji) (Buchanan et al., 1997). These dogs were genetically selected for over 15 years showing allergy to pollen and foods and eventually these wheat-sensitized dogs developed

vomiting and/or diarrhea upon feeding with wheat (Buchanan et al., 1997). All four types of wheat proteins were capable of eliciting IgE and skin reactions in the following order of potency: gliadins, glutenins, albumins, and globulins. However, rat/murine models are much more common today.

Table 2.2 Dog and rat models of wheat allergy: experimental approaches used to study wheat allergy (Source: This table is reproduced from this publication where Jorgensen is a co-author: Jin et al., 2019).

Model/Developers	Wheat Protein Used	Sensitization (Route, Dose, Age, Gender, Adjuvant)	Elicitation of Reaction (Route, Dose and Age)	Immune Markers	Disease Phenotype
Dog model *Spaniel/Basenji* inbred dog colony/Buchanan et al. (1997)	Wheat + cow's milk + beef extract	SC injection (on days of age: days 1, 22, 29, 50, 57, 78, and 85) 1 ug each of the food allergens + 0.2 mL alum; SC injection distemper-hepatitis vaccine on days 21, 49, and 77; Booster at bimonthly intervals with 10 ug each of the food allergens; Bleedings at 3, 4 months	At 6 months: Feeding challenge with 200 g wheat flour gruel or cow's milk	Specific IgE, Skin prick test	Vomiting and/or diarrhea (increased number of loose or watery stools for 2–4 days after the feeding challenge)
Rat models *Brown Norway* inbred rats (bred on gluten-free diet for three generations)/Kroghsbo et al. (2014) *Brown Norway* inbred rats/Bellegaard et al. (2019)	Gluten (Unmodified, acid hydrolyzed, Enzyme hydrolyzed) Native gluten vs. acid hydrolyzed gluten	IP sensitization: day 0: 200 ug adsorbed on Alhydrogel/rat in PBS; Days 14, 21, and 28: 20 ug in 0.9% NaCl; 0.2 mL volume/bleeding on day 35	None	Specific IgE, IgG Rat Basophilic Leukemia cell degranulation in vitro	None
		Oral sensitization: Female BN rats; Days 1 to 35: gavage with 0.2, 2, and 20 mg suspension in 0.5 mL PBS; Bleeding on days 0, 14, 28, and 42	None	Specific IgE, IgG Rat Basophilic Leukemia cell degranulation in vitro	None
		Skin sensitization: damage to skin then apply gluten without adjuvant 3 times per week for 3–5 weeks	None	Specific IgE, IgG antibodies	None

IP = intra-peritoneal injection; SC = subcutaneous injection.

In rats, there are two models reported in the literature relevant to studying the mechanisms of wheat gluten allergic sensitization (Table 2.2). The first of which, in 2014, Brown Norway (BN) rats were bred for three generations on a strict gluten-free diet (Kroghsbo et al., 2014). Immunogenicity (IgG) and allergenicity (IgE) of native gluten, acid hydrolyzed gluten, and enzyme gluten was compared. The findings were that enzyme hydrolyzed gluten was more immunogenic followed by acid hydrolyzed gluten, and lastly native gluten was found to be the least immunogenic (Kroghsbo et al., 2014). The second finding was that enzyme hydrolyzed gluten was the most allergenic in the oral exposure study (Kroghsbo et al., 2014). A second study utilized skin sensitization and investigated the allergenicity of native gluten juxtaposed to acid hydrolyzed gluten in wheat fed rats versus rats fed a wheat-free diet (Ballegaard et al., 2019). Both acid hydrolyzed gluten and native gluten elicited allergenic responses in the wheat-free rats. However, only the acid hydrolyzed gluten elicited allergenic response, in much lower levels, in the wheat-tolerant rats (Ballegaard et al., 2019). Using inhibitor assays, acid hydrolysis was

found to create new epitopes thereby eliciting antibody response in rats that were tolerant to native gluten (Ballegaard et al., 2019; Jin et al., 2019).

Table 2.3 Wheat food allergy mouse models: experimental approaches of allergic sensitization to hydrolyzed wheat gluten at the molecular epitope level (Source: This table is reproduced from this publication where Jorgensen is a co-author: Jin et al., 2019).

Model/Developers	Wheat Protein Used	Sensitization (Route, Dose, Age, Gender, Adjuvant)	Elicitation of Reaction (Route, Dose and Age)	Immune Markers	Disease Phenotype
B10.A model by Kozai et al. (2006)	Water/saline- soluble protein extract, Alcohol-soluble protein extract, alkali-soluble protein	IP (day 0, 14, 28, and 42) 10 ug + 1 mg alum/mouse Female B10.A	20 mg/0.5 mL/mouse oral feeding plus acute or moderate exercise	Specific IgE	Time to exhaustion, mucosal lesions in the small intestine, wheat protein leakage into the liver
Balb/cJ B10.A C3H/HeJ model by Bodinier et al. (2009)	Gliadin (Hardi)	IP (day 0, 10, 20, and 30) 10 or 20 ug + 1 mg alum/mouse 3-week females Balb/CJ 4–5-week females B10.A, C3H/HeJ	Nasal administration (10 ug on day 40)	Specific IgE, IgG1; IL-4, IL-5, IL-10, GM-CSF, IL-12 in lungs; cell counts in lung fluids	Eosinophil influx to lungs upon challenge
B10.A model by Tanaka et al. (2011)	Gliadin, purified ω5-gliadin	Gliadin 100 ug/mouse first IP injection; 50 ug/mouse for next 5 injections at weekly interval + 1 mg alum/mouse Female B10.A 5 weeks age	Gliadin at 0.1 and 0.8 mg/mouse/0.5 mL acetic acid; ω5 gliadin at 0.1 mg/mouse/0.5 mL acetic acid Oral feeding plus acute exercise	Specific IgE	Anaphylaxis by hypothermia shock response, voluntary exercise performance, leakage of wheat proteins into the blood
Balb/cJ model by Denery-Papini, et al. (2011)	Gliadins extract (Hardi) LTP1	IP (day 0, 10, 20, and 30) 10 ug of gliadins or LTP1 + 1 mg alum/mouse 3-week females	As in Bodinier et al. (2009)	Specific IgE	None reported
Balb/cJ model by Gourbeyre et al. (2012)	Deamidated gliadins (acid hydrolysis) (Hardi)	IP (day 0, 10, 20, and 30) 10 ug + 1 mg alum/mouse, 6-week females	IP injection with 1 mg + 1 mg alum on day 38	Total IgE, specific IgG1, IgG2a	None reported
Balb/c model by Adachi et al. (2012)	Acid hydrolyzed gluten	Skin sensitization (days 1–3, 8–10, 15–17, and 22–24) 0.5 mg	IP injection with 1 mg on days 18 or 25	Specific IgE, IgG1, plasma histamine levels	Hypothermia shock response
Balb/c model by Abe et al. (2014)	Native gliadin and deamidated gliadin by carboxylated cation exchange resin	IP (day 0, day 14) 50 ug of native gluten with 1 mg alum/mouse 5-week males	Intra-gastric administration of deamidated gliadin, 10 mg on days 28, 30, 32, 34, 36, 38, and 40	Specific IgE; peritoneal mast cells, histamine (gut and plasma)	Intestinal permeability, mast cell degranulation
Balb/cJ model by Jin et al. (2017)	Saline-soluble wheat protein (duram)	IP (days 0, 10, 24, and 40), 10 ug + 1 mg alum, 6–8-week females	IP injection with 0.5 mg, 1 week after last sensitization and repeated	Specific IgE, IgG1, total IgE murine mast cell protease-1, correlation analysis among readouts, cytokines, chemokines, adhesion molecule in skin lesion	Hypothermia shock response, atopic dermatitis, skin mast cell degranulation, mucosal mast cell mediator release

IP = intraperitoneal injection; LTP = lipid transfer protein.

Table 2.4 Pathogenic IgE-binding peptide epitopes present in wheat identified using a mouse model (Source: This table is reproduced from this publication where Jorgensen is a co-author: Jin et al., 2019).

Protein	Pathogenic IgE Binding Peptide Epitopes
Salt-soluble protein	
LTP1 *	(1) QARSQSDRQS; (2) GIARGIHNLN
Alcohol-soluble proteins	
α-gliadin	(1) PLVQQQ; (2) QQQFPGQQQQ #; (3) YLQLQLP #; (4) YPQQQPQYLQ; (5) SFQQPQQQYP
ω2-gliadin	(1) FPTPQQQFPE; (2) QQSFPLQPQQ #; (3) QQLFPELQ
ω5-gliadin	(1) QQFPQQQ #; (2) QQLPQQQ #; (3) QQSPQQQ #; (4) QQEFPQQQ; (5) QQQFPQQEFP

* LTP1 = Lipid transfer protein 1. #: Epitope is present in both the human and mouse model; Amino acids: Q = Glutamine; I = Isoleucine; P = Proline; F = Phenylalanine; G = Glycine; S = Serine; Y = Tyrosine; L = Leucine; E = Glutamic Acid; V = Valine; R =Arginine; N = Asparagine. Reference: [29].

Table 2.5 Major lessons ascertained from animal models on wheat protein allergenicity (Source: This table is reproduced from this publication where Jorgensen is a co-author: Jin et al., 2019).

Species	Wheat Allergen	Exposure Route	Sensitization	Elicitation of Reaction
Dog	Gliadins	IP	IgE	ND
	Glutenins, Albumins, Globulins	Oral	ND	Vomiting, Diarrhea
		Skin	ND	Skin Reaction
	Thioredoxin Modified Gliadins & Glutenins	Skin	ND	Reduced Skin Reaction
Mouse	Gliadins	IP	IgE	Anaphylaxis, EIA
	Gluten	Skin	No IgE	No Anaphylaxis
	Gluten + Detergent	Skin	IgE	Anaphylaxis
	Acid-Hydrolyzed gluten (AHG)	Skin	IgE	Anaphylaxis
	AHG + Detergent	Skin	Increased IgE	Increased Anaphylaxis
	Deamidated-Gliadins (DG)	IP	Increased IgE	ND
	DG	Oral	ND	Reduced Gut Reactions
	Albumins + Globulins	IP	IgE	Anaphylaxis Dermatitis (Th1, Th2, Th17 Cytokines + Allergenic Chemokines)
Rat	Gluten and AHG	IP	IgE	ND
		Skin	IgE	ND
		Oral	IgE	ND
	Enzyme Hydrolyzed Gluten	IP	IgE	ND
		Oral	IgE	ND

Abbreviations: IP = intraperitoneal injection; ND = not done; EIA = exercise-induced anaphylaxis.

Several studies have investigated the allergenic potential of wheat proteins using a mouse model. In 2006, a mouse model was used to investigate the molecular mechanisms of wheat-dependent exercise-induced anaphylaxis (WDEIA) (Kozai et al., 2006; Table 2.3-2.5). B10.A mice were sensitized to albumin/globulin, gliadin, and glutenin fractions. The challenge was conducted with oral feeding after exercise with the mentioned protein fractions. This model demonstrated that gliadins and glutenin were capable of sensitizing mice as well as inducing WDEIA, and that salt-soluble proteins (albumins/globulins) were not capable of eliciting sensitization nor WDEIA. Finally, gliadin and glutenin proteins were found leaking into the liver after exercise due to mucosal lesions forming after oral challenge. Thus, gliadin and glutenin were found to be linked with WDEIA.

In 2011, a study was conducted to determine if ω-5 gliadin was capable of eliciting anaphylaxis without the need of exercise (Tanaka et al., 2011). This study utilized B10.A mice

with gliadin and alum adjuvant as the route of sensitization. The mice were challenged with gliadin or ω-5 gliadin and monitored for anaphylaxis. The findings were that a majority of the IgE antibodies bind to ω-5 gliadin and that the mice demonstrated identical anaphylactic reactions upon feeding with gliadin or ω-5 gliadin (Tanaka et al., 2011). Therefore, both ω-5 gliadin and total gliadins were capable of eliciting anaphylaxis without the need of exercise in a mouse model.

One study looked to determine the differences in allergic responses seen between humans and mice (Bodinier et al., 2009). Three mice strains (B10.A, C3H/HeJ, and Balb/c) were tested with gliadin extract and compared against wheat allergic patients (both children and adults). Total gliadin and purified gliadin antibody responses (α/β, γ, ω1, 2, ω5) were compared via IgE response. Balb/c mice were found to exhibit the strongest allergic response, while the other two mice strains (C3H/HeJ and B10.A) had very little immune response to gliadin. Secondly, all five fractions of gliadins were found to be allergenic in both Balb/c mice as well as wheat allergic children. In adults, ω-5 gliadin was determined to be the major allergen, while in children and mice, the most allergenic fraction was α/β, with ω-5 gliadin being the least allergenic.

Denery-Papini et al. set to map the IgE epitopes on gliadin and lipid-transfer protein 1 (LTP1) in Balb/c mice versus humans (Denery-Papini et al., 2011b; Jin et al., 2019; Table 2.3). IgE epitopes were mapped using the pepscan technique. On LTP1, they discovered only one continuous IgE epitope shared by mice and humans. In both species, other IgE epitopes on LTP1 were conformational (or discontinuous). On the other hand, they discovered many continuous (or linear) epitopes in both species on ω5-gliadin (Denery-Papini et al., 2011b; Jin et al., 2019). Therefore, Balb/c mice and wheat allergic humans are similar regarding IgE epitopes for gliadins and LTP1.

As gluten is often modified industrially by deamidation to increase its solubility and enhance its use as a food ingredient, a study was conducted to compare the sensitization and elicitation potentials of native and deaminated gliadins. (Gourbeyre et al., 2012; Table 2.3 and 3.5). Both proteins were found to elicit anaphylaxis, however, mice sensitized with deamidated gliadin secreted higher levels of total IgE, IL-4, gliadin-specific IgE and IgG1 than mice sensitized with native gliadin. Mice sensitized with native gliadin produced higher levels of gliadin-specific IgG2a and IFN-γ by contrast. After the challenge, deamidated gliadin-sensitized mice had higher levels of histamine. Therefore, deamidated gliadin is capable of sensitizing mice more efficiently than native gliadins (Gourbeyre et al., 2012).

Another study examined the allergenicity of deamidated gliadins in a mouse model of wheat-gliadin allergy (Abe et al., 2014). Oral administration of deamidated gliadin to gliadin-sensitized mice suppressed enhanced intestinal permeability, serum allergen levels, serum allergen specific IgE levels, and serum and intestinal histamine levels when compared to native gliadin (Abe et al., 2014). Therefore, the author suggests that deamidation of gliadin by cation exchange treatment is a potential method for the creation of hypoallergenic wheat products with better expansibility (Jin et al., 2019).

Hydrolyzed wheat protein, mainly consisting of hydrolyzed gluten, is widely used in cosmetics, therefore, Adachi et al. sought to investigate the sensitizing potential of hydrolyzed wheat protein (HWP) (Adachi et al., 2012; Figure 2.1). They employed a published methodology that had previously been used for other food allergies to expose mice to native gluten vs. HWP vs. HWP plus detergent (0.5 percent sodium dodecyl sulfate) vs. native gluten plus detergent via the skin (Adachi et al., 2012; Navuluri et al., 2006; Birmingham et al., 2007; Gonipeta et al., 2009; Parvataneni et al., 2009; Jin et al., 2019). Interestingly, they found that skin exposure to

native gluten alone does not induce sensitization for anaphylaxis, however, in the presence of a detergent such as SDS, native gluten was capable of eliciting sensitization sufficient to cause anaphylaxis. HWP was found to induce sensitization sufficient to cause anaphylaxis both with and without SDS. Thus, skin exposure to gluten with a detergent such as SDS, and HWP with or without SDS is a plausible route of sensitization in humans for wheat anaphylaxis (Jin et al., 2019; Figure 2.1).

Finally, in 2017, a study was conducted in Balb/c mice to determine if sensitization as well as allergic disease elicitation was possible using salt-soluble wheat proteins (SSWPs) (Jin et al., 2017). Interestingly, they found that injection with SSWPs resulted in time-dependent sIgE antibody responses, thus sensitization to SSWPs and anaphylaxis to SSWPs after challenge was confirmed. They also found, for the first time, that anaphylaxis is linked to IgE-mediated mucosal mast cell degranulation in mouse model (Jin et al., 2017, 2019).

In summary, it is apparent that published mouse models of wheat allergenicity typically use alum-adjuvant or detergent to enhance the allergic reactions seen due to wheat proteins. Many studies utilize the method of creating skin-wounds by tape-stripping the stratum corneum to artificially enhance sensitization. Ballegaard et al. (2019) used 400 grit sandpaper to create skin wounds every week on rats. Adachi et al. (2012) used tape-stripping for a similar effect in a mouse model. Thus, our TS/SE model is more humane as there is no harm to the skin of the mice (Adachi et al., 2012; Jin et al., 2019). Thus, an adjuvant-free mouse model without causing skin wounds is unavailable at present for gluten proteins. The TS/SE mouse model looks to fill this void as a validated adjuvant-free mouse model without skin wounding for wheat gluten allergenicity.

2.7 The importance of the TS/SE mouse model being adjuvant-free and skin wound-free

Mouse models are most widely used in immunological research as mice have short generation time, large litter sizes, breed well, and in general have low purchasing and maintenance costs (Gonipeta et al., 2015). Also, large number of animals per experimental group can be studied allowing for improved statistical analysis, thus making mice the most economical model for studying allergic responses for food allergy (Gonipeta et al., 2015). In food allergy animal studies, both adjuvant-based as well as adjuvant-free models have been established (Jin et al., 2019, 2020).

Thus, animal models of food allergy, including wheat allergy, are divided into two categories: adjuvant-based and adjuvant-free models (Gonipeta et al., 2015; Jin et al., 2019, 2020). The adjuvant-based models utilize adjuvants such as alum as this can provide robust phenotypes, enhancing the allergenic response to food proteins (Jin et al., 2019, 2020). However, while it is reported the use of adjuvants provides a convenient way to exacerbate readouts of allergenicity such as IgE response to food proteins, the adjuvant related effects may exaggerate or even mask the intrinsic allergenic potential of food proteins (Gonipeta et al., 2015; Jin et al., 2020). The mechanism of how the alum adjuvant masks or exaggerates the intrinsic potential of food proteins is currently unknown (Abe et al., 2014; Jin et al., 2017, 2020; Kozai et al., 2006; Tanaka et al., 2011). Therefore, adjuvant-based models are not considered suitable to evaluate the intrinsic allergenicity of GM/GE foods as adjuvants such as alum can enhance sensitivity as well as reducing specificity in individuals with food allergy (Gonipeta et al., 2015; Jin et al., 2019; Ladics et al., 2015; Selgrade et al., 2009).

In contrast to adjuvant-based models, adjuvant-free models are desirable as the data interpretation of the intrinsic allergenicity of food proteins such as wheat becomes more

accessible (Gonipeta et al 2015, Jin et al 2019, Jin et al 2020, Adachi et al., 2012; Ballegaard et al., 2019). Several studies have suggested that an adjuvant-free model that utilizes transdermal sensitization may be useful in the evaluation of the intrinsic allergenic potential of food proteins, including wheat (Birmingham et al., 2007; Gonipeta et al., 2009; Jin et al., 2020; Parvataneni et al., 2009, 2016). Therefore, this TS/SE mouse model will utilize an adjuvant-free approach to sensitization as this may more closely simulate the human wheat allergenicity mechanism in transdermal skin exposure. Again, with GM/GE wheat varieties on the horizon, it is important to understand and evaluate the intrinsic allergenic potential of these potentially allergenic proteins.

How relevant is the adjuvant-free TS/SE mouse model to natural human disease? Sensitization to food allergens are typically thought to occur via oral routes, however exposure to food allergens through non-oral routes, particularly via skin, is increasingly recognized as a potentially important factor in the rising rate of food allergies (Jin et al., 2019, Jin et al., 2020, Renz et al., 2018, Berin & Sampson, 2013; Gonipeta et al., 2015; Ungar et al., 2017). In 2020, we demonstrated that transdermal sensitization to salt-soluble wheat proteins was successful in eliciting clinical disease upon IP injection with salt-soluble protein without the need of an adjuvant (Jin et al., 2020). In humans, the sensitization to wheat allergens can occur via the eye, nose, oral, and skin routes in the context of a dysregulated host-microbiome in a genetically susceptible individual (Jin et al., 2019). As adjuvant-free and adjuvant-based models were both capable of eliciting robust IgE as well as mucosal mast cell protein-1 responses, and that alum adjuvant-based Balb/c mouse models showed IgE response in mice similar to that of wheat allergic human subjects, it provides strong justification that adjuvant-free models may closely simulate the human wheat allergenicity mechanism from skin exposure (Denery-Papini et al., 2011b; Jin et al., 2020).

Summary: By validating the TS/SE mouse model, which more closely reflects human allergies than existing models, a new in vivo reference standard without the need of adjuvant and skin wounds as artificial enhancers of allergenicity may be utilized to evaluate the allergenic potential of any new GM/GE wheat strains. Simultaneously, we will perform the first research of the intrinsic allergenic variability of the world's wheat genotypes in an animal model. The TS/SE model can potentially be utilized to provide novel wheat allergy treatment strategies and prevention (e.g., vaccines, immunotherapies).

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CHAPTER 3 ADVANCES IN GLUTEN HYPERSENSITIVITY: NOVEL DIETARY-BASED THERAPEUTICS IN RESEARCH AND DEVELOPMENT

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3.1 Abstract

Gluten hypersensitivity is characterized by the production of IgE antibodies against specific wheat proteins (allergens), and a myriad of clinical allergic symptoms including life-threatening anaphylaxis. Currently, the only recommended treatment for gluten hypersensitivity is the complete avoidance of gluten. There have been extensive efforts to develop dietary-based novel therapeutics for combating this disorder. There were four objectives for this study: i) to compile the current understanding of the mechanism of gluten hypersensitivity; ii) to critically evaluate the outcome from preclinical testing of novel therapeutics in animal models; iii) to determine the potential of novel dietary-based therapeutic approaches under development in humans; and iv) to synthesize the outcomes from these studies and identify the gaps in research to inform future translational research. We used Google Scholar and PubMed databases with appropriate keywords to retrieve published papers. All material was thoroughly checked to obtain the relevant data to address the objectives. Our findings collectively demonstrate that there are at least five promising dietary-based therapeutic approaches for mitigating gluten hypersensitivity in development. Of these, two have advanced to a limited human clinical trial and the others are at the preclinical testing level. Further translational research is expected to

offer novel dietary-based therapeutic options for patients with gluten hypersensitivity in the future.

3.2 Introduction

The major source of dietary gluten in the human diet is wheat as it is one of the three main staple crops besides rice and corn, consumed globally, with expected consumption to increase by 11% by 2031 (FAO, 2022). Besides serving as a nutritional source, gluten is among the major food allergens regulated by multiple countries including the USA, Canada, European Union, United Kingdom, Australia, Japan, and New Zealand. However, because of its distinctive viscoelastic properties, gluten plays a vital role as a thickener and structure holder in food matrices, thus making it very challenging to exclude gluten from individual's daily diets (García-Manzanares & Lucendo, 2011; Pasha et al., 2016; Sievers et al., 2020).

Gluten allergens are seed storage proteins that are comprised of gliadins and glutenins. Aside from these gluten allergens, wheat also contains non-gluten allergens known as albumins (water-soluble) and globulins (saline-soluble) which have metabolic and structural functionalities in wheat plant biology (de Sousa et al., 2021). Gliadins are prolamin proteins that are ethanol-soluble, and glutenins are glutelin proteins that are soluble in weak acid (acetic acid) solution (Cabanillas, 2020). Both gliadin and glutenin allergens are linked to gluten hypersensitivity (or allergy) in humans (Pastorello et al., 2007; Ricci et al., 2019). Non-gluten allergens can also elicit similar types of disease.

Based on the electrophoretic mobility and on the similarity of amino acid sequences, gliadin allergens can be classified into three major subtypes: α-gliadins, γ-gliadins, and ω-gliadins (Matsuoka et al., 2022; Shewry et al., 1995; Wieser, 2007). All three types of gliadin

allergens can elicit hypersensitivity reactions. Unlike the monomeric units of gliadin, glutenin allergens are proteins with multiple linked components held together via disulfide bonds. These glutenin subunit polymers possess viscoelastic properties. They are insoluble in water-alcohol mixtures unless these bonds are broken under specific conditions (Matsuoka et al., 2022). Based on their electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), glutenin allergens can be further divided into high-molecular-weight glutenin subunits (HMW-GS) (70 to 120 kDa) and low-molecular-weight glutenin subunits (LMW-GS) (30-45 kDa) (Li et al., 2021; Liu et al., 2009; Peng et al., 2015; Tosi et al., 2004; Zhang et al., 2008). Despite making up approximately 10% of gluten protein, the HMW-GS plays a significant role in the end use quality (Li et al., 2021; Wieser, 2007). Both ω-5 gliadin and HMW-GS have been shown to be major allergens associated with wheat-dependent exercise-induced anaphylaxis (WDEIA) (Scherf et al., 2016). Many gluten-allergic individuals have been shown to be sensitized to both HMW-GS and LMW-GS (Baar et al., 2012; Kotaniemi-Syrjänen et al., 2010; Ricci et al., 2019). Thus, both gliadins and glutenins are major gluten allergens that are naturally present in wheat that can elicit hypersensitivity reactions in humans.

Gluten proteins can elicit multiple immune system mediated diseases in humans. These include not only gluten hypersensitivity (commonly called allergy), but also celiac disease, and non-celiac gluten sensitivity (NCGS) (Cabanillas, 2020). Gluten hypersensitivity is characterized by the production of IgE (Immunoglobulin E) antibodies against specific gluten proteins (i.e., allergens), and a myriad of clinical allergic symptoms including life-threatening systemic anaphylaxis (**Figure 3.1**) (Cianferoni, 2016). Celiac disease is an enteropathy with autoimmune characteristics and is triggered by gluten-containing foods in susceptible individuals that possess the human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8 haplotypes (Cárdenas-Torres et al.,

2021; Lebwohl et al., 2018). NCGS is described as a condition in which individuals experience distress after consumption of gluten, but does not show characteristics of the other two conditions (Cárdenas-Torres et al., 2021). Improvements are experienced after following a gluten-free diet. Notably, gluten hypersensitivity, but not the other two diseases, is mediated by gluten specific IgE antibodies and it is the focus of the research in this study (Cabanillas, 2020; Fasano et al., 2015).

When genetically susceptible individuals produce IgE antibodies against one or more gluten proteins they are deemed to be sensitized to gluten (**Figure 3.1**). However, gluten sensitized subjects typically do not have clinical symptoms of disease unless they are subsequently exposed to gluten via the oral, nasal, eyes, or skin routes (**Figure 3.1**). Currently, antihistamines, steroids, and epinephrine (adrenaline) are the only treatments recommended to manage clinical symptoms of allergic reactions to gluten , as is done for other food allergies such as peanut allergy. Notably, for life-threatening systemic anaphylaxis, epinephrine is the only life-saving emergency medication available at present (Cianferoni, 2016).

Currently, the only recommended dietary-based treatment for gluten hypersensitivity is the complete avoidance of exposure to gluten. However, avoiding gluten exposure is extremely challenging given the widespread use of wheat in foods, feed, and cosmetics including skin care products. Nevertheless, extensive efforts are underway towards creating novel dietary-based therapeutics for gluten hypersensitivity disorders (Gao et al., 2021). Here, we provide a comprehensive and up-to-date review of preclinical and clinical studies testing the novel dietary-based therapeutic approaches in animal models, and gluten hypersensitive human subjects.

A current and all-encompassing review regarding the advances in therapies combating wheat allergy is not currently available. To address this lack of knowledge, an extensive

literature search was conducted without imposing date restrictions, utilizing the PubMed database. We employed various combinations of keywords such as IgE, wheat, processing, allergy, allergenicity, immunogenicity, antigenicity, food allergy, anaphylaxis, adverse reaction, and hypersensitivity. Articles written in English were retrieved and thoroughly examined, leading to the creation of summary tables. The focus of this study was specifically on IgE-mediated diseases associated with gluten, necessitating the exclusion of articles addressing non-IgE-mediated wheat disorders (including celiac disease, non-celiac gluten/wheat sensitivity, eosinophilic enterocolitis, and food protein-induced enterocolitis) or therapies focusing on non-gluten wheat fractions. Our findings and interpretations are detailed in the subsequent sections.

There were four objectives for this study: i) to compile the current understanding of the mechanism of gluten hypersensitivity, including the roles of genetic and environmental factors; ii) to critically evaluate preclinical testing of novel dietary-based therapeutics in animal models; iii) to determine the potential of such novel therapeutics currently under research and development in gluten hypersensitive humans; and iv) to synthesize the outcomes from these studies, and identify gaps in the research to inform future translational research. We used Google Scholar and PubMed databases with appropriate keywords to retrieve published research papers. All material was thoroughly checked to obtain the relevant data to address the objectives. Our findings collectively demonstrate that there are at least five promising dietary-based therapeutic approaches for mitigating gluten hypersensitivity in research and development. Of these, two approaches have advanced to a limited human clinical trial and the others are at the preclinical testing level. Further translational research is expected to offer dietary-based therapeutic options for patients with gluten hypersensitivity.

3.3 Mechanism of gluten hypersensitivity

Gluten hypersensitivity is clinically classified as immediate (or Type-I) hypersensitivity reaction mediated by the immune system in response to specific gluten proteins known as gluten allergens. Mechanisms of gluten hypersensitivity are incompletely understood at present. However, akin to other types of food allergies, it is thought to develop in two sequential phases (**Figure 3.1**): (i) the first phase of sensitization, where genetically susceptible individuals produce IgE antibodies specific to gluten upon initial exposures only under environmental conditions that are incompletely understood; once produced these IgE antibodies attach to mast cells in the tissues and to basophils in the blood via the high affinity IgE receptor; such gluten-sensitized subjects do not have clinical symptoms of disease when they are not exposed to gluten; and (ii) the second phase, known as clinical elicitation of allergic reactions, occurs when sensitized individuals exhibit clinical symptoms of allergic reaction upon oral or other routes of re-exposure to gluten. Among the gluten hypersensitivity reactions, systemic anaphylaxis is potentially fatal and requires emergency medical management to save life. In case of WDEIA, exercising within 1-4 hours upon consuming gluten results in systemic anaphylaxis (Jin et al., 2019; Sicherer & Sampson, 2018).

Specific genetic and environmental factors leading to the genesis of gluten hypersensitivity are incompletely understood at present. However, emerging evidence shows that it is a complex genetic disorder like other food allergies, and it involves both susceptible immune gene variants as well as immune modulating environmental factors, both of which are beginning to be unraveled. Recent research has identified several genetic factors as potential risk factors for developing gluten hypersensitivity disorders (**Figure 3.1**). These include the genes encoding for the skin immune barrier function gene (filaggrin), genes involved in allergen presentation by the

antigen presenting cells such as dendritic cells, macrophages/monocytes and B cells (MHC class II genes), the T helper (Th)-2 immune response regulator genes (IL-4, and IL-4 receptor) that are required for developing IgE antibody production, and genes encoding proinflammatory cytokine (Il-18) and innate immune receptor (TLR4) (Cai & Yin, 2013; Cho et al., 2011; Fukunaga et al., 2021; X. Gao et al., 2020; Hur et al., 2013; Iga et al., 2013; Mizuno et al., 2015; Noguchi et al., 2019; von Mutius, 2007). Such genetic variants work in concert with poorly known environmental factors to create conditions for the development of gluten hypersensitivity disorders. Currently, the following four types of environmental factors are implicated: i) early-life exposure to pets such as cats; interestingly such exposure appears to offer protection from developing gluten hypersensitivity; ii) deficiency of vitamin D—a major immune function modulating nutrient, has been shown to increase the risk for developing gluten hypersensitivity; iii) gut microbial composition has major impact on food allergy development including gluten hypersensitivity; and iv) use of antacids/antiulcer mediations have been shown to increase the risk of developing gluten hypersensitivity (**Figure 3.1**) (Baek et al., 2014; De Zorzi et al., 2007; Okabe et al., 2023; Savage et al., 2018; Untersmayr et al., 2005).

During the first phase, susceptible individuals are exposed to gluten via various routes: eyes, nose, skin, and oral. These allergens are captured by antigen-presenting cells (i.e., macrophages, dendritic cell), processed and presented to T cells. Coupled with several co-factors such as dysregulated host microbiome and other environmental factors (e.g., detergents in allergen-containing cosmetic products), primed Th-2 cells in sensitized subjects activate B cells to produce allergen-specific Immunoglobulin E (IgE) antibodies. These allergen-specific IgE antibodies then bind to the high-affinity IgE receptor (FcεRI) present on the surface of mast cells and basophils. Upon re-exposure of sensitized individuals, gluten allergens cross-link the IgE on

mast cells and basophils and activate them to release histamine and other mediators (Cianferoni, 2016). These mediators cause clinical symptoms of allergic disease.

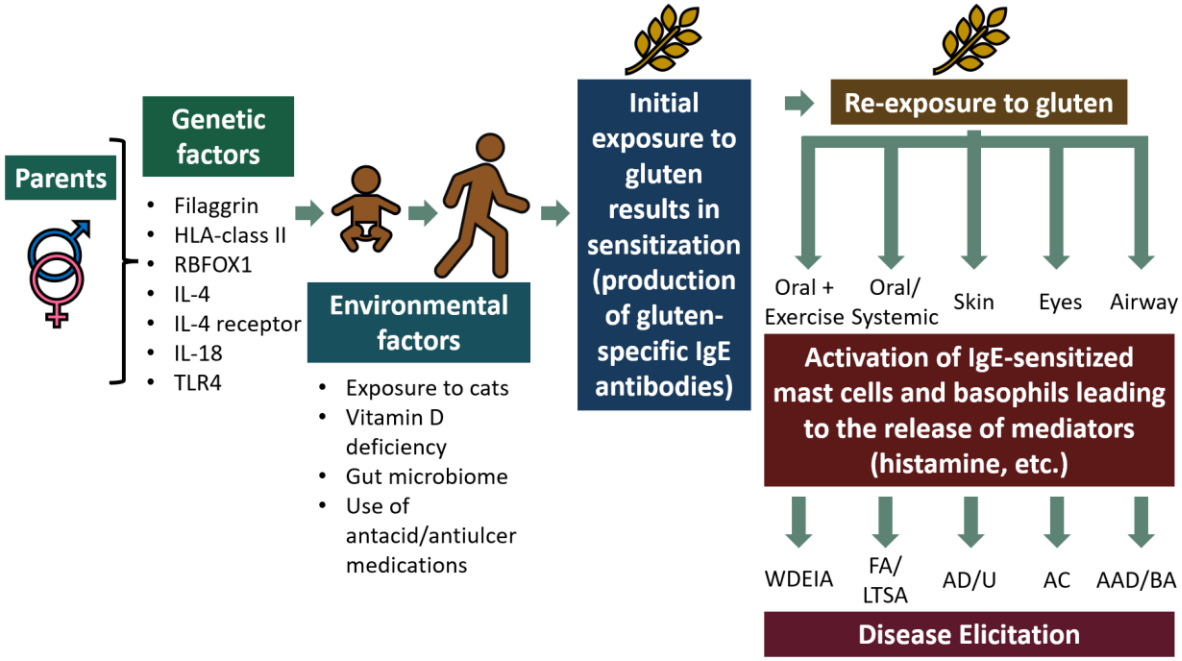

Figure 3.1 Pathogenesis of gluten hypersensitivity: role of genetics and environmental factors.

Inheritance of susceptibility gene variants from parents render the offspring propensity to develop atopic sensitization to gluten that is modulated by co-exposure to environmental factors. Some of the known genetic and environmental factors are illustrated in the figure. Upon re-exposure to gluten, results in the development of gluten hypersensitivity reactions. The routes of sensitization can be oral, skin, eyes, and airways. The routes of disease elicitation can be oral, skin, eyes, airways, and blood transfusion. Exercise upon ingestion of gluten can result in WDEIA within one to four hours. WDEIA: wheat-dependent exercise-induced anaphylaxis; FA: food allergy; LTSA: life-threatening systemic anaphylaxis; AD: atopic dermatitis; U: urticaria; AC: allergic conjunctivitis; AAD: allergic airways disease; BA: baker's asthma; IgE: immunoglobulin E.

Depending on the dose and route of exposure, symptoms of allergic reactions vary from rashes, hives, vomiting, diarrhea, airway hyper-responsiveness, conjunctivitis, to severe reactions such as life-threatening systemic anaphylaxis and asthma attacks known as Baker's asthma. Individuals who suffer from allergic rhinitis and conjunctivitis exhibit increased mucus secretion, itching and sneezing (Pawankar et al., 2015). Whereas gluten allergens when get into the bloodstream upon ingestion can cause systemic allergic reactions known as systemic anaphylaxis that involves multiple organs including the gut, skin, heart, and lungs. Such reactions capable of causing airway constriction such as difficulty in breathing (asthma attacks) as well as severe hypotension resulting in anaphylactic shock that can be deadly (Finkelman et al., 2005; Sicherer & Leung, 2015). Exercising within 1-4 hours after gluten consumption can cause WDEIA in sensitized subjects. Late-phase reactions can occur 6-8 hours after the initial immediate reaction due to new mediators' release by mast cells/basophils and may persist for up to 24 hours (Stone et al., 2010).

3.4 Preclinical development of novel dietary-based therapeutics using animal models of gluten hypersensitivity

Several novel therapeutic approaches are being developed using canine and rodent models of gluten hypersensitivity for future clinical application in humans. These are reviewed below.

3.4.1 Animal testing

Animal models for gluten hypersensitivity were first developed using a dog model, then subsequently using rat, guinea pig, and mouse models. Here, several animal models are

systematically evaluated for potential development of immunotherapies to combat gluten hypersensitivity.

3.4.1.1 Canine model: potential of thioredoxin and heat-killed Listeria monocytogenes to reduce gluten allergenicity

Using inbred dogs, a novel animal model of food allergy including gluten hypersensitivity was developed and used to test the potential of novel therapeutics (Buchanan et al., 1997; Frick et al., 2005). In 1997, Buchanan et al. aimed to investigate whether altering the biochemical and physical properties of wheat proteins including gluten through reduction with thioredoxin could impact their allergenic properties (Buchanan et al., 1997). They used inbred high IgE responder dogs (spaniel/basenji) and developed a complex protocol to study the impact of thioredoxin treatment on the molecular nature of gluten allergenicity (**Table 3.1**). They found that thioredoxin treatment increased the amount of gluten protein required to induce skin allergic reaction upon skin injection suggesting positive outcome reduced gluten allergenicity. A potential limitation of this study would be the administration of the allergen via subcutaneous sensitization but not the oral route to elicit allergic reaction. Further research is needed to address this limitation and validate the potential of thioredoxin treatment to reduce or eliminate oral gluten allergenicity.

Table 3.1 Potential of thioredoxin and heat killed Listeria monocytogenes for treating gluten hypersensitivity in a dog model.

Model and Therapy	Protein Used	Sensitization Phenotype	Disease Phenotype
Mitigation of gluten (wheat) allergenicity using thioredoxin treated wheat flour (Buchanan et al., 1997)	Wheat gluten	Specific IgE	Wheal and flair reaction upon SPT
Mitigation of gluten (wheat) allergenicity using HKL treatment of dogs (Frick et al., 2005)	Wheat flour	Specific IgE	Increase in minimum dose require to elicit positive SPT reaction

*****HKL: heat killed listeria; SPT: skin prick test**;** IgE Immunoglobulin E

Another study using the same canine model explored the use of heat-killed *Listeria monocytogenes* (HKL) as a potential therapy (Frick et al., 2005). Briefly, subcutaneous immunization of dogs was conducted with a mixture of wheat and cow's milk extract in conjunction with HKL (**Table 3.1**). The findings revealed that as the immunizations progressed, the mean dose required to trigger a positive skin prick test increased, suggesting the potential effectiveness of this therapy in mitigating gluten allergic responses. However, they did not study the effect of HKL on oral gluten induced allergic reactions. Furthermore, whether HKL would be an acceptable dietary component for administering gluten in humans remains to be investigated. This warrants further investigation and refinement in future studies focusing on these two general issues.

3.4.1.2 Evidence from rat models: potential of gluten genetic deletion, deamidation, and enzymatic digestion to reduce gluten allergenicity

In rat models, there have been several studies exploring potential therapeutics for gluten hypersensitivity (**Table 3.2**). One such study aimed to investigate the allergenicity of 1BS-18 (Deletion in the 1B short arm) Hokushin wheat (a wheat variety deficient in ω-5 gliadin gene) utilizing a rat model of anaphylaxis as quantified by hypothermic shock responses (HSR) (Yamada et al., 2019). They found that rats sensitized with ω-5 gliadin experienced significantly lower HSR when challenged with 1BS-18 gluten that is deficient in ω-5 gliadin as compared to control Hokushin gluten containing ω-5 gliadin. They also found that 1BS-18 gluten-sensitized rats exhibited no HSR when challenged with ω-5 gliadin, whereas those sensitized with Hokushin gluten and challenged with ω-5 gliadin displayed significant HSR. There are some limitations to this model: i) The reported significant changes in HSR were not substantial; ii) use of adjuvant limits generalization on intrinsic allergenicity interpretations on the gene deficient wheat line; and iii) the very low reported specific IgE optical densities (OD) in sensitized mice (stated as OD of 0.09), may raise questions about the study's robustness and applicability to real-world scenarios.

Table 3.2 Potential of gluten gene editing, deamidation, and enzyme treatment for gluten hypersensitivity in rat and guinea models.

Model and Therapy	Protein Used	Sensitization Phenotype	Disease Phenotype
Injection of 1BS-18 gluten lacking ω-5 gliadin reduced anaphylaxis in BN rats (Yamada et al., 2019)	Gluten from Hokushin and 1BS-18 wheat	Specific IgE	HSR decreased
Induction of oral tolerance by early ingestion of 1BS-18 gluten that is lacking ω-5 gliadin in BN rats (Yamada et al., 2022)	Commercial gluten, gluten prepared from Hokushin, and 1BS-18H wheat flours	Specific IgE	HSR decreased
Induction of oral tolerance using deamidated gliadin in BN rats (Kumagai et al., 2007)	Gliadin and deamidated gliadin	Specific IgE	None
Enzyme treated gluten reduced airway allergenicity in BN rats (J. Watanabe et al., 2001)	Gluten and hydrolyzed gluten with cellulase and actinase	Specific IgE	Reduced BAL immune cells (neutrophils, lymphocytes, and eosinophils)
Mitigation of oral allergy using 1BS-18 gluten lacking ω-5 gliadin in Guinea Pigs (Kohno et al., 2016)	Commercial gluten and 1BS-18 gluten	None	Significant decrease in allergy scores

*BN: Brown Norway Rat; HSR: hypothermic shock response; BAL: broncho-alveolar lavage; IgE: Immunoglobulin E; 1BS-18: Deletion in the 1B short arm

A subsequent study aimed to determine whether gluten prepared from 1BS-18 wheat would induce oral tolerance (OT) to both gluten and ω-5 gliadin (Yamada et al., 2022). The

findings revealed that gluten-specific IgE decreased in OT-induced rats that received 1BS-18 gluten. Moreover, hypersensitivity reactions were not observed in OT-induced rats challenged with 1BS-18 gluten. Similarly, ω-5 gliadin-specific IgE levels decreased in OT-induced rats that received 1BS-18 gluten, and hypersensitivity reactions were not observed when challenged with ω-5 gliadin. The study's strength includes its exploration of the potential of 1BS-18 gluten to establish OT to both gluten and ω-5 gliadin, which has implications for understanding and managing wheat allergies. However, the research did report minimal changes in rectal temperature and very low optical density for specific IgE antibody levels, potentially raising questions about the robustness of the results. Combining these findings with the previously reported study by Yamada et al (2029) indicate the lower allergenicity of 1BS-18 gluten offering promising insights into the development of a ω-5 gliadin-deficient line for allergy management.

The potential of deamidation as a method to reduce gluten allergenicity has been tested in rats (Kumagai et al., 2007). The study found that deamidated gliadin had the potential to induce oral tolerance in rats. The reported positive impact of IgE responses. However, the low optical density values for specific IgE antibody seen in the rat testing, raising questions regarding the robustness of their findings. They did not study disease phenotypes of gluten hypersensitivity. Another study in rats aimed to investigate whether hypoallergenic gluten containing wheat flour could be created to mitigate the effects of airway inflammation associated with gluten allergy (Watanabe et al., 2001). The hypoallergenic flour was prepared by digesting the wheat flour with cellulase and actinase. The research revealed that hypoallergenic gluten elicited lower IgE levels. Reduced immune cell counts (eosinophils, lymphocytes, and neutrophils) were observed in the broncho-alveolar lavage fluid after intranasal challenge with the modified gluten. The merits of this research demonstrate the potential of hypoallergenic wheat to induce oral tolerance,

subsequently reducing allergy-related immune cell counts in the airways. However, it would be interesting to assess the intrinsic allergenicity of this novel hypoallergenic gluten using adjuvant-free mouse models of gluten hypersensitivity and in food allergy related disease phenotypes.

3.4.1.3 Guinea pig model: potential of gluten genetic deletion to reduce gluten allergenicity

In guinea pigs, Kohno et al. utilized the 1BS-18 wheat line lacking the ω-5 gliadin locus to determine if it was capable of causing sensitization (Kohno et al., 2016) (**Table 3.2).** The findings revealed that the allergic scores associated with WDEIA (commonly associated with ω-5 gliadin), were significantly lower in the 1BS-18 line. However, while the decreased allergic scoring is promising, the notable lack of specific IgE data which is crucial when assessing the sensitization capacity of wheat is concerning. Additionally, the study could benefit from providing more detailed information on the scoring system used in the challenge test. Further research is needed to test the effect of this gene targeted wheat on other food allergenicity related phenotypes.

3.4.1.4 Mouse models: potential of probiotics, enzymatic digestion, L-arabinose, deamidation, polyphenols from fruits, and phosphorylation of gluten to reduce wheat allergenicity

Several mouse model studies explored six novel dietary-based therapies in gluten hypersensitivity (**Table 3**). One such study by Fu et al. explored the effects of *Pediococcus acidilactici* XZ31 in mitigating gluten allergenicity (Fu et al., 2022). Fermentation of with *P. acidilactici* promotes the digestion of gluten by pepsin and trypsin reducing antigenic reactions. The findings indicated that *P. acidilactici* treated gluten showed decreased IgE, as well as anaphylactic scores when compared to the control gluten sensitized and challenged group.

Histological scores within the duodenum showed no differences between treated vs. control groups. While the study demonstrated several strengths, such as its ability to highlight the regulation of Th1/Th2 imbalance and lower gluten specific IgE levels, it also revealed some weaknesses, notably the lack of effects on histology scores, which warrants further examination.

Table 3.3 Potential of probiotics, enzymatic hydrolysis, deamidation, and phosphorylation in mouse models of gluten hypersensitivity.

Model and Therapy	Protein Used	Sensitization Phenotype	Disease Phenotype
Mitigation of gluten anaphylaxis using oral administration of *Pediococcus acidilactici* XZ31 in Balb/c mice (Fu et al., 2022)	Commercial wheat gluten	Specific IgE	Clinical symptom scores of systemic anaphylaxis
Enzyme hydrolyzed gluten reduced sensitization in Balb/c mice (Mohan Kumar et al., 2019)	Hydrolyzed wheat gluten with *Aspergillus niger*-derived prolyl endopeptidase	Specific IgE	None
Oral treatment with L-arabinose reduced anaphylaxis symptoms in Balb/c mice (Wang et al., 2021)	Commercial gliadin	Total IgE	Clinical symptom scores of systemic anaphylaxis, allergic enteritis (histology, jejunum)
Hydrolysis and deamidation of gliadin reduced sensitization in Balb/c mice (X. Li et al., 2020)	Hydrolyzed and deamidated gliadin	Specific IgE	None
Phosphorylated gliadin, and enzyme treatment of gliadin reduced sensitization in Balb/c mice (Xue et al., 2019)	Gliadins, phosphorylated gliadins, hydrolyzed gliadins with alcalase or papain	Specific and total IgE	None
Repeated oral administration of deamidated gliadin reduced allergenicity in native gliadin sensitized Balb/c mice (Abe et al., 2014)	Native gliadin and deamidated gliadin by carboxylation cation exchange resin	Specific IgE	Blood elevation of histamine

*AN-PEP: Aspergillus niger-derived prolyl endopeptidase; IgE: Immunoglobulin E

Another study in mice aimed to assess the allergenicity of gluten derived from hydrolyzed wheat flour (HWF) treated with *Aspergillus niger* fungi derived prolyl endopeptidase (AN-PEP) compared to the standard wheat gluten (Mohan Kumar et al., 2019). The study found positive effects of reduced IgE by AN-PEP-HWF. Furthermore, various inflammatory markers including INF-γ, TNF-α, IL-4, IL-6, and IL-15 were elevated in mice exposed to regular wheat flour, but not in those exposed to AN-PEP-HWF. This paper demonstrates the potential of AN-PEP as a novel treatment method to produce hypoallergenic gluten. However, the one weakness is the absence of gluten hypersensitivity disease phenotype, which should be addressed in future work.

Another study sought to investigate the antiallergic activities and the underlying mechanisms of L-arabinose in a mouse model of gliadin hypersensitivity (Wang et al., 2021). They found that the anaphylactic scores in the group receiving the L-arabinose and gliadin were lower than those mice receiving the gliadin. The IgE levels and histamine levels were also lower suggesting a positive outcome of treatment. Therefore, L-arabinose appears as a candidate therapy. However, a notable weakness would be the use of alum adjuvant which does not allow for the testing of the intrinsic allergenicity potential of gluten. Also, while the histamine levels did decrease, the reported levels in this model were notably low when compared to other models suggesting a milder type of gluten hypersensitivity reactions observed in this model (Abe et al., 2014).

Abe and co-workers investigated the effects of deamidation of gliadin on allergenicity in a mouse model (Abe et al., 2014). Oral administration of gliadin or deamidated gliadin was compared between mice sensitized with gliadin in the presence of alum adjuvant. The results elucidate that deamidation lowered histamine levels, decreased IgE levels, and reduced intestinal

permeability. While this study provides another promising approach via the utilization of deamidation of gliadins, it is important to acknowledge its limitations. The use of alum adjuvant does not permit evaluation of effects of treatment on the intrinsic gluten allergenicity. Additionally, the study reported very low optical densities for IgE levels which may raise concerns regarding the robustness of the results.

Another study investigated the interaction between plant polyphenol extracts with gliadins to reduce gluten allergenicity (Pérot et al., 2017). The study used Balb/cJ mice sensitized with gliadin. In this model cranberry was the only polyphenol to decrease gliadin recognition by both IgG and IgE antibodies as well as prevent the degranulation process in mast cells. However, the use of alum adjuvant limited the exploration of the effects on the intrinsic allergenicity of gluten. Disease phenotypes were not studied.

Xue and co-workers sought to treat gliadins with phosphorylation, alcalase, and papain hydrolyses to determine the effects on gliadin allergenicity (Xue et al., 2019). Mice were sensitized with either native gliadin, phosphorylated gliadin, hydrolyzed gliadin with alcalase, and hydrolyzed gliadin with papain via intraperitoneal injections. Total IgE, specific IgE, histamine, and select cytokines were measured. Mice sensitized with treated gliadin exhibited significantly lower levels of total and specific IgE. Histamine, Serum IFN-γ, and serum IL-4 were decreased in the mice exposed to treated gliadin when compared to non-treated gliadin. Thus, phosphorylation and alcalase or papain digestion appear as promising ways to reduce gluten allergenicity.

Li and co-workers investigated the use of pepsin and trypsin treated gluten to reduce the adverse effects suffered during oral immunotherapy (Li et al., 2020). In one portion of the study, IgE-binding capacity in mice sensitized with gliadin via intraperitoneal injections was shown to

have a lower affinity for any of the treated gliadins. In the oral tolerance model, mice received oral administration of gliadin or pepsin treated gliadin followed by subsequent intraperitoneal immunization. The oral challenge was conducted with gliadin. The gliadin specific IgE and IgG1 were significantly decreased by oral administration of gliadin or pepsin treated gliadin, suggesting the development of oral immune tolerance. Disease phenotypes were not studied.

Thus, in several mouse models novel dietary-based therapeutic approaches are being researched and developed for potential future human application. However, for future research more disease phenotypes need to be considered, and the use of adjuvant-free models are needed to interpret impacts of treatment on intrinsic gluten hypersensitivity reactions.

3.5 Testing novel dietary-based therapeutics for gluten hypersensitivity in humans

Several therapeutic approaches discussed in the previous section have been applied to human gluten hypersensitivity. Most are at the preclinical testing stage, with two studies at a limited clinical testing stage (Table 3.4).

Table 3.4 Potential of fermentation, gluten gene targeting, deamidation, thioredoxin, and enzyme treatment for gluten hypersensitivity in humans.

Model and Therapy	Protein Used	Sensitization Phenotype	Disease Phenotype
In vitro model, soy sauce fermentation reduced/eliminated gluten allergenicity (Kobayashi, 2005)	Non-gluten and gluten	Specific IgE	None
In vitro model, gene targeting to remove conventional gluten reduced gluten allergenicity (Altenbach et al., 2015; Denery-Papini et al., 2007; Lee et al., 2022; Jacek Waga & Skoczowski, 2014)	Gluten from gene targeted wheats using various techniques (Gene Translocation, Gene inactivation, gene deletion and gene silencing)	Specific IgE	None
In vitro model, thioredoxin treatment of gluten reduced it's allergenicity (J. Waga et al., 2008)	Alcohol-soluble gluten extract was treated with thioredoxin	Specific IgE	None
In vivo and *in vitro* model, diploid genotype (AA) reduced gluten allergenicity (Lombardo et al., 2015)	Alcohol (40%) soluble gluten extract	Specific IgE	Negative SPT reaction in WDEIA 13/14 patients
In vivo and *in vitro* model, enzyme hydrolyzed wheat flour reduced gluten allergenicity (Tanabe 2008)	Enzyme hydrolyzed wheat flour	Specific IgE	13/15 AD patients tolerated cupcakes made from treated flour: 2/15 developed severe urticaria

*SPT: Skin prick test; AD: atopic dermatitis; WDEIA: wheat-dependent exercise-induced anaphylaxis; IgE: Immunoglobulin E

3.5.1 Soy sauce fermentation can reduce/eliminate human gluten allergenicity: in vitro evidence

Traditionally, soy sauce is produced using wheat and soy beans used in a 1:1 ratio. There is extensive evidence that fermentation process in general has the potential to reduce or eliminate the allergenicity of food proteins (Kobayashi, 2005). In particular, seminal studies by Japanese investigators demonstrate the allergenicity reducing/eliminating power of soy sauce fermentation processing (Kobayashi, 2005). During soy sauce fermentation, molds, yeast, and bacteria work on soy and wheat proteins to create the final product. Depending on the method of soy sauce fermentation used in different countries, different types of molds, yeasts, and bacteria have been identified in the process of soy sauce production (**Table 3.5**). Previous studies examined the effect of soy sauce fermentation after every major step in the production process. For example, the effect on allergenicity after roasting, mold treatment, yeast and bacterial treatments have been demonstrated (**Figure 3.2**). Major findings from these studies are illustrated below:

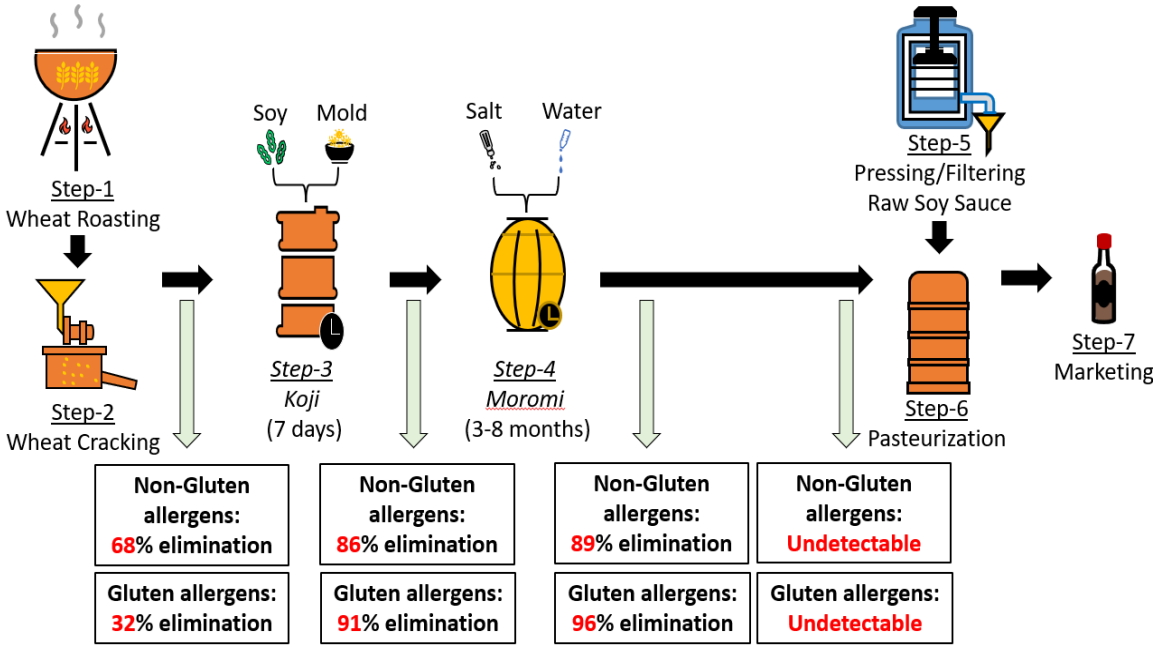

Figure 3.2 Key steps in traditional soy sauce production and the progressive reduction and elimination of gluten and non-gluten allergens.

This figure shows the various steps involved in the soy sauce production. Also, the effects on gluten and non-gluten allergens based on the research reported in the literature is summarized. Salt-soluble non-gluten allergen content as measured by direct ELISA is progressively reduced by approximately 68%, 86%, 89%, and undetectable by the end of step 2, *koji*, *moromi* day 10, and *moromi* day 48 respectively as measured by direct ELISA. Salt-insoluble wheat allergen (gluten) content as measured by direct ELISA is progressively reduced by approximately 32%, 91%, 96%, and undetectable by the end of step 2, *koji*, *moromi* day 10, and *moromi* day 48 respectively as measured by direct ELISA.

Kobayashi and co-workers studied the effects of soy sauce fermentation on wheat protein including allergenicity in vitro using ELISA (Kobayashi, 2005). They reported a progressive decrease in detectable salt-soluble (non-gluten) as well as salt-insoluble (gluten) allergens during soy sauce fermentation processing (**Figure 3.2**). They used pooled serum from five wheat-allergic children as source of anti-wheat IgE antibodies in their assay.

Salt-soluble non-gluten allergens were present during the first two stages (raw material and koji) of production at comparable levels suggesting no marked effect of mold treatment during koji stage. However, dramatic reduction in salt-soluble wheat allergens was noted progressively during the *moromi* stage of fermentation (with yeast and lactic acid bacteria) with 50% of the allergens lost by day 48, and over 91% lost by day 67 of *moromi* fermentation. Interestingly, no allergens were detectable in the raw soy sauce to the final product.

They also investigated the effect of soy sauce fermentation on salt-insoluble (gluten) allergens using a direct IgE ELISA method. They found that as opposed to salt-soluble allergens, gluten allergens were decreased during the koji stage of production, because of their solubilization due to enzymatic degradation. Furthermore, by day 10 of *moromi* stage, nearly all

the gluten proteins were undetectable, suggesting that gluten proteins are more susceptible to degradation by mold (koji stage), yeast and bacteria (*moromi* stage) than the salt-soluble wheat allergens. Nevertheless, both gluten and non-gluten allergens were absent in raw soy sauce suggesting that the final product may be hypo/non-allergenic with complete degradation of both types of wheat allergens.

Researchers have examined extensively the microorganisms involved in soy sauce fermentation (**Table 3.5**). In general, soy sauce fermentation involves the first step of mold activity leading to the formation of *koji*. This is followed by yeast and lactic acid bacterial fermentation during the *moromi* stage. Depending on the country of origin, different groups of molds, yeasts, and bacteria have been identified in soy sauce fermentation process (**Table 3.5**). However, this research show that there are some common genera of molds, yeasts, and bacteria independent of the country of origin producing the soy sauce (**Table 3.5,** see bold faced microbes).

These studies together demonstrate that: i) microbial fermentation of wheat has the potential to reduce and possibly even eliminate the allergenicity of both non-gluten and gluten proteins; ii) specific effects of mold versus yeast versus bacteria on wheat gluten versus non-gluten protein allergenicity needs further investigation urgently; and iii) since microbial composition of soy sauce produced in different countries can significantly differ, precise identification of microbes responsible for reducing and/or eliminating wheat gluten and non-gluten allergenicity is required.

Table 3.5 Diversity of microorganisms in different types of soy sauce based on country of origin.

Soy Sauce Country of Origin	Mold	Yeast	Gram Positive Bacteria	Gram Negative Bacteria
Korea [66]	*Tetrapisispora* *Cryptococcus* *Penicillium* ***Aspergillus: Aspergillus sp., A. flavu: (6/6)***	*Wickerhamomyces* *Torulaspora* *Tetrapisispora* *Rhodotorula* *Pichia* *Microbotryum* *Debaryomyces* ***Candida: (6/6)*** *Zygosaccharomyces*	*Not Studied*	*Not Studied*
China [67]	***Aspergillus***	*Starmerella* *Wickerhamiella* *Saturnispora* ***Candida***	*Weisella* ***Bacillus: (3/5)*** ***Lactobacillus: (3/5)*** *Leuconostoc* *Lactococcus* *Pediococcus* *Enterococcus* *Micrococcus* *Streptococcus* ***Staphylococcus: (3/5)*** *Propionibacteriacea*	*Xanthomonas* *Salmonella* *Pseudomonas* *Pantoea* *Lebsiella* *Dechloromonas* *Cupriavidus* *Arsenophonus* *Acidobacteriaceae*
Japan [68]	***Aspergillus: A. oryzae*** ***Geotrichum***	*Zygosacchormyces* ***Candida etchellsii*** ***C. nodaensis*** ***C. versatilis*** ***C. catenulata*** *Wickerhamomyces* *Pichia* *Trichosporon*	*Weisella* ***Lactobacillus*** ***Staphylococcus gallinarum*** ***S. xylosus*** ***S.arlettae*** ***S. saprophyticus*** ***S. succinus*** ***S. cohnii*** ***S. caprae*** ***S. kloosii*** *Pediococcus* *Tetragenococcus*	Not studied

Table 3.5 (cont'd).

Not specified [69]	***Aspergillus:*** ***Aspergillus sojae*** ***A. parasiticus*** *Peronospora*	*Sacchoramycopsis* *Millerozyma* *Pichia* ***Candida sp.*** ***C. rugosa*** ***C. orthopsilosis*** ***C. tropicalis***	***Staphylococcus*** *Kurthia* ***Bacillus*** *Paenibacillus* *Corynebacterium*	*Klebsiella* ***Enterobacter***
China (LSSF) [70]	***Aspergillus oryzae***	*Wickerhmomyces* *Saccharomycopsis* *Kluyveromyces* ***Candida rugosa*** ***C. glabrata*** ***C. tropicalis*** *Pichia* *Trichosporon*	*Weisella* ***Bacillus subtilis*** ***B. licheniformis*** ***B. pumilus*** ***Staphylococcus sciuri*** ***S. gallinarum*** ***S. succinus*** ***S. aureus*** ***S. cohnii*** *Corynebacterium* *Kurthia* *Enterococcus* ***Lactobacillus*** *Rothia* *Arhrobacter* *Pediococcus*	*Escherichia* ***Enterobacter***
China (Xianshi) [71]	***Aspergillus niger*** *Cladosporium* *Fusarium* *Lichtheimia* *Absidia*	*Meyerozyma* ***Candida parapsilosis*** *Sterigmatomyces*	***Bacillus amyloliquefaciens*** ***B. subtilis*** ***B. lincheniformis*** ***B. methylotrophicus*** ***B. aerius*** ***B. halmapalus*** ***B. flexus*** ***B. thuringiensis*** ***B. coagulan*** *Scopulibacillus* *Shimwellia* *Weissella* *Lactococcus* *Clostridium* *Streptomyces* *Microlunatus*	*Klebsiella* *Pantoea* ***Enterobacter*** *Erwinia* *Trichodesmium*

Table 3.5 (cont'd).

**Bold faced mold, yeast, and bacteria are the organisms used most in soy sauce preparation independent of country of origin*

3.5.2 Gluten gene targeting can be used to develop hypoallergenic wheat lines: in vitro evidence from human studies

Several *in vitro* models have investigated the effects of the deletion of specific genes to engineer potential hypoallergenic wheat lines. One such study by Denery-Papini et al. explored the genetic variability at the Gli-B1 locus (responsible for encoding ω-5 gliadins), and how this would affect the responsiveness of IgE antibodies in individuals with WDEIA and urticaria (Denery-Papini et al., 2007). One of the wheat cultivars Clément (produced via replacing the short arm of the 1B chromosome with the portion of the short arm of the 1R chromosome from rye; 1BL/1RS), yielded minimal/no IgE reactivity in immunoblotting with serum from WDEIA and Urticaria patients. However, it did react with reduced binding with serum from an individual suffering from anaphylaxis. Thus, the 1BL/1RS translocation may reduce wheat allergenicity.

Waga et al (2014) investigated the IgE-binding capacity of glutens extracted from a wheat line with inactivated gene variants in the three gliadin containing loci (Gli A1, Gli B1, and Gli D1) by traditional plant breeding. Sera from wheat allergic patients (n = 10, specific type of allergy not specified) was used in an ELISA testing as the source of IgE antibodies (Jacek Waga & Skoczowski, 2014). A significant decrease (approximately 30%) in IgE-binding compared to the control wheat was noted. Further *in vivo* testing is needed to evaluate allergenicity of this new wheat line.

Lee et al (2022) elucidated the use of a deletion line (ω5D) as a method for reducing allergenicity in gluten (Lee et al., 2022). This new cultivar has selective deletions in the 1B chromosome Glu-B3 locus, therefore causing it to lack ω-5 gliadin as well as some LMW glutenins and γ-gliadins (Lee et al., 2022). They used serum from 14 WDEIA patients and 7 classical wheat allergy patients for testing IgE reactivity using ELISA and inhibition CAP system. They found significant reduction in IgE-binding of gliadins and glutenins from this line as measured by immunoblotting, and inhibition ELISA. They concluded that oral challenge testing is needed to confirm potentially hypo-allergenicity of this line in WDEIA patients. They did not discuss the relevance of their findings regarding classical wheat allergy patients.

Altenbach and co-authors. sought to explore the use of a transgenic wheat line which utilized RNA interference to silence the ω-5 gliadin gene as a potential hypoallergenic wheat line (Altenbach et al., 2015). Sera from patients suffering from WDEIA (and some with urticaria or rhinitis; $n = 11$) was used in IgE immunoblotting. A reduction in IgE-binding was seen in the transgenic lines when compared to a traditional wheat line. However, while a reduction in IgE reactivity was noted in the transgenic wheat lines, it was not eliminated completely, thus further *in vivo* testing is needed to evaluate the allergenicity of this wheat line.

Waga and co-workers investigated the effects of thioredoxin on both immunoreactivity and dough rheological properties in ten winter wheat genotypes (J. Waga et al., 2008). Sera from patients suffering from Baker's asthma ($n = 2$), chronic atopic dermatitis and food intolerance ($n = 2$), and chronic urticaria and angioedema ($n = 1$) was used in a direct and sandwich ELISA. Both ELISAs revealed a reduction (>50%) in IgE-binding in all wheats treated with thioredoxin when compared to the native samples. Treatment with thioredoxin did not significantly impact

dough rheological properties suggesting thioredoxin as a promising method of reducing allergenicity without impacting wheat quality.

3.5.3 Gluten gene targeting and enzyme hydrolysis to develop hypoallergenic wheat products: clinical evidence from testing in gluten hypersensitive subjects

Naturally ω-5 gene encoding chromosome B deficient ancient wheat known as *Triticum monococcum* (Einkorn, AA genotype) offers a unique opportunity to develop hypoallergenic wheat products for ω-5 gliadin allergic subjects such as WDEIA patients where ω-5 gliadin is the major allergen. Lombardo et al. sought to study the immunoreactivity of proteins from *Triticum monococcum* (Einkorn, AA genotype), a diploid ancestral wheat lacking the B chromosome; notable as ω-5 gliadin (the major wheat allergen in WDEIA) is encoded on the B chromosome (Lombardo et al., 2015). Using skin prick testing with both *Triticum monococcum* and *Triticum aestivum* (commercial wheat, AABBDD genotype), revealed no positive reactions in *Triticum monococcum*, whereas 43% of WDEIA patients tested positive when exposed to *Triticum aestivum.* Patient sera revealed a lack of IgE-immunoreactivity to ω-5 gliadin observed in *Triticum monococcum.* However, future studies testing oral reactivity are needed to confirm suitability of Einkorn wheat for WDEIA patients.

Watanbe and co-workers have spent a great deal of effort to successfully develop a novel dietary-based treatment for gluten hypersensitivity. They first created a hypoallergenic wheat flour using both cellulase and actinase as hydrolyzing agents (Watanabe et al., 2000). Later, they investigated the safety of the hypoallergenic wheat flour by creating a hypoallergenic cupcake and testing the health outcomes by administering to children suffering from gluten-induced atopic dermatitis (AD) (Tanabe, 2008). They reported that upon consumption, 13 of the 15

children showed no adverse response, with only two of the patients showing an immediate reaction in the form of severe urticaria (Tanabe, 2008). Interestingly, more than half of the patients were able to consume normal wheat products after consuming the cupcakes over a period of more than six months suggesting development of oral tolerance. Therefore, the consumption of such hypoallergenic wheat products offers a promising method of creating oral immunotolerance in individuals suffering from gluten induced AD type of hypersensitivity. However, whether such products are safe for subjects with other types of gluten hypersensitivity such as life-threatening systemic anaphylaxis remains to be established.

3.5.4 Most commonly used thermal food processing methods can potentially reduce gluten allergenicity

Several studies have investigated the effects of thermal processing on gluten allergenicity. One such study by Lupi et al., 2019 examined the impact of boiling (100°C) on the allergenic properties of purified alcohol-soluble glutens. The study employed wheat flour extract of total gliadins, further isolating α-gliadins through a reversed phase high-performance liquid chromatography method. IgE-based dot blotting was performed using pooled serum from a cohort of wheat allergic subjects (composed of five groups containing specific IgE antibodies against gliadin ranging from 27 ng/mL to 167 ng/mL in the testing). Mast cell degranulation effects of the gliadin were tested *in vitro* via cell line assay. The findings indicated a complete loss of IgE reactivity and mast cell degranulation potential in boiled gliadins. However, the validation of non-allergenicity in animal models and humans remains a crucial step.

In contrast to the above findings, another study reported that boiling wheat flour had no significant impact on IgE reactivity (Pastorello et al., 2007). They obtained serum samples from

22 wheat allergic subjects and tested each sample individually using the IgE western blot method. The discrepancy between the two papers suggests that whereas purified gliadins are susceptible to boiling, gliadins contained in wheat flour appear to retain their IgE reactivity despite boiling.

A study conducted by De Angelis et al., 2007 compared impact of pepsin and pancreatin digestion on the allergenicity of conventional yeast bread in contrast to yeast plus VSL#3 fermented sourdough bread, the latter prepared with a cocktail of selected lactic acid bacteria (LAB) as previously described. The study focused on allergenicity testing of albumins, globulins, and gliadins extracted from the digested bread. The results revealed that the enzymatic digestion of yeast bread led to a reduction in IgE reactivity specifically for 31 to 45 kDa prolamins, with no significant effect observed on 14, 60, and 97 kDa proteins. In contrast, the enzymatic digestion of VSL#3 sourdough bread demonstrated markedly diminished IgE reactivity of albumins, globulins, and gliadins, while glutenins were not investigated. This suggests the intriguing possibility that VSL#3 sourdough bread may be tolerated by individuals allergic to albumins, globulins, and gliadins. The study's noteworthy strength lies in presenting a novel method for producing potentially hypo-/nonallergenic sourdough bread.

Finally, in 2005, Kobayashi et al. delved into the impact of soy sauce production on the allergenicity of both non-gluten and gluten components. Notably, the initial stage of soy sauce production involves the high-temperature roasting of wheat. Leveraging pooled serum from five children allergic to wheat as a source of anti-wheat IgE antibodies, they conducted ELISA testing. The results revealed a significant 32% reduction in gluten allergens following the roasting and cracking of wheat. The findings from the literature imply that thermal processing holds the potential to mitigate the allergenicity of gluten components.

In summary, these findings suggest the following effects of thermal processing on gluten allergenicity: i) baking temperatures used in bread making have the potential to reduce gluten allergenicity; ii) boiling has different effects on gluten allergenicity depending on whether gluten proteins are in pure form or contained within the wheat flour matrix; and iii) roasting and cracking steps during soy sauce production has the potential to reduce gluten allergenicity. Future testing using preclinical rodent models and human clinical testing are needed to confirm these effects.

3.6 Conclusion and Future Directions

Extensive animal pre-clinical testing and a limited human pre-clinical study have identified at least five promising dietary-based novel therapeutic possibilities for gluten hypersensitivity: i) fermentation; ii) probiotics; iii) enzyme hydrolysis; iv) thioredoxin, phosphorylation, and deamidation; and v) gene targeting of gluten loci in wheat plant. However, there are only two human clinical testing of such novel therapeutics reported in the literature. Our findings collectively suggest the following research agenda to advance translational science in gluten hypersensitivity:

i) Challenges and opportunities to consider in animal model testing: preclinical dose-response studies in animal models using quantitative readouts of disease phenotypes of gluten hypersensitivity noted in humans are urgently needed; although few studies have investigated models of anaphylaxis, more work is needed to carefully test the effects on validated quantifiable readouts of life-threatening systemic anaphylaxis, such as hypothermic shock responses, mucosal mast cell degranulation responses, and histamine responses; further, mechanisms of effects of treatments on gluten hypersensitivity also need to be investigated; all animal models testing the

effects of novel therapeutics have utilized adjuvants to create gluten hypersensitivity in their studies. Recent research shows that mechanisms underlying adjuvant-based versus adjuvant-free models of food allergy can be substantially different (Jin et al., 2020); since human gluten allergenicity is pathogenesis typically does not involve adjuvants such as alum, adjuvant-free models might be more helpful in developing therapeutics for human application. Adjuvant-free models of gluten hypersensitivity reported recently can be employed for this purpose (Jorgensen, et al., 2023a,b); furthermore, most animal models have focused on systemic anaphylaxis only; animal models of other types of hypersensitivities caused by gluten (atopic dermatitis, baker's asthma, etc.) are urgently needed for preclinical testing of novel diet-based therapeutics in development (**Figure 3.1**).

ii) Challenges and opportunities to consider in humans: most studies currently have focused on testing binding of IgE antibodies to altered gluten proteins. Since IgE binding only demonstrates sensitization at best, and not elicitation of clinical reactions, more work is needed to develop methods for clinical testing in humans or humanized cell lines/humanized animal models; there are only two *in vivo* testing of novel therapeutics (use of wheat naturally deficient in ω5 gliadin, such as Einkorn for WDEIA; and enzyme treated wheat flour for gluten induced AD) reported for human gluten hypersensitivity (Lombardo et al., 2015; Tanabe, 2008). Furthermore, most focus of human preclinical and clinical research has been on WDEIA and AD as the types of gluten hypersensitivity disorders for developing diet-based therapeutics. Therefore, more translational research is needed for other promising approaches in gluten hypersensitivity disorders not only for AD and WDEIA, but also for all other allergic conditions including life-threatening systemic anaphylaxis, typical gluten induced food allergy (vomiting,

diarrhea, urticaria), airways/conjunctival allergies, and baker's asthma caused by gluten (**Figure 3.1**).

In summary, evidence from the current literature demonstrates at least five promising dietary-based therapeutic approaches for mitigating gluten hypersensitivity that are in various stages of research and development. Further progress in future translational research promises potential novel dietary-based therapeutic options for the management of gluten hypersensitivity in humans.

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CHAPTER 4 A MOUSE-BASED METHOD TO MONITOR WHEAT ALLERGENS IN NOVEL WHEAT LINES AND VARIETIES CREATED BY CROSSBREEDING: PROOF-OF -CONCEPT USING *DURUM* AND *Ae. TAUSCHII* WHEATS

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4.1 Abstract

Wheat allergies are potentially life-threatening because of the high risk of anaphylaxis. Wheat belongs to four genotypes represented in thousands of lines and varieties. Monitoring changes to wheat allergens is critical to prevent inadvertent introduction of hyper-allergenic varieties via breeding. However, validated methods for this purpose are unavailable at present. As a proof-of- concept study, we tested the hypothesis that salt-soluble wheat allergens in our mouse model will be identical to those reported for humans. Groups of Balb/cJ mice were rendered allergic to durum wheat salt-soluble protein extract (SSPE). Using blood from allergic mice, a mini hyper-IgE plasma bank was created and used in optimizing an IgE Western blotting (IEWB) to identify IgE binding allergens. The LC-MS/MS was used to sequence the allergenic bands. An ancient *Aegilops tauschii* wheat was grown in our greenhouse and extracted SSPE. Using the optimized IEWB method followed by sequencing, the cross-reacting allergens *in Ae. tauschii* wheat were identified. Database analysis showed all but 2 of the durum wheat allergens and all *Ae. tauschii* wheat allergens identified in this model had been reported as human

allergens. Thus, this model may be used to identify and monitor potential changes to salt-soluble wheat allergens caused by breeding.

4.2 Introduction

Wheat allergy is a major food safety issue that affects wheat products. Contamination with major food allergens including wheat is a leading cause of food recalls in the USA (FDA, 2022). Prevalence of wheat allergies along with other major types of food allergies such as nut allergies, has been increasing not only in the USA but also in many other developed countries including Canada, Australia, Japan, European Union countries, and the United Kingdom (Leonard & Vasagar, 2014; Venter al., 2006A; Venter et al., 2006B; Sicherer et al., 2020). Because food allergens including wheat can trigger life-threatening systemic anaphylaxis, they are a serious concern for the food industry, and public health (Sicherer et al., 2018; Warren et al., 2020). Therefore, preventing inadvertent creation of hyper-allergenic wheats by wheat breeding is a major challenge facing wheat breeders.

Food allergies currently affect 8% of children and 10.8% of adults in the USA (Sicherer et al., 2018; Gupta et al., 2018). Sensitization to wheat (i.e., presence of IgE antibodies in the blood that bind to wheat proteins) in the United States is around 3.6% (Biagini et al., 2004). Clinically confirmed wheat allergies affect 0.4% of the population in the USA (Vierk et al., 2007); prevalence at the global level is estimated to be ~0.9% (Venter et al., 2006B); and affects both adults and children of both genders (Leonard & Vasagar, 2014; Cianferoni, 2016). Thus, wheat allergy is a major growing global public health problem that must be addressed immediately.

There are two major types of immune system-mediated diseases caused by wheat consumption: i) IgE antibody-mediated allergic diseases that include, classical food allergies (with symptoms of the gastrointestinal tract, and systemic anaphylaxis), atopic dermatitis, urticaria, baker's asthma, and allergic rhino-conjunctivitis; and ii) non-IgE-mediated immune diseases that include celiac disease (an autoimmune disease), non-celiac gluten sensitivity, and eosinophil-mediated diseases (e.g., eosinophilic esophagitis, eosinophilic gastroenteritis etc.) (Matsumura et al., 1994; Baur et al., 1998; Salcedo et al., 2011; Mimura et al., 2015; Beaudouin et al., 2006; Morita et al., 2009; Scherf et al., 2016; Furuta et al., 2015; Kottyan & Rothenberg, 2014). Notably, IgE-mediated allergic reactions such as systemic anaphylaxis and allergic asthma are potentially lethal (Sicherer et al., 2020; Warren et al., 2020). Therefore, identification of specific wheat allergens associated with life-threatening diseases is a critical first step towards diagnosis and management of wheat allergies.

Wheat proteins are classified based on the solubility of the non-glutens (water/salt-soluble albumins/globulins) and the glutens (alcohol-soluble gliadins and acid-soluble glutenins) (Shewry, 2009; Jin et al., 2019). The non-glutens represent 15-20% of total proteins, while the glutens comprise the rest (Pastorello et al. 2007; Palosuo et al., 1999; Morita et al., 2003). Notably, both types of wheat proteins can trigger IgE-mediated allergic reactions in humans and in animal models (Leonard & Vasagar, 2014; Cianferoni, 2016; Jin et al., 2019).

Wheat allergy develops in two phases: sensitization to wheat allergens, and wheat allergy disease elicitation in sensitized individuals (Sicherer et al., 2018; Renz et al., 2018; Platts-Mills, 2015). Sensitization occurs when genetically susceptible subjects are exposed to wheat products via physiologic routes such as oral, nasal, dermal, or conjunctival thus commencing the production of IgE antibodies to wheat allergens, resulting in sensitization (Sicherer et al., 2018;

Jin et al., 2019). The second phase of development of a wheat allergy is characterized by disease elicitation upon re-exposure to wheat allergens. Re-exposure to wheat allergens in sensitized individuals results in the binding of allergens to the IgE now present on the mast cells and basophils that trigger release of histamine and other mediators causing disease symptoms including life-threatening anaphylaxis (Cianferoni, 2016; Jin et al., 2019; Renz et al., 2018; Gao et al., 2021). Therefore, it is possible to identify wheat allergens that cause allergic reactions based on their ability to bind to IgE antibodies obtained from wheat-allergic humans and animal models such as mice.

Wheat is among the top 3 cereals (the others being rice and corn) consumed world-wide as a staple food by billions of people (FAO, 2020). Wheat production and consumption has been increasing worldwide in general. For example, wheat production has increased from 731.4 million tonnes in 2018, to 776.1 tonnes in 2020 (FAO, 2020). However, recent data shows that while production is increasing, its consumption has taken a downward trend in the USA for reasons yet to be determined (USDA, 2016). One potential reason for this trend may be the avoidance of wheat products due to real or perceived health concerns (Leonard et al., 2017; Pilolli et al., 2019). Therefore, identification and systematic monitoring of wheat allergens in various wheat varieties and wheat lines has become more urgent than ever before.

Commonly consumed wheats can be classified into three distinct genotypes, namely, AA, AABB, and AABBDD (Shewry 2016; Gao et al., 2021). The ancient *Ae. tauschii* wheat of the DD genome is not commercially available. The commonly consumed wheats produce wheat products meant for human and animal usage (Gao et al., 2021). Using conventional cross-hybridization, and back-crossing, wheat breeders have produced thousands of wheat varieties and wheat lines primarily to enhance agronomical phenotypes and for enhancing profitability

(Shewry et al., 2006; Lupi et al., 2013). However, the impact of these changes on allergenicity properties of wheat such as composition of wheat allergens has not been well studied. A major reason for this is the unavailability of validated and reliable methods to monitor changes to wheat allergens resulting from wheat breeding. There are efforts to generate wheat lines lacking specific allergens such as ω-5 gliadin (Kohno et al., 2016). Although genetically modified (GM) wheat by recombinant DNA technology is not commercially available, future researchers may consider such an approach. An international expert panel organized by the FAO/WHO had previously provided a decision-tree framework for assessing allergenic potential of GM foods so that they could be compared to the native varieties in determining 'substantial equivalence' between the GM vs. non-GM varieties (Domingo et al., 2016; Hollingworth et al., 2003; Selgarde et al., 2009). A similar approach may also be used to compare allergenicity of novel wheat varieties and lines developed by conventional wheat breeding with the currently established wheats. Currently, methods used to identify potential wheat allergens include using IgE from human allergic subjects and using database approaches by sequence comparisons. Use of appropriate animal models for pre-clinical assessment of novel foods has been suggested (Gao et al., 2021). However, a fully validated animal model is not yet available although efforts are underway in that direction.

There are many animal models of wheat allergenicity including dogs, rats, and mice that have been valuable for studying mechanisms of wheat allergenicity (Jin et al., 2019; Gao et al., 2021; Jin et al., 2020). Among these models, mice are most widely studied because of their short generation time, availability of gene knockout mice and reagents, and for economic reasons (Gonipeta et al., 2015). In addition, mouse models can also potentially serve as a valuable in vivo tool for monitoring changes to wheat protein allergenicity introduced by wheat breeding and

food processing. However, the mouse models must be extensively validated to simulate human wheat allergenicity as closely as possible to demonstrate their power to predict human allergenicity hazards. We have previously shown that salt-soluble protein extract (SSPE) from du-rum wheat can be used to clinically sensitize mice for life-threatening systemic anaphylaxis (Jin et al., 2020; Jin et al., 2017). However, it is unclear whether specific wheat allergens that elicit IgE antibodies and consequently cause sensitization for systemic analysis in this mouse model are identical to those that trigger human wheat allergies—the focus of this study. It is critical to validate this in this mouse model so that the model can be pro-actively applied to monitor changes to wheat allergens potentially due to wheat breeding.

Here, we tested the hypothesis that specific wheat allergens in our mouse model will be similar to human wheat allergens reported in the database. There were 4 objectives in this study: i) to optimize and validate an IgE Western blot (IEWB) method using hyper IgE plasma obtained from durum wheat-allergic mice; ii) to determine the IgE-binding protein bands in the IEWB followed by allergen identification by LC-MS/MS sequencing; iii) to identify the cross-reacting allergens present in the ancient *Ae. tauschii* wheat using the IgE antibodies from the durum wheat-allergic mice; and iv) to compare the mouse model wheat allergens to the human wheat allergens reported in the database. Our results collectively demonstrate that all, except two, of the allergens identified in this mouse model, are indeed reported as human wheat allergens. Therefore, these data provide the proof-of-concept that the mouse model may be used to identify and monitor changes to wheat allergens due to wheat breeding.

4.3 Results

4.3.1 Identification of IgE Binding Protein Bands in Boiled/Reduced and Native SSPE from *Durum* Wheat

The overall experimental approach used in this study is shown in Figure 4.1. Specific IgE antibody levels were measured using an ultra-sensitive ELISA method we have de- scribed before (Jin et al., 2017; Birmingham et al., 2003). The IgE antibody titer of the plasma was 2560. As evident, using the optimized conditions of the IgE Western blot, we found 5 distinct IgE-binding protein bands in the boiled/reduced durum SSPE (Figure 4.2). These were labeled Tetraploid Boiled (TB) 1 through TB5 and approximately corresponded to the following sizes, respectively: 45–48 kDa, 40–43 kDa, 30–33 kDa, 26–28 kDa, and 22–23 kDa (Figure 4.2). Three were 7-distinct IgE binding bands in the native durum SSPE (Figure 4.2) that were labelled as Tetraploid Native (TN) 1 through TN7. Their approximate sizes were, respectively: 35–37 kDa, 32–35 kDa, 28–30 kDa, 25–26 kDa, 18–20 kDa, 16–17 kDa, and 12–15 kDa (Figure 3.2). Notably, the last two bands were observed only in the native SSPE but not in the boiled/reduced SSPE. Native SSPE exhibited relatively stronger background activity compared to the boiled/reduced SSPE.

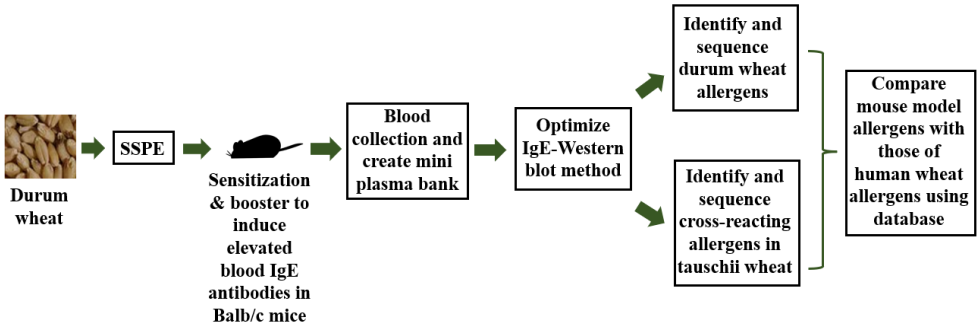

Figure 4.1 Overall experimental approach used in this study. The durum wheat flour was used to produce salt-soluble protein extract (SSPE).

Groups of Balb/c mice (*n* = 20) were sensitized to SSPE using a published method. After booster injections, blood was collected at biweekly intervals. Plasma was separated and pooled to create a mini plasma bank. The anti-SSPE IgE antibody levels in the plasma were quantified by ELISA (titer: 1/2560). Then it was used to optimize an IgE-Western blot method. The IgE antibody binding protein bands present in durum wheat SSPE were identified in the Western blot. These protein bands were then sequenced by LC-MS/MS method and durum wheat allergens were identified. Using *Ae. tauschii* wheat SSPE, the cross-reacting allergens that bind to anti-durum wheat IgE antibodies were identified. The mouse model allergens were then compared to the human wheat allergens reported in the database.

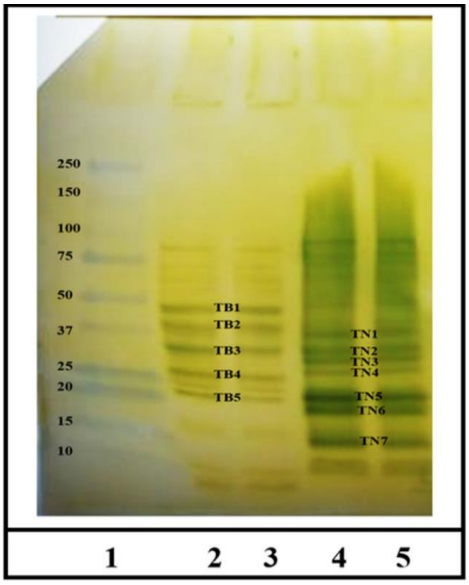

Figure 4.2 Western blot analysis of IgE binding protein bands in the salt-soluble proteins extracts from durum wheat.

Figure 4.2 depicts data from analysis of boiled/reduced durum wheat protein extract and native durum wheat protein extract. Lane 1 = molecular weight marker (kDa); 2 and 3 = Durum wheat (AABB) boiled/reduced protein extract in duplicates. The IgE binding protein bands are labelled

as Tetraploid Boiled (TB) 1 to TB5. 4 and 5 = Durum wheat (AABB) native protein extract in duplicates. The IgE binding protein bands are labelled as Tetraploid Native (TN) 1 to TN7.

4.3.2 Sequencing and Identification of Allergens in Boiled/Reduced SSPE and Native SSPE from Durum Wheat

Allergens present in the boiled/reduced IgE binding protein bands TB1 to TB5 are depicted in Figure 4.3A–E. The TB1 band contained 4 allergens. The TB2 band contained 6 allergens of which 2 were unnamed protein products. The TB3 band contained 1 allergen. The TB4 and TB5 bands contained 4 and 1 allergens, respectively. Overall, there were 9 allergens in boiled/reduced durum SSPE of which 2 were unnamed protein products, resulting in a total of 7 mouse allergens. Globulin 1, Globulin 3, and Serpin allergens appeared at multiple sizes. Detailed information on peptide sequences, position etc. is provided in Supplementary Table S3.1.

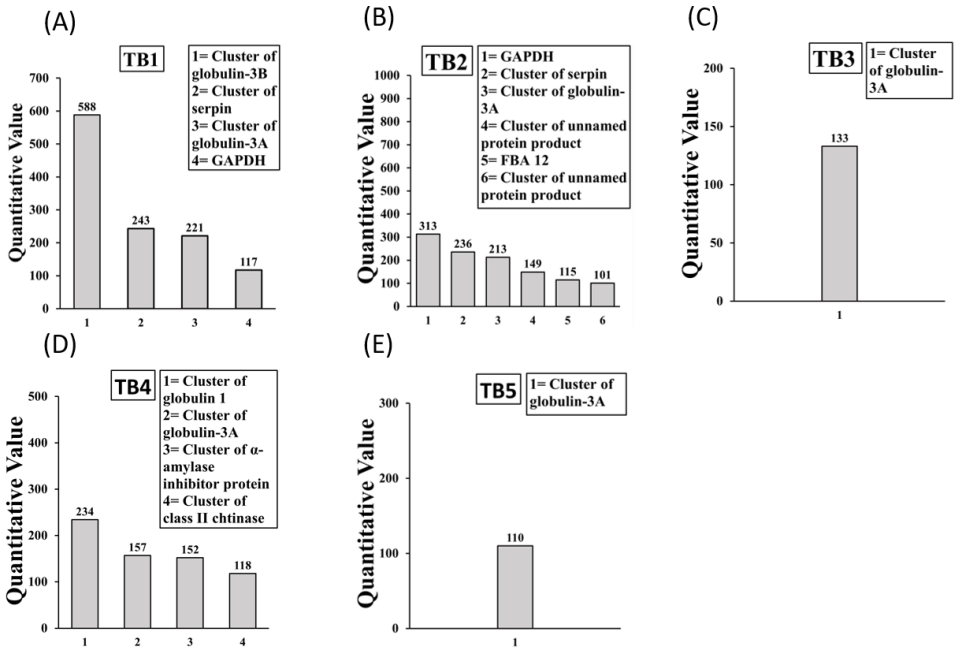

Figure 4.3 Allergens (IgE binding proteins) present in the boiled/reduced durum wheat salt-soluble protein extract.

The subfigures (A–E) show the results from LC-MS/MS analysis of IgE binding protein bands TB1 to TB5 in durum wheat respectively. Specific allergens found are listed in the boxes.

The most abundant allergenic proteins present in the non-reduced non-boiled durum SSPE IgE binding protein bands TN1 to TN7 are shown in Figure 4.4A–G. The TN1 band contained 3 allergens. The TN2 band contained 3 allergens. The TR3 band contained 1 allergen. The TN4 band contained 4 allergens. The TN5 band contained 2 allergens. The TN6 band contained 3 allergens. The TN7 band contained 2 allergens. Overall, there were 12 allergens in native durum SSPE. Globulin 3A, Serpin, and GAPDH allergens appeared at multiple sizes. Notably, the following allergens present in native durum SSPE were absent in boiled/reduced durum SSPE: Tritin, Peroxidase, cluster of dehydroascorbate reductase, alpha amylase/subtilisin inhibitor, endogenous alpha amylase/subtilisin inhibitor, Histone H4, and cluster of heat shock protein 17.3. Detailed information on peptide sequences, position etc. is provide in Supplementary Table S4.2.

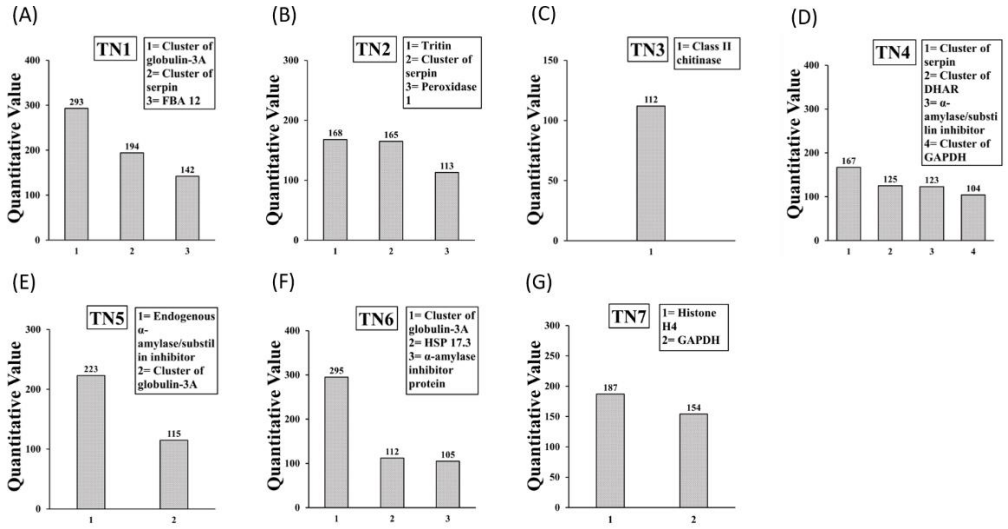

Figure 4.4 Allergens (IgE binding proteins) present in the native durum wheat salt-soluble protein extract.

The subfigures (A–G) show the results from LC-MS/MS analysis of IgE binding protein bands TN1 to TN7 in durum wheat respectively. Specific allergens found are listed in the boxes.

4.3.3 Identification of IgE Binding Protein Bands in Boiled/Reduced SSPE and Native SSPE from the *Ae. tauschii* Wheat

As evident, we found 2 IgE binding protein bands in the boiled/reduced *Ae. tauschii* SSPE (Figure 4.5). There were named as Diploid Boiled (DB)1 and DB2 that approximately corresponded to 40–43 kDa and 33–37 kDa, respectively. There were also few fine lines showing reactivity at the higher sizes (Figure 4.5). In contrast, there were 3 IgE binding bands in the native *Ae. tauschii* SSPE (Figure 4.5) that were labelled as Diploid Native (DN) 1 through DN3. Their approximate sizes were: 33–37 kDa, 24–26 kDa, and 15–16 kDa, respectively (Figure 4.5). Notably, DN2 and DN3 were present only in the native *Ae. tauschii* SSPE but not in the boiled/reduced *Ae. tauschii* SSPE. On the other hand, DB1 was present only in the boiled/reduced SSPE, and there was no equivalent band in the native SSPE.

4.3.4 Sequencing and Identification of Allergens in Boiled/Reduced and Native *Ae. tauschii* Wheat SSPE

Allergens present in the boiled/reduced DB1 and DB2 bands are shown in Figure 4.6 A,B. The DB1 band contained 4 allergens (3 of which were isoforms of serpin, and Globulin 3), and the DB2 band contained 2 allergens. Overall, there were only 3 allergens (Serpins of 3 isoforms, peroxidase 1, and Globulin 3) in the boiled/reduced *Ae. tauschii* wheat SSPE. Detailed information on peptide sequences, position etc. is provided in Supplementary Table S4.3.

Allergens present in the native *Ae. tauschii* wheat SSPE IgE-binding protein bands DN1

to DN3 are shown in Figure 4.7 A–C. The DN1 band contained 1 allergen. The DN2 band contained 7 allergens. The DN3 band contained 2 allergens of which 1 was an unnamed product. Overall, there were 9 allergens in native *Ae. tauschii* wheat SSPE with one unnamed protein product resulting in 8 allergens altogether. Notably, the following allergens present in native *Ae. tauschii* SSPE were absent in boiled/reduced *Ae. tauschii* SSPE: Cluster of dehydroascorbate reductase, Class II Chitinase, endogenous alpha-amylase subtilisin inhibitor (17–20 kDa), gamma gliadin, cluster of dimeric α-amylase inhibitor precursor, and an unnamed protein product. Detailed information on peptide sequences, position etc. is provided in Supplementary Table S4.4.

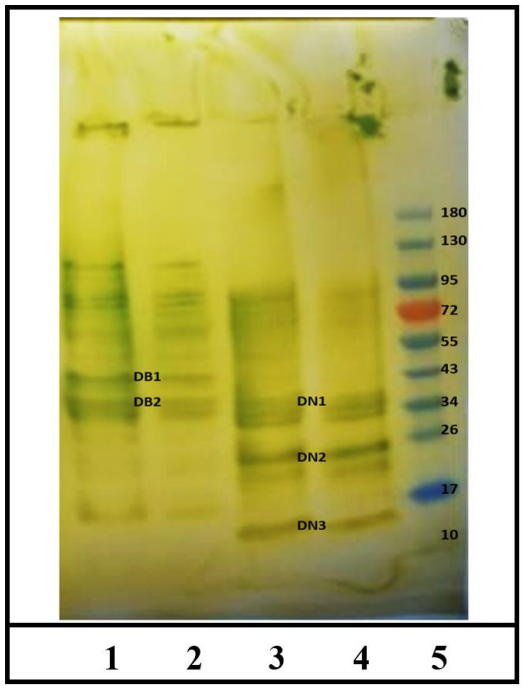

Figure 4.5 Western blot analysis of IgE binding protein bands in the salt-soluble proteins extracts from *Ae. tauschii* wheat.

Figure 4.5 depicts data from analysis of boiled/reduced and native *Ae. tauschii* wheat protein extract. Lanes 1 & 2 = *Ae. tauschii* wheat (DD) boiled/reduced protein extract in duplicate. The IgE binding protein bands are labelled as Diploid Boiled (DB) 1–2. Lanes 3 & 4 = *Ae. tauschii*

wheat (DD) native protein extract in duplicate. The IgE binding protein bands are labelled as Diploid Native (DN) 1–3. Lane 5 = molecular weight marker (kDa).

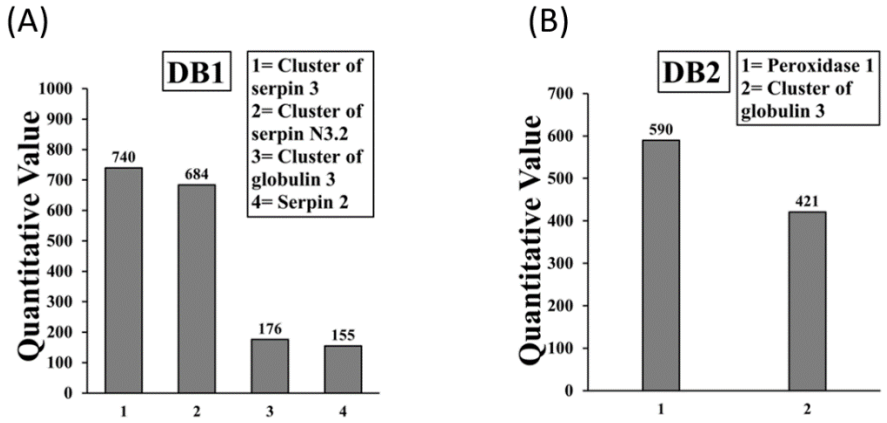

Figure 4.6 Allergens (IgE binding proteins) present in the boiled/reduced *Ae. tauschii* wheat salt-soluble protein extract.

The subfigures (A,B) show the results from LC-MS/MS analysis of IgE binding protein bands DB1 and DB2 in *Ae. tauschii* wheat respectively. Specific allergens found are listed in the boxes.

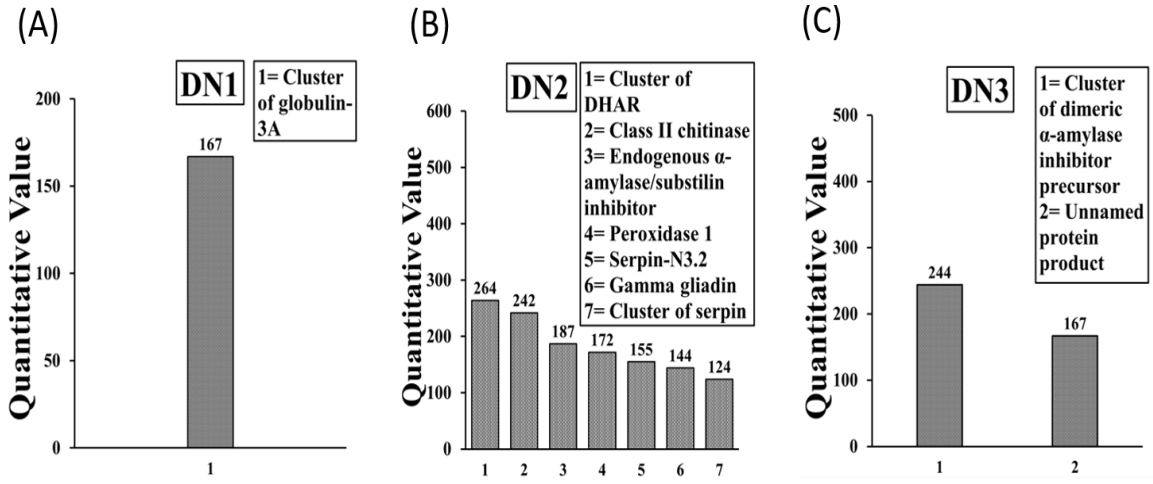

Figure 4.7 Allergens (IgE binding proteins) present in the native *Ae. tauschii* wheat salt-soluble protein extract.

The subfigures (A–C) show the results from LC-MS/MS analysis of IgE binding protein bands DN1 to DN3 in *Ae. tauschii* wheat respectively. Specific allergens found are listed in the boxes.

4.3.5 Comparison of the Wheat Allergens in the Mouse Model to Those Reported as Human Wheat Allergens in the Database

PubMed, Google Scholar and allergome.com databases were searched for evidence of the reports of human allergies to the proteins identified as allergens in this mouse model. We found that all 7 allergens identified in this mouse model in boiled/reduced durum SSPE had been reported as human allergens (Table 4.1). Two allergens were unnamed protein products and therefore, their relevance to human wheat allergen could not be determined. Overall, 10 out of 12 allergens found in this mouse model in the native durum SSPE had been reported as human allergens (Table 4.1). We found that all 3 allergens identified in the boiled/reduced *Ae. tauschii* SSPE had been reported as human wheat allergens (Table 4.2). Similarly, we found that all 8 allergens identified in this mouse model in the native *Ae. tauschii* SSPE had been reported as human wheat allergens (Table 4.2). One mouse model allergen was an unnamed protein product and therefore, its relevance to human wheat allergen could not be determined.

Table 4.1 Durum wheat allergens identified in the mouse model vs. reported wheat allergens in humans.

Durum Mouse Allergens	Accession Number	Human Allergen
Boiled/reduced SSPE (7)		
Tri Glo (Globulin 3 A, B)	AFM30909.1 [3], ACJ65515.1 [5]	Yes
Cluster of Globulin 1 *,**	ABG68034.1 [2]	Yes
Tri a 33 (Serpin)	CAB52710.1 [5], CAA90071.1 [5]	Yes
Tri a 34 (GAPDH)	ANW11922.1 (+1), ALE18232.1 [3]	Yes
Tri a aASI (Endogenous α-amylase/substilin inhibitor)	AAR10959.1 [4], IAAS_WHEAT (+1)	Yes
Tri a Chitinase	AAX83262.1 [13]	Yes
Cluster of fructose-1,6-bisphophate aldolase 12 **	AVL25144.1 [5]	Yes
Native SSPE (12)		
Tri Glo (Globulin 3 A)	AFM30909.1 [4]	Yes
Tri a 33 (Serpin)	CAB52710.1 [7], CAA90071.1 [6]	Yes
Tri a Tritin	BAA0248.1	Yes
Tri a Peroxidase 1	AAM88383.1 (+4)	Yes
Tri a Chitinase	AAX83262.1	Yes
Cluster of dehydroascorbate reductase **	AAL71851.1 [2]	Yes
Tri a aASI (Endogenous α-amylase/substilin inhibitor)	AAR10959.1 (+2)	Yes
Tri a aASI (Endogenous α-amylase/substilin inhibitor)	P16347.1 (+1)	Yes
Cluster of GAPDH **	ALE18233.1 [3], ANW11922.1 (+1)	Yes
Histone H4 **	AAA34292.1 (+21)	?
Cluster of fructose-1,6-bisphophate aldolase 12 **	AVL25144.1 [5]	Yes
Cluster of heat shock protein 17.3 **	CAA41218.1 [5]	?

SSPE: Salt-soluble protein extract; * These proteins showed IgE binding only in boiled/reduced SSPE but not in native SSPE; underlined allergens were present only in the native SSPE; ** For this protein technical allergen name (Tria a #) is not available at present.

Table 4.2 Ancient *Ae. tauschii* wheat allergens identified in the mouse model vs. reported wheat allergens in humans.

A. tauschii Mouse Allergens	Accession Number	Human Allergen
Boiled/reduced SSPE (3)		
Tri a 33 (Serpin 2 *, 3 *, N3.2)	ACN59484.1 (+1), ACN59485.1 [6], AFC89429.1 [5]	Yes
Tri Glo (Globulin 3)	ACJ65514.1 [4]	Yes
Tri a Peroxidase 1	AAM88383.1 (+4)	Yes
Raw SSPE (8)		
Tri Glo (Globulin 3A)	AFM30909.1 [4]	Yes
Cluster of dehydroascorbate reductase **	ACV89491.1 [2]	Yes
Tri a Chitinase	AAX83262.1	Yes
Tri a aASI (Endogenous α-amylase/substilin inhibitor)	P16347.1 (+1)	Yes
Tri a Peroxidase 1	AAM8838.1 (+4)	Yes
Tri a 33 (Serpin N3.2)	AFC89429.1	Yes
Tri a 20 (Gamma gliadin)	ABO37959.1	Yes
Tri a 28 (dimeric α-amylase inhibitor precursor)	ABF93411.1 [8]	Yes

SSPE: Salt-soluble protein extract; * These proteins showed IgE binding only in boiled/reduced SSPE but not in native SSPE; underlined allergens were present only in the native SSPE; ** For this protein technical allergen name (Tri a #) is not available at present.

4.4 Discussion

Monitoring changes to wheat allergens in novel wheat varieties and wheat lines is critical to prevent inadvertent introduction of hyper-allergenic varieties via wheat breeding. However, validated methods for this purpose are unavailable at present. Towards this end, as a proof-of-concept study, here we tested the hypothesis that salt-soluble wheat allergens in our mouse model would be identical to those reported for human wheat allergy. Our data obtained using the commonly consumed durum wheat shows that mostly the same set of proteins are recognized as allergens in the mouse model as well as in humans. Furthermore, we also demonstrate that anti-durum wheat IgE antibodies also bind to similar allergens present in an ancient *Ae. tauschii* wheat, and those cross-reacting wheat allergens are also reported as human wheat allergens.

Thus, collectively our data provides the proof-of-concept in support of our hypothesis that this mouse model may be used to monitor changes to salt-soluble wheat allergens present in the durum wheat and *Ae. tauschii* wheats that might be introduced by breeding.

There are seven novel findings from this study: (i) it is possible to obtain detailed information on salt-soluble wheat allergens using a mouse model of durum wheat allergy; (ii) based on susceptibility to heating, three types of allergens are present in durum wheat salt-soluble protein extract (SSPE): (a) allergens (6) that are present in the native SSPE, but not in boiled/reduced SSPE; (b) allergens (6) that are present in native SSPE, but are resistant to boiling/reducing conditions; and (c) allergens (2) that do not bind to IgE in native SSPE, but bind to IgE only in boiled/reduced SSPE (2); (iii) durum wheat-elicited IgE antibodies can identify cross-reacting allergens present in the ancient *Ae. tauschii* wheat; (iv) based on susceptibility to heating, similar to the durum wheat, three types of allergens are present in the *Ae. tauschii* wheat: (a) allergens (5) that are present in the native SSPE but are not present in boiled/reduced SSPE; (b) allergens (3) that are present in the native SSPE, but are resistant to boiling/reducing conditions; and (c) allergens (2) that do not bind to IgE in native SSPE, but bind to IgE only in boiled/reduced SSPE; (v) allergens identified in the ancient *Ae. tauschii* wheat are also present in the durum wheat; (vi) durum wheat contains 3 allergens (GAPDH, tritin, fructose-1,6-bisphosphate aldolase 12) that are absent in the *Ae. tauschii* wheat; (vi) all, but 2, durum wheat allergens, and all *Ae. tauschii* wheat allergens identified by us in the mouse model have been reported as human wheat allergens in the database; and (vii) two allergens (Histone H4 and cluster of heat shock protein 17.3) that are present only in the durum wheat but not in the *Ae. tauschii* wheat, have not been reported as human wheat allergens so far.

We used *durum* wheat in this study because: (i) it is a commonly consumed tetraploid

wheat (AABB genome); and (ii) previously we had used durum wheat SSPE for developing and characterizing the mouse model of wheat allergy (Jin et al., 2020; Jin et al., 2017). We also used the ancient *Ae. tauschii* wheat because: (i) the ancient *Ae. tauschii* wheat, which is a diploid wheat (DD genome), has been part of the history of wheat evolution that has resulted in today's common bread wheat (hexaploid, AABBDD genome); and (ii) we had access to the seeds of this ancient wheat at our university repository in the wheat genetics and breeding program. Using these two types of wheats, we were able to specifically identify the allergens from these two distinct types of wheats and were able to identify the allergens present in the ancient wheat that showed IgE cross-reactivity with the durum wheat. It is remarkable that the ancient wheat contains only some of the allergens present in the durum wheat. This finding may explain our previous report where we demonstrated significantly lower IgE binding of *Ae. tauschii* wheat SSPE compared to the durum wheat SSPE in an IgE inhibition ELISA (Gao et al., 2019). These in vitro data together support the idea that *Ae. tauschii* wheat SSPE may be relatively hypoallergenic compared to durum wheat in durum wheat-sensitized hosts. However, this remains to be tested in future *in vivo* studies using the mouse model.

We used the Balb/cJ mouse to generate the hyper-IgE plasma used in this study because: (i) this strain of mouse is genetically prone to develop food allergies; (ii) allergic responses to several food proteins in this mouse strain are similar to humans with food allergies to hazelnut, cashew nut, sesame, shellfish and wheat gliadins and non-gluten allergens including lipid transfer protein (Gao et al., 2021; Jin et al., 2020; Birmingham et al., 2005; Birmingham et al., 2007; Parvataneni et al., 2009; Gonipeta et al., 2010; Gonipeta et al., 2015); and (iii) we have extensively characterized the allergic response to salt-soluble wheat proteins in this model previously (Jin et al., 2020; Jin et al., 2017; Gao et al., 2019). It is remarkable that all 7 thermal-

resistant salt-soluble wheat allergens which elicit IgE responses in this model are also reported to elicit and bind to human IgE antibodies, and thus act as allergens in both species. An elegant previous study showed that purified lipid transfer protein (LTP) from wheat elicited IgE antibody response when injected into Balb/cJ mice and that subjects who were allergic to this protein showed a highly similar epitope binding structure suggesting again the similarity between the Balb/cJ mice and the human allergic responses (Denery-Papini et al., 2011). Thus, together, previous studies along with findings from the present study further support and justify the use of the Balb/cJ mouse model for wheat allergenicity research.

The rationale for choosing salt-soluble wheat proteins in this study is as follows: wheat allergens belong to both glutens and non-gluten protein families. Salt-soluble proteins are non-glutens. Both types of allergens are important in human wheat allergic disease and therefore, both types of proteins need to be researched (Jin et al., 2019; Gao et al., 2021). However, in this 'proof-of- concept' study we researched salt-soluble wheat proteins as a model wheat allergen. Using this concept, it should also be possible to study gluten allergens.

We have extensively characterized the wheat protein extracts and ensured that high quality proteins are present. We have provided the SDS-PAGE images of the two protein extracts used in this study (Supplementary Figure S4.1). Furthermore, we have studied their allergenicity using an IgE inhibition ELISA method and found that *Ae. tauschii* is indeed less allergenic (Gao et al., 2019). This was our baseline comparison. Based on this data, we set out to identify the specific allergens using the IgE Western blot method we have described in this paper.

In the native durum SSPE we identified two mouse model wheat allergens (Histone

H4, and cluster of heat shock protein) that have not been reported as human allergens. It is possible that since these 2 allergens are destroyed by boiling of SSPE, they may not be able to elicit human IgE responses since they are not expected to be present in the thermally processed wheat food products to elicit an IgE response. Since we used native SSPE to sensitize mice, these 2 proteins could elicit and bind to IgE antibodies in our study. However, it is possible for human exposures to native SSPE (and therefore to these 2 proteins) to occur via non-oral routes (eyes, airways, and skin) from non-heat-treated wheat flour at home or in the baking industries. Therefore, these two proteins may pose potential allergenicity problems in humans that are yet to be identified.

Previous studies show that depending on the type of processing, wheat allergenicity may increase or reduce or may not change (Gao et al., 2021). For example, while boiling wheat flour has no significant effect on allergenicity of albumins and globulins (salt-soluble wheat proteins), boiling of pasta reduced their allergenicity by about 50% as measured by human IgE Western blot analysis (Pastorello et al., 2007). We also found that boiling/reducing of SSPE did not reduce allergenicity of globulins and several other salt-soluble allergens. However, we also identified several other allergens present in the native SSPE that were inactivated by boiling/reducing. In addition, we found that peroxidase present in durum wheat SSPE was inactivated by boiling/reduction; however, peroxidase present in *Ae. tauschii* wheat SSPE did not lose allergenicity by such processing suggesting differences in the property of the same allergen encoded by AABB vs. DD genomes. Whereas Globulin 3A (present in both durum and *Ae. tauschii* wheats) was resistant to boiling/reduction conditions, Globulin 1 & 3B allergens (present only in durum wheat but not in *Ae. tauschii* wheat) showed allergenicity only upon boiling/reducing. Interestingly, Serpins present in both durum and *Ae. tauschii* wheats were

resistant to boiling/reducing conditions. Thus, the effects of boiling on wheat allergenicity appears to be complex. Nevertheless, our data demonstrate that the approach described here can be proactively used to identify boiling/reduction sensitive vs. resistant allergens in novel wheat varieties and wheat lines that might be created by breeding of durum and *Ae. tauschii* wheats (Zhou et al., 2021). Furthermore, this approach can also be used to screen existing lines and varieties to establish baselines profiles of boiling/reduction sensitive vs. resistant wheat allergens. This type of information can not only help prevent inadvertent introduction of hyper allergenic wheats but also can inform the development of novel hypo/non-allergenic wheat lines and varieties (Gao et al., 2021).

Wheat allergens belong to both non-gluten and gluten protein families. To validate a mouse model for human wheat allergenicity, it will be necessary to test both non-gluten and gluten (gliadins and glutenins) allergens in the mouse model. Here, we validate the similarity of non-gluten wheat allergens in this model to those reported for human wheat allergenicity. Previous elegant studies demonstrated that the alcohol-soluble gliadins and lipid transfer protein show allergenicity in Balb/c mice like that in human wheat allergic subjects (Denery-Papini et al., 2011; Bodinier et al., 2009). However, the allergenicity potential of acid-soluble glutenin in mice remains to be tested.

Currently many different wheat (sub-)species/cultivars are available on the market. There are only a few studies at present that report potential differences among them in causing human wheat allergy. Nakamura et al. (2005) studied 324 wheat varieties and reported that some are potentially hypoallergenic based on IgE binding in an ELISA. Larre et al. (2011) compared salt-soluble protein allergens in diploid vs. hexaploid wheats using serum from wheat allergic subjects. They reported that IgE binding was much lower for the diploid wheat. Kohno et al.

2016 developed a new wheat line lacking ω-5 gliadin locus and showed that it is less allergenic in a guinea pig model (Kohno et al., 2016). Gao et al. (2019) showed that salt-soluble protein extract from an ancient diploid wheat is significantly less allergenic than that from the tetraploid durum wheat and the hexaploid Ambassador wheat based on *in vitro* IgE binding. Thus, there is emerging evidence that different wheats might be different in allergenicity. However, *in vivo*, or clinical evidence is lacking. Therefore, there is urgent need to further research this problem and carefully map the variation in clinical allergenicity among various wheat species, sub-species, and cultivars. Such research has the potential to develop hypo/non-allergenic wheat lines.

In this study, as a proof-of-concept, we used IgE enriched hyper immune plasma from durum wheat salt-soluble protein sensitized mice and studied the allergens present in du- rum wheat as well as cross-reacting allergens present in the ancient diploid *Ae. tauschii* wheat. A similar approach can be used to sensitize mice with whichever wheat variety/line one would be interested in and study the allergens using the approach we have reported here.

The mouse method described here is a novel approach to monitor changes to wheat allergens potentially caused by crossbreeding and/or genetic modification. It is not intended to replace or substitute other methods such as using human serum from wheat allergic subjects for allergen identification. However, the mouse method we describe has several advantages compared to the screening for wheat allergens using serum from wheat allergic subjects including the following: (i) in mice, exposure to wheat can be controlled completely. For example, mice can be sensitized to a particular species/sub-species/genotype (e.g., diploid vs. tetraploid vs. hexaploidy), wheat variety/lines etc. Therefore, mono sensitized IgE antibodies can be produced by this method. In contrast, such controlled exposure of humans to one wheat cultivar/species/sub-species/genotype etc., is not possible. Therefore, having mono sensitized

human serum from exposure to a single type of wheat exposure is not possible to obtain from wheat allergic subjects; (ii) humans produce IgE antibodies against many grass allergens that are known to cross-react with wheat allergens (Larre et al., 2011). Therefore, human serum containing wheat-binding IgE antibodies could be in theory from either exposure to wheat or to grass or to both. Such exposure to grass does not occur in mice where environmental and dietary exposure can be strictly controlled; (iii) the ancient diploid *Ae. tauschii* wheats are not commercially available. Therefore, obtaining *Ae. tauschii* specific IgE from humans is not possible; in this study we elicited IgE antibodies using durum wheat; however, using the same approach, mice can also be exposed to *Ae. tauschii* wheat in controlled conditions, and it is possible to obtain *Ae. tauschii* specific IgE antibodies from such mice which will not be possible to obtain from humans; and (iv) when a novel wheat cultivar/line is developed, it can also be tested in this mouse model to monitor and identify the changes to allergens. Such an approach is not possible with human serum testing simply because humans would not have been exposed to the novel wheat cultivar/line at that point in time.

In this study we used our optimized IgE Western blot method to identify the allergens that bind to IgE antibodies. In most cases, for a given IgE binding band, more than one protein was identified in MS analysis. It is possible that one or more or all the proteins identified within band might contribute to the IgE reactivity. Therefore, the relative contribution of multiple proteins to IgE binding reactivity in Western blot remains to be determined in future studies. Therefore, the allergens identified here should be deemed as 'putative' allergens. Future studies can be conducted using single isolated proteins to determine their relative contribution to the IgE reactivity in Western blot.

There are several limitations of using a mouse model to study wheat allergy including the following: (i) human wheat allergies result from natural exposure to wheat via oral, nasal, conjunctival, or skin routes; here we sensitize mice to wheat protein using intraperitoneal injections with alum adjuvant; (ii) digestion of wheat proteins in the gut is an additional factor that influences wheat allergenicity in humans; notably, this feature is not represented in the mouse model; and (iii) some wheat-sensitized humans develop anaphylaxis after performing exercise following wheat ingestion and this is known as exercise-dependent wheat induced anaphylaxis (Scherf et al., 2016); in this mouse model to induce anaphylaxis exercise is not required; intraperitoneal injection with the wheat allergen is sufficient to elicit systemic anaphylaxis.

It is very important to validate and improve the existing mouse models of wheat allergenicity so as to simulate the allergic response as closely as possible to that in humans for a number of reasons, including the following: (i) validated mouse models of wheat allergenicity are essential to conduct pre-clinical studies testing the allergenicity of novel wheat lines, and wheat varieties developed by conventional breeding; it is important to note that validated animal models of food allergenicity have been incorporated in the decision-tree approach recommended by the FAO/WHO expert committee for establishing 'substantial-equivalence' of novel foods (Domingo, 2016; Hollingworth et al., 2003; Selgarde et al., 2009); currently GM wheats are not commercially sold yet, but if future researchers were to consider developing GM wheats, this method would be useful (Shewry et al., 2006; Lupi et al., 2013; Beale et al., 2009; Venter et al., 2016; Yadav et al., 2015; Meyer et al., 2018); (ii) validated animal models also have applications in the development and validation of novel hypo/non-allergenic wheat products based on food and industrial processing methods (Gao et al., 2021); and (iii) validated mouse models will be

valuable to advance the knowledge on the mechanisms of wheat allergenicity so that new methods to prevent and treat wheat allergies become available.

4.5 Materials and Methods

4.5.1 Chemicals and Reagents

The following chemicals and reagents were obtained from the sources indicated in parentheses: Biotin conjugated rat anti-mouse IgE paired antibodies and isotype standards (BD BioSciences, San Jose, CA, USA); para-Nitrophenylphosphate (Sigma, St Louis, MO, USA); streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA); protein estimation reagents: bovine serum albumin standard and reagents A and B (Sigma, St Louis, MO, USA). Pre-made SDS-PAGE gels were purchased from Bio-Rad (catalog #4561094). The cellulose membranes for Western blot were purchased from Bio-Rad (cat- alog #1620145); as were the molecular weight markers (Bio-Rad Precision Plus Protein catalog #161-0373; Thermo Scientific PageRuler Prestained Protein Ladder Product #26616); substrate buffer (Southern Biotech TMB membrane substrate catalog #0304-01); blocking buffer (5% BSA).

4.5.2 Mice Breeding Generation of a Plant-Protein-Free Mice Colony

Balb/cJ mice (female) were generated on a plant-protein free diet (AIN-93G) and purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in the animal facility of the Trout Food Science and Human Nutrition Building at Michigan State University. Mice were maintained on the plant protein-free diet (AIN-93M). All mice used in this study were 4–6 weeks old at the onset of the studies. All animal procedures used were in

accordance with Michigan State University policies and approved by the animal use and care review committee.

4.5.3 Preparation of Salt-Soluble Wheat Protein Extract from Durum Wheat

Durum wheat (variety Carpio) flour was used in the preparation of salt-soluble protein extract (SSPE) using the following standard method (Jin et al. 2017). Briefly, 10 g of flour in 100 mL of 0.5 M NaCl was stirred continuously for 2 h at 20 ◦C followed by centrifugation (5000× g, 10 min) at 20 ◦C. The supernatant was frozen overnight at −16 ◦C and then freeze-dried. The protein content was quantified according to Bradford dye-binding method (Bradford et al., 1976).

4.5.4 Growing Ancient *Ae. tauschii* Wheat and Preparation of Salt-Soluble Wheat Protein Extract

Using seeds stored with the Michigan State University wheat breeding program, *Ae. tauschii* wheat was grown at the university greenhouse. Upon harvesting, the berries were threshed, then milled. Using the whole meal flour and following the same method described above for durum wheat, *Ae. tauschii* SSPE was prepared and quantified.

4.5.5 Preparing a Hyper-IgE Plasma Mini-Bank from Durum Wheat-Allergic Mice

As described before, mice (n = 20) were sensitized with the durum wheat SSPE to induce IgE antibody responses using the published method (Gao et al., 2019). Briefly, animals were injected with SSPE (0.01 mg/mouse) plus alum (1 mg/mouse) four times by intraperitoneal (IP) route, on days 0, 10, 24, and 40. Specific IgE levels were measured and clinical sensitization

for anaphylaxis was confirmed by IP challenge (0.5 mg/mouse) followed by rectal thermometry and determination of mucosal mast cell degranulation responses. After booster injections, blood was collected at bi-weekly intervals. Pooled plasma was prepared, and aliquots were stored at −70 ◦C until used in Western blot analysis. IgE levels were quantified by an optimized ELISA method as described before (Gao et al., 2019).

4.5.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Pre-made SDS-PAGE gels (4–20%) were purchased and used in the study. The SSPE samples were analyzed under non-reduced and reduced/boiled conditions. Freeze-dried samples of durum SSPE were prepared with sample buffer (Bio-Rad catalog #161-737) and diluted with deionized water to desired concentration to produce non-reduced, non-boiled SSPE samples (hereafter referred to as “native SSPE”). Freeze-dried samples of durum SSPE were prepared with sample buffer, 5% β-mercaptoethanol, diluted with deionized water to desired concentration, and boiled at 100 ◦C for 10 min to produce reduced/boiled SSPE samples (hereafter referred to as “boiled SSPE”). Mini-PROTEAN TGX gels were loaded with native and entire boiled SSPE with one marker on each side of the gel and run in the running buffer at 100 volts until the dye front reached the reference line. Gels were stained using Coomassie brilliant blue dye. The method was optimized for protein quantity and run times.

4.5.7 Optimization of an IgE Western Blot Method

After SDS-PAGE the protein was transferred to a nitrocellulose membrane (Bio-Rad catalog #1620145) overnight at 4 ◦C. Ponceau staining was used to ensure the transfer of proteins. The membrane was washed five times using TBST, one minute each time. Blocking

was done in 5% BSA for one hour at room temperature. The membrane was washed five times with TBST, one minute each. Then the membrane was incubated with the primary antibody (i.e., mouse hyper IgE plasma) in blocking buffer for three nights at 4 ◦C. Then the membrane was washed five times with TBST, one minute each. The membrane was then incubated with the secondary antibody (Southern Biotech Goat anti-mouse IgE-HRP catalog #1110-05) in blocking buffer for one hour. Then the membrane was washed five times with TBST, one minute each. The membrane was then incubated with the substrate solution (Southern Biotech TMB membrane substrate catalog #0304-01) for 10 min. Excess substrate was removed with TBST wash and then the signals were photographed. The method was optimized for incubation times, antibody quantities, and developing times. Plasma sample from non-allergic control mice was used as the negative control.

The pooled hyper IgE plasma was used in optimizing the IgE Western blot method. In order ensure that the durum wheat extract used in this study indeed contain human IgE reacting epitopes, we have tested and confirmed the IgE reactivity of the extracts used in this study using plasma from wheat allergic subject. Durum wheat SSPE was used with and without boiling/reducing and subjected to SDS-PAGE analysis. The following conditions were carefully optimized: (1) hyper IgE plasma dilution to use in the Western blot; the optimized amount of 35 ul of the hyper IgE plasma per 10 mL of blocking buffer was found to give a clear signal with minimal background activity; (2) incubation period for the primary antibody was tested at 1 day vs. 3 days duration and the latter was found to yield the best outcome; and (3) amount of secondary antibody was optimized using different amounts, and 40 µL per 10 mL blocking buffer was found to yield the signals.

4.5.8 Identification of Wheat Allergens by LC-MS/MS Sequencing Method

Protein bands corresponding to IgE binding bands in the Western blot were removed using sterile scalpel blades. A separate scalpel blade was used for each band to prevent cross-contamination. Protein bands were stored in 100 µL of 5% acetic acid until used in LC-MS/MS method at the Michigan State University Proteomics Core Facility as follows.

Gel bands were digested according to Shevchenko et al. (1996) with modifications. Briefly, gel bands were dehydrated using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate at a pH of ~8 at 56 ◦C for 45 min. The bands were then dehydrated again and incubated in darkness with 50mMiodoacetamide in 100 mM ammonium bicarbonate for 20 min. Gel bands were then washed with ammonium bicarbonate and dehydrated again. Sequencing grade modified trypsin was prepared to 0.01 µg/µL in 50 mM ammonium bicarbonate and ~50 µL of this was added to each gel band, completely submerging the band. Bands were then incubated at 37 ◦C overnight. Peptides were extracted from the gel via water bath sonication in a solution of 60% ACN/1% TCA and vacuum dried to 2 µL. Peptides were then re-suspended in 2% acetonitrile/1% TFA to 20 µL. From this, 5 µL were injected by a Thermo EASYnLC 1000 onto a Thermo Acclaim PepMap RSLC 0.075 mm × 250 mm C18 column and eluted over 35 min with a gradient of 6% B to 32% B in 24 min, ramping to 90% B at 35 min and held at 90% B for the duration of the run at a constant flow rate of 300 nL/min. (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid). Column temperature was maintained at a constant 50 ◦C using an integrated column heater (PRSO-V1, Sonation GmbH, Biberach, Germany).

Eluted peptides were sprayed into a ThermoFisher Q-Exactive mass spectrometer using a FlexSpray ion source. Survey scans were taken in the Orbi trap (70,000 resolution, determined at

m/z 200) and the top ten ions in each survey scan were then subjected to automatic higher energy collision induced dissociation (HCD) with fragment spectra acquired at a resolution of 17,500. The resulting MS/MS spectra were converted to peak lists using Mascot Distiller, v2.7 and searched against a database containing protein sequences from *T. aestivum* appended with common laboratory contaminants. The Mascot output was analyzed using Scaffold v. 4.8.8 to probabilistically validate protein identifications. Assignments validated using the Scaffold 1% FDR confidence filter were considered true. Most abundant proteins were identified with a quantitative value of ≥100.

4.5.9 Comparison of Mouse Wheat Allergens to Human Wheat Allergens Reported in the Literature

Each protein identified as an allergen in the mouse model was checked in the allergome.com, Google Scholar, and PubMed databases. If it was found to be reported as an allergen in humans, then the information was recorded as a 'reported human wheat allergen'. If it was not listed in any of these databases, then it was deemed to be 'not-reported' as a human wheat allergen.

4.6 Conclusions

Here we demonstrate that all but two salt-soluble wheat allergens in durum wheat and all salt-soluble IgE cross-reacting allergens in an ancient *Ae. tauschii* wheat, all identified through a wheat-allergic mouse model, are indeed identical to those reported as allergens in human wheat-allergic subjects. This study also further supports the use of the Balb/cJ mouse model to advance scientific knowledge on wheat allergenicity and to use it as a pre-clinical

testing tool to assess and monitor changes to wheat allergens occurring either naturally by random mutations or by human intervention such as breeding of new wheat varieties. This method will be a useful tool to assess the effects of food processing on wheat allergenicity, advance mechanisms of wheat allergenicity, and develop novel methods to prevent/treat wheat allergies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23126505/s1.

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CHAPTER 5 CHRONIC APPLICATION OF ALCOHOL-SOLUBLE GLUTEN EXTRACT OVER UNDAMAGED SKIN CAUSES CLINICAL SENSITIZATION FOR LIFE-THREATENING ANAPHYLAXIS VIA ACTIVATION OF SYSTEMIC TH2 IMMUNE RESPONSES IN MICE

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5.1 Abstract

Gluten allergy is a major public health problem that is growing at an alarming rate. Specific mechanisms underlying sensitization to gluten remain incompletely understood. Currently, it is unclear whether chronic exposure to alcohol-soluble gluten extract via undamaged skin has the capacity to clinically sensitize mice for life-threatening anaphylaxis. Using an adjuvant-free mouse model, here we tested the hypothesis that chronic application of alcohol-soluble durum gluten (ASDG) extract will clinically sensitize mice for life-threatening anaphylaxis. This study was conducted in a gluten-free Balb/c mouse colony that was established and maintained on a plant protein-free diet. Groups of adult female mice were exposed dermally to ASDG extract or vehicle once a week for 9-weeks. Specific (s) and total (t) IgE levels were quantified. Mice were challenged systemically with ASDG to measure symptoms of systemic anaphylaxis. Hypothermic shock response (HSR) and mucosal mast cell degranulation response (MMCR) were determined upon challenge. Spleen Th1, Th2, and other immune markers were

quantified. We found that chronic exposure to ASDG elicited robust elevation of sIgE and tIgE. Systemic challenge with ASDG, but not vehicle, elicited life-threatening anaphylaxis associated with dramatic HSR and MMCR. Correlation analysis demonstrated direct positive inter-relationships among IgE, HSR, and MMCR. Anaphylaxis was associated with significant elevation of prototypic Th2 but not Th1 immune markers in the spleen. Our study collectively demonstrates that ASDG is intrinsically allergenic; and chronic exposure to ASDG via undamaged skin can clinically sensitize mice for life-threatening anaphylaxis via activating the systemic Th2 immune responses.

5.2 Introduction

Gluten is a major group of proteins found in cereal grains such as wheat, barley, and rye. Traditionally, glutens are classified based on their solubility properties into two general groups such as ethanol-soluble prolamin proteins (gliadins, 30-40% of the total proteins), and weak acid-soluble proteins (glutenins, 45-50% of the total protein). Non-gluten proteins (being albumins/globulins) are the remainder proteins that are soluble in aqueous solutions (Cabanillas 2020; Jin et al., 2019). Gliadins exist as individual proteins that interact through hydrogen bonds and primarily have intramolecular disulfide bonds. In contrast, glutenins are polymeric proteins that form connections through both intermolecular and intramolecular disulfide bonds. Additionally, gliadins can also be linked to the glutenin network through intermolecular disulfide bonds (Gao et al., 2021). Within the group of alcohol-soluble proteins, ω−1, 2, 5 gliadin, and α/β/γ-gliadins have been extensively characterized and are known to elicit allergic reactions in susceptible individuals (Cianferoni, 2016; Juhász et al., 2018).

There is extensive evidence that wheat gluten has the capacity to elicit several immune-mediated diseases. These include gluten hypersensitivity, celiac disease (CD), and non-celiac gluten sensitivity (NCGS) (Cabanillas, 2020). Among these, gluten hypersensitivity (also known as gluten food allergy or wheat food allergy) is potentially deadly (Cianferoni, 2016; Quirce, Boyano-Martínez, and Diáz-Perales, 2016; Ricci et al., 2019). Hypersensitivity reactions to wheat are caused by inappropriate activation of immune system by wheat proteins that includes both gluten as well as non-gluten proteins (Cianferoni, 2016; Leonard & Vasagar, 2014).

The underlying immune mechanisms as well as clinical presentations of gluten hypersensitivity are completely different from that of celiac disease (CD) and non-celiac gluten sensitivities (NCGS). Gluten hypersensitivity is primarily due to the production of IgE antibodies against gluten during initial exposures to gluten that sensitizes mast cells. Subsequent exposures to gluten results in mast cell degranulation leading to potentially deadly anaphylactic reactions (Gao et al., 2021; Rubin & Crowe, 2020). In contrast, gluten-triggered CD is an autoimmune chronic inflammatory disease that affects primarily the small intestine in most cases; in some cases, gluten also causes celiac disease associated with dermatitis and brain damage (Croall et al., 2020; Kaunisto et al., 2022). The NCGS show up clinically as a chronic digestive disorder caused by unknown mechanisms, although innate immune system activation is implicated (Cárdenas-Torres et al., 2021).

The prevalence of all three gluten-induced disorders have been increasing at an alarming rate worldwide (Caio et al., 2019; Gupta et al., 2019). Current estimates of gluten hypersensitivity in the United States are 0.4-3% (Venter et al., 2008; Venter et al., 2006; Vierk et al., 2007). The prevalence of CD and NCGS are 1% and 3-6%, respectively (Ludvigsson et al., 2015; Molina-Infante et al., 2015). Currently there is no cure for these diseases other than a

complete avoidance of gluten-containing foods (Seth et al., 2020). Consequently, gluten-free and wheat-free diets are the primary mode of treatment and management of these diseases.

While clinical sensitization to gluten is required for manifestation of anaphylaxis, the specific mechanisms (for example, route of sensitization) underlying gluten hypersensitivity are incompletely understood at present (Jin et al., 2020). It is generally presumed that oral exposure to dietary gluten leads to sensitization resulting in gluten hypersensitivity (Cabanillas 2020). However, there is also evidence that exposure to gluten can occur via non-oral routes including: skin, airways, and eyes (Jin et al., 2019; Platts-Mills, 2015; Renz et al., 2018; Sicherer & Sampson 2018). Therefore, it is critical to determine whether non-oral exposure to gluten can result in clinical sensitization. It is interesting to note that exposure through skin to food proteins such as tree nuts, sesame, milk, shellfish, etc., has been implicated in sensitization to the respective food hypersensitivities (Birmingham et al., 2007; Parvataneni et al., 2009). However, whether chronic exposure to gluten via undamaged skin can lead to clinical sensitization is unknown at present.

There are several animal models of gluten hypersensitivity reported in the literature including dogs, rats, guinea pigs and mice (Ballegaard, Madsen, and Bøgh 2019; Buchanan et al., 1997; Frick et al., 2005; Kohno et al., 2016; Kroghsbo et al., 2014; Yamada et al., 2019). A common feature of most of these models has been the use of adjuvants to elicit sensitization to gluten. Although such an approach is very popular as it elicits robust sensitizations to gluten, it is not useful when evaluating the intrinsic allergenicity of gluten since adjuvants artificially exacerbate immune responses to gluten. There is one previous mouse model study involving skin exposure to gluten; in this study, mouse skin was deliberately damaged by removing the outer layer of stratum corneum using a tape-stripping method; gluten was applied over the damaged

skin along with a detergent to induce sensitization (Adachi et al., 2012). Thus, there are two specific gaps in knowledge in gluten hypersensitivity: i) whether gluten is intrinsically capable of sensitizing mice without using adjuvants; and ii) whether chronic application of gluten over undamaged skin can elicit clinical sensitization in mice. These two questions were the foci of this study.

Using an adjuvant-free mouse model, here we tested the hypothesis that chronic application of alcohol-soluble durum gluten (ASDG) extract will clinically sensitize mice for anaphylaxis. There were eight objectives for this study: 1) to establish a colony of gluten-free Balb/c mice; 2) to test the intrinsic sensitization capacity of ASDG when repeatedly applied over undamaged skin by measuring IgE antibody responses; 3) to study clinical symptoms of anaphylaxis upon systemic ASDG challenge; 5) to quantify hypothermic shock responses (HSR); 6) to quantify mucosal mast cell degranulation responses (MMCR); 7) to determine inter-relationships among IgE, HSR, and MMCR; and 8) to characterize the systemic T-helper (Th)-1, and Th2 immune responses in this model.

This study collectively demonstrates that ASDG is intrinsically allergenic, and chronic exposure to ASDG via undamaged skin can clinically sensitize mice for life-threatening anaphylaxis via activating the systemic Th2 immune responses.

5.3 Materials and Methods

5.3.1 Chemicals and Reagents

Biotin-conjugated rat anti-mouse IgE-paired antibodies were purchased from BD BioSciences (San Jose, CA, USA). p-nitro-phenyl phosphate was obtained from Sigma (St Louis, MO, USA). Streptavidin alkaline phosphatase was obtained from Jackson ImmunoResearch

(West Grove, PA, USA). Folin reagent was purchased from BioRad (Hercules, CA). The following reagents were obtained as listed: IgE Mouse Uncoated ELISA Kit with Plates; Streptavidin-HRP, TMB substrate; MCPT-1 (mMCP-1) Mouse Uncoated ELISA Kit with Plates; Avidin-HRP, TMB substrate (all from Invitrogen, MA, USA); Tissue Protein Extraction Reagent (T-PERTM, a proprietary detergent in 25mM bicine, 150mM sodium chloride (pH 7.6) (from ThermoFisher Scientific, MA, USA); protease (serine, cysteine, and acid proteases, and aminopeptidases) inhibitor cocktail (Sigma-Aldrich, MO, USA).

5.3.2 Mice breeding and establishment of a plant-protein-free mouse colony

Adult Balb/c breeder pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). Once received, the mice were placed on a strict plant-protein-free diet (AIN-93G, Envigo, Madison, MI). Once acclimated after one week, breeding was set up using standard procedure. Adult female mice from the litter (6-8 weeks) were used in this study. All mice were maintained on the strict plant-protein-free diet (AIN-93G) throughout the entirety of the study. All animal procedures were in accordance with Michigan State University policies.

5.3.3 Preparation of Alcohol-Soluble Protein Extract from Durum Wheat Flour

Durum wheat flour (AABB genotype, Carpio variety) was used in protein extraction. Alcohol-soluble durum gliadin was extracted using an Osborne method of sequential extraction (Chen & Bushuk, 1969). Briefly, flour and sterile 0.5 M NaCl in a 1:10 ratio (m/v) were mixed continuously for 2 hours followed by centrifugation at 20000g for 30 minutes. Pellets were stored and used in alcohol extraction. Salt-insoluble pellets were then mixed in a 1:10 ratio with 70% ethanol for 2 hours followed by centrifugation at 20000g for 15 minutes. The supernatant

was frozen at -70°C overnight and freeze-dried the next day. Lyophilized alcohol-soluble durum gluten (ASDG) was reconstituted with 70% ethanol to a concentration of 1 mg protein per 100 uL for topical application. For IP injection challenges, ASDG was reconstituted with phosphate buffered saline (PBS) to a concentration of 0.5 mg/mouse. Protein content was determined using the LECO total combustion method (LECO, St. Joseph, MI). Protein quality was tested by SDS-PAGE.

5.3.4 Skin Sensitization, Bleeding, and Plasma Sample Preparation

Adult female Balb/c mice were used in experimentation. For transdermal allergen applications, ASDG dissolved in 70% ethanol was used in skin application (100 uL/mouse applied over a 2 cm2 area on the rump). For transdermal control applications without allergen, 70% ethanol was used in skin application as a vehicle application (100 uL/mouse over a 2 cm2 area on the rump). The application site of allergen or vehicle was over the rump skin of mice, once a week for nine weeks. The rumps of the mice were clipped bilaterally to remove hair (Philips, Amsterdam, Netherlands). The ASDG was applied on the rump at a dose of 1 mg/mouse or vehicle (70% ethanol). The applied area was then covered with a non-latex bandage (Johnson & Johnson, New Brunswick, New Jersey) for one day. This was repeated once a week for a total of nine weeks. Blood was collected before the first exposure and after the 6th exposure via the saphenous vein in anti-coagulant (lithium heparin) coated tubes (Sarstedt Inc MicrovetteCB 300 LH, Germany). The blood was then centrifuged to obtain plasma. Plasma was stored individually at -70°C until needed for testing.

5.3.5 Elicitation of Systemic Anaphylaxis and Clinical Symptom Scoring

Two weeks after the final skin exposure to ASDG or vehicle mice were challenged via intraperitoneal (IP) injection with either 0.5 mg ASDG or vehicle (PBS). Mice were observed for signs of systemic anaphylaxis during the 30 minutes after the IP injection as described previously (Birmingham et al., 2007). Scores were recorded based on the following criteria: 0 = no symptoms; 1 = scratching and rubbing of the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity and/or decreased activity with a noted increase of respiratory rate; 3 = wheezing, labored breathing, cyanosis around the tail and the mouth; 4 = no activity after prodding, tremors, and convulsions; 5 = death.

5.3.6 Elicitation of Oral Anaphylaxis

Two weeks after the final exposure to ASDG, mice were orally challenged with ASDG (20mg per mouse in 300 µl sterile saline) using curved feeding needles (22-gauge, length: 1.4 in, Kent Scientific, Torrington, CT, United States) as reported previously (Gao et al., 2022).

5.3.7 Determination of Hypothermic Shock Responses

Rectal temperature (°C) was recorded before the challenge and every 5 minutes after the challenge, up to 30 minutes using a rectal thermometer (DIGI-SENSE, MA, USA). Actual temperatures and the change in rectal temperature (Δ°C) at every 5-minute increment compared to the before-challenge temperatures for each respective mouse were utilized in analyses.

5.3.8 Measurement of Specific IgE Antibody Levels

The ASDG-specific (s) IgE antibody levels were measured using a previously reported ultrasensitive ELISA method with modifications (Gao et al., 2019; Jin et al., 2020; Yining Jin et al., 2017). Briefly, 96-well Corning 3369 plates were coated with ASDG followed by blocking with 5% gelatin. The plate was then washed, plasma sample was added, washed again, biotin-conjugated anti-mouse IgE antibody was added, washed, addition of streptavidin alkaline phosphatase and finally p-nitro-phenyl phosphate to allow for quantification as previously reported (Gao et al., 2019; Jin et al., 2017). Mice samples were screened individually in quadruplicate.

5.3.9 Measurement of Specific IgG1 Antibody Levels

The levels of ASDG-specific IgG1 antibodies were measured using a modified ultrasensitive ELISA method previously reported (Birmingham et al., 2003; Gao et al., 2019). In brief, 96-well Corning 3369 plates were coated with ASDG and blocked with bovine serum albumin. Subsequently, the plate was washed, and plasma samples were added. After another wash, biotin-conjugated anti-mouse IgG antibodies were added, followed by additional washing steps. Streptavidin alkaline phosphatase was then added, and p-nitro-phenyl phosphate was used for quantification, as previously described (Birmingham et al., 2003; Gao et al., 2019).

5.3.10 Measurement of Total Plasma IgE Concentration

Total (t)IgE concentration was determined using a commercial ELISA kit (Invitrogen, Waltham, MA). Briefly, 96-well Corning Costar 9018 plates were coated with capture antibody (anti-mouse IgE) followed by the addition of plasma samples and standards (recombinant mouse

IgE). A secondary antibody (anti-mouse IgE) and then a detection system of Streptavidin-HRP and TMB substrate was added as described previously (Gao et al., 2019; Jin et al., 2017). Assay limit of detection is 4 ng/mL. The standard range used for analysis is 4-250 ng/mL. Samples were tested individually from each mouse in quadruplicate.

5.3.11 Quantification of Mucosal Mast Cell Protease-1 (MMCP-1) level

Blood was collected one hour after challenge and was used in measurement of mucosal mast cell protease-1 levels (MMCP-1) in the plasma using an ELISA-based method developed by Invitrogen as described previously (Gao et al., 2019; Jin et al., 2017). Briefly, 96-well Corning Costar 9018 plates were coated with capture antibody (anti-mouse MMCP-1) followed by the addition of samples and standards (recombinant mouse MMCP-1). Biotin-conjugated anti-mouse MMCP-1 was then added as a secondary antibody. Detection was completed utilizing an avidin-HRP/TMB substrate system. The limit of detection for this assay is 120 pg/mL and the standards ranged from 120-15000 pg/mL. Individual mouse plasma was tested in quadruplicate.

5.3.12 Spleen Extract Preparation and Analysis of Immune Markers

After one-hour post-challenge, mice were euthanized, and the spleens were harvested. The spleens were snap-frozen in liquid nitrogen and stored at -70°C. Tissue was extracted as described previously (Jin et al., 2020). Briefly, spleen tissue was immersed in a tissue protein extraction reagent (T-PER) buffer with protease inhibitor. For every 100 mg of tissue, 10 µL of protease inhibitor per 1 mL T-PER buffer was used. Ultra-sonication was utilized to homogenize the spleen tissue for two 30-second intervals with a resting period of 5 minutes in between. After the second homogenization, the samples were rested for 15 minutes and placed for centrifugation

(13500 x g) at 4°C for 10 minutes. The supernatant was then collected and aliquoted for storage at -70°C. Immune markers were quantified by RayBiotech service utilizing the Quantibody microarray (RayBiotech, Atlanta, GA) (https://www.raybiotech.com/mouse-cytokine-array-q2000/). Samples were analyzed in quadruplicate.

5.3.13 Histopathology

The formalin-fixed skin tissues underwent processing at the Michigan State University Histopathology and Cytology Service Laboratory using well-established methods as described previously (Gonipeta et al., 2015; Jin et al., 2017). Paraffin sections were prepared using the Reichert Jung 2030 rotary microtome and stained with Hematoxylin and Eosin.

5.4.14 Quantification of plasma histamine levels

Histamine concentration was determined using a commercial ELISA kit (IBL America, MN). Briefly, acylated standards, controls, and samples, along with the solid phase-bound analyte, compete for limited antiserum binding sites. After reaching equilibrium, free antigen and antigen-antiserum complexes are washed away. The antibody bound to the solid phase is then detected using an anti-rabbit IgG-peroxidase conjugate, with TMB serving as the substrate for the enzymatic reaction. The resulting color change was measured at 450 nm. The concentration of unknown samples was determined by comparing their absorbance with a reference curve generated using known standard concentrations. Assay limit of detection was 0.2 ng/mL. The standard range used for analysis was 0-50 ng/mL.

5.3.15 Statistics

An online software service was used in these analyses (https://www.socscistatistics.com/tests/). A student's t-test was used to compare two groups, and ANOVA was used for multiple group comparisons. The statistical significance level was set at $p < 0.05$. Pearson correlation coefficient calculation was conducted using excel built-in program. The following formula was used to calculate r-sc ores:

$$r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\bar{x})^2 \sum(y-\bar{y})^2}}$$

Using the r scores, and n-values significance was calculated with $p < 0.05$.

5.4 Results

5.4.1 Chronic application of alcohol-soluble durum gluten (ASDG) onto undamaged skin elicits robust specific IgE antibody response in Balb/c mice

The ability of ASDG to elicit sensitization upon chronic application over the skin was tested as follows: Groups of female adult mice ($n = 10$/group) were exposed topically to ASDG or to vehicle once per week for nine weeks as described in the methods. Specific (s) IgE levels were measured in blood collected before (pre) exposure and after 6 skin exposures using an ELISA method pre-optimized for this purpose. As shown in Figure 4.1A, skin exposure to ASDG, but not vehicle elicited robust sIgE response. Serial two-fold dilution of plasma sample demonstrated specific IgE levels at 1/320 dilution (Supplemental Figure 1).

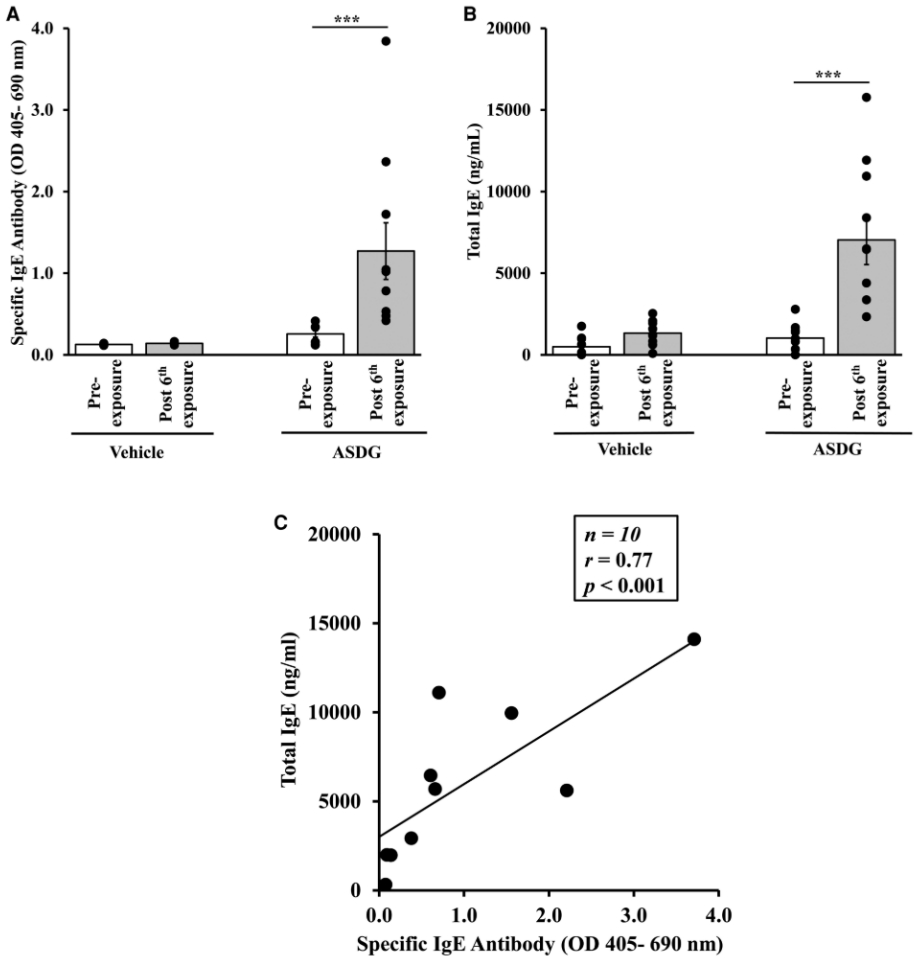

Figure 5.1 Chronic skin exposure to alcohol-soluble durum gluten (ASDG) elicited specific IgE antibody responses and elevation of total IgE in balb/c mice.

Mice were exposed to ASDG (1 mg in 100 µL 70% ethanol/mouse, n = 10) or to vehicle (70% ethanol, 100 µL/mouse, n = 10) as described in Materials and Methods. Blood was collected before 1st exposure (Pre) and after 6th exposure. Plasma was used in measurement of ASDG-specific IgE levels (OD 405–690 nm) using an ELISA method described previously. (A) ASDG-specific IgE antibody levels in vehicle-exposed mice and ASDG-exposed mice before and after 6th exposure. (B) Total IgE levels in vehicle-exposed mice and ASDG-exposed mice before and after 6th exposure. Student'stwo-tailed t-test: ***p < 0.0001. (C) Pearson correlation analysis between alcohol-soluble durum gluten (ASDG)-specific IgE antibody levels and total IgE levels.

Pearson correlation analysis was used to test the relationship between ASDG-specific IgE antibody and total IgE levels in the plasma after 6th transdermal exposure to ASDG.

5.4.2 Chronic application of alcohol-soluble durum gluten (ASDG) onto undamaged skin also elevates total IgE levels, which correlate with sIgE levels

Total IgE levels in the blood collected before (pre) exposure and after exposure to ASDG or vehicle were measured by ELISA method. As evident, chronic exposure to ASDG significantly elevated tIgE levels, which were not noted in vehicle-exposed mice (~7-fold increase in ASDG-exposed mice versus vehicle-exposed mice) (Figure 5.1B). Pearson correlation coefficient analysis was conducted using individual mouse data for sIgE and tIgE. A significant positive correlation was found between the two readouts (Figure 5.1C).

5.4.3 ASDG-sensitized, but not vehicle-sensitized mice, exhibit life-threatening symptoms of anaphylaxis upon systemic challenge with ASDG

The parallel groups of mice skin-sensitized with ASDG versus vehicle-only were challenged with intra-peritoneal ASDG to evaluate for systemic anaphylaxis. Clinical symptom scores were determined using published methods (Birmingham et al., 2007). As evident, severe life-threatening clinical symptoms were noted only in the ASDG-sensitized mice but not in vehicle groups (Figure 5.2). Most common symptoms included altered respiration, scratching, and rubbing of nose, face, and/or head, and lack of activity upon prodding.

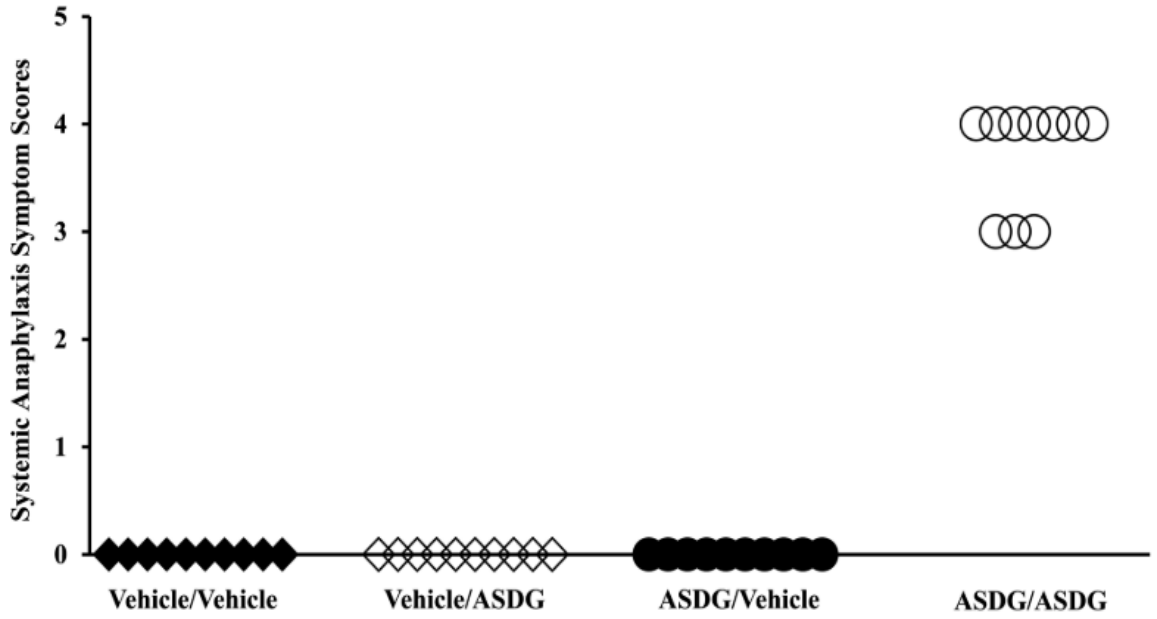

Figure 5.2 Chronic skin exposure to alcohol-soluble durum gluten (ASDG) is sufficient to clinically sensitize balb/cmice for systemic anaphylaxis.

Groups of Balb/c mice were sensitized with either vehicle or ASDG as described in Methods. After 9 transdermal exposures mice were challenged with either vehicle or ASDG (0.5 mg/mouse) by intraperitoneal injection. Mice were monitored for symptoms of systemic anaphylaxis using a published method described in the text. Each symbol represents an individual mouse. Horizontal axis shows different groups as follows: Vehicle/Vehicle= skin exposure with vehicle followed by vehicle challenge; Vehicle/ASDG= skin exposure with vehicle followed by ASDG challenge; ASDG/Vehicle= skin exposure with ASDG followed by vehicle challenge; ASDG/ASDG= skin exposure with ASDG followed by ASDG challenge.

5.4.4 Mice with systemic anaphylaxis symptoms upon systemic challenge with ASDG exhibit dramatic hypothermic shock responses (HSR)

Anaphylactic reactions were also quantified using rectal thermometry to evaluate for any hypothermic shock responses (HSR). As evident, systemic challenge with ASDG resulted in life-threatening HSR of ASDG-sensitized, but not of vehicle control mice (Figure 5.3A-B). Actual temperature changes are shown in Figure 5.3A. Absolute change in temperature is depicted in Figure 5.3B. There was no HSR observed in vehicle-challenged mice or in vehicle-sensitized mice challenged with ASDG.

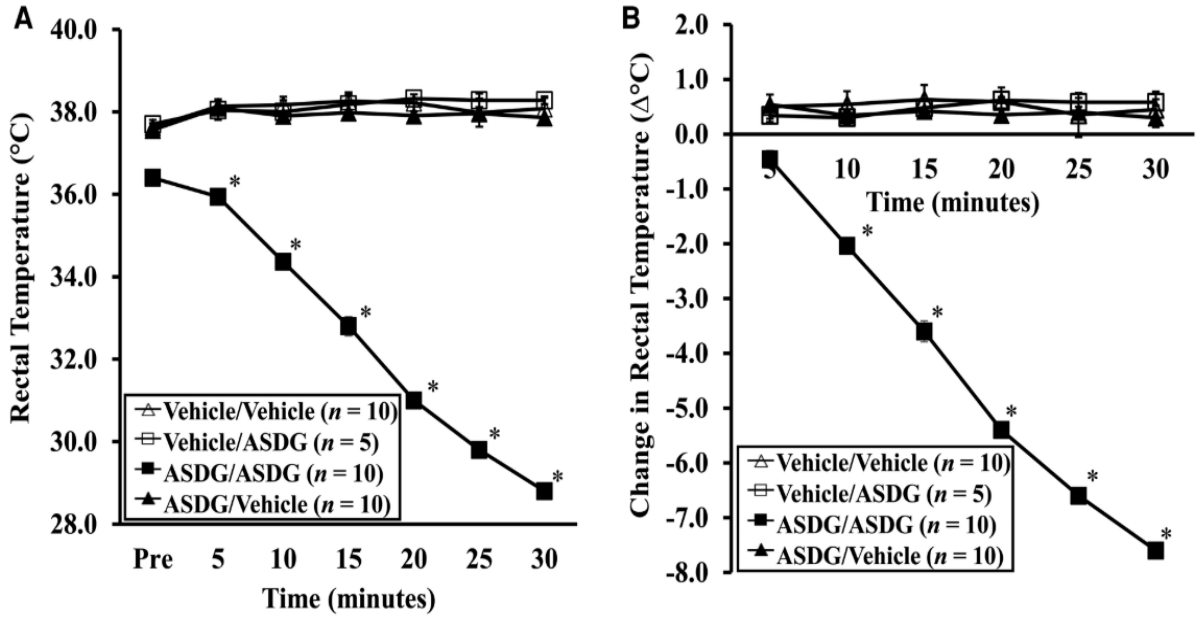

Figure 5.3 Induction of hypothermic shock responses upon systemic challenge with alcohol-soluble durum gluten (ASDG).

Mice exposed to ASDG or to vehicle were systemically challenged by intraperitoneal injection as described in Materials and Methods. (A) Rectal temperatures (°C) at indicated time points in vehicle-sensitized and ASDG-sensitized mice challenged with ASDG or vehicle. (B) Change in rectal temperature (Δ°C) at indicated time points in vehicle-sensitized and ASDG-sensitized mice challenged with ASDG or vehicle. Vehicle/Vehicle = skin exposure with vehicle followed

by vehicle challenge; Vehicle/ASDG = skin exposure with vehicle followed by ASDG challenge; ASDG/Vehicle = skin exposure with ASDG followed by vehicle challenge; ASDG/ASDG = skin exposure with ASDG followed by ASDG challenge. Student's two-tailed t-test: *$p < 0.05$.

5.4.5 Hypothermic Shock Responses elicited by ASDG correlates significantly with sIgE levels

In order to determine the relationship between the hypothermic shock response and specific IgE response we conducted Pearson correlation analysis. The rationale underlying this was to test whether specific IgE antibody was involved in causing temperature drop at a specific time point. The results are shown in Figure 5.4. As evident, except for 5 minutes, temperature drops were significantly correlated with IgE levels at all other timepoints suggesting that IgE mediated allergic reactions likely contributed to the observed temperature drops.

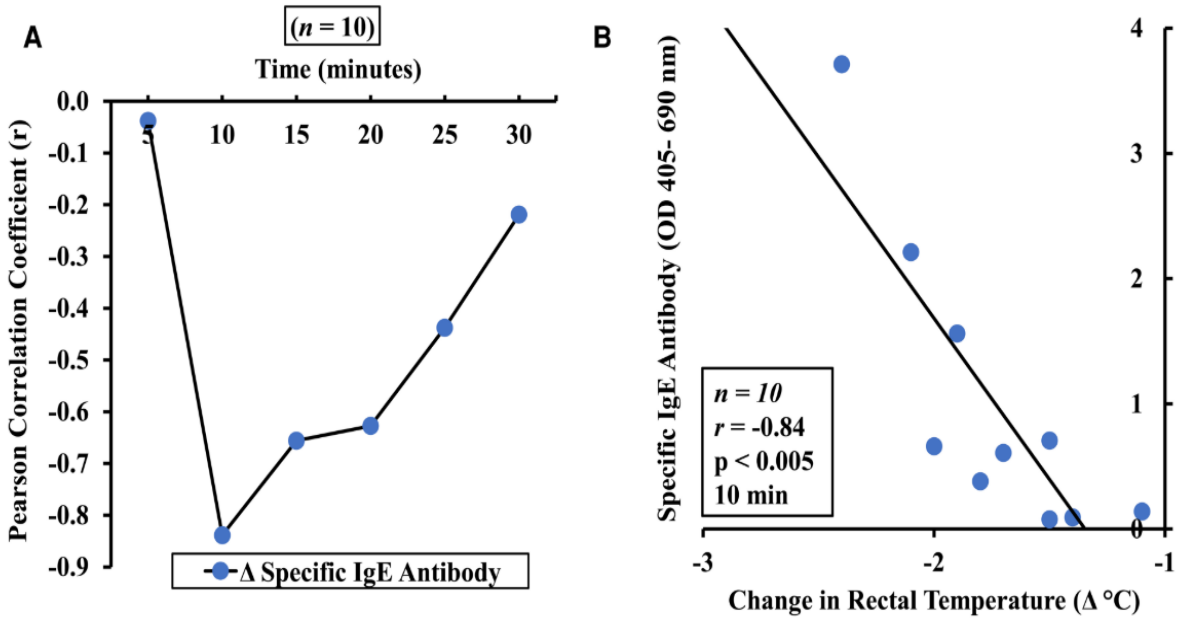

Figure 5.4 Pearson correlation analysis of hypothermic shock response and alcohol-soluble durum gluten (ASDG)-specific IgE antibody levels in this model.

Mice were exposed to ASDG or vehicle and challenged as described in Materials and Methods. Pearson correlation coefficient (r) between ASDG-specific antibody levels and change in rectal

temperature (Δ°C) in mice sensitized and systemically challenged with ASDG (0.5 mg/mouse). (A) Pearson correlation coefficient (r) at indicated time points. (B) Pearson correlation analysis between ASDG-specific IgE antibody levels and Δ°C at 10 min post challenge

5.4.6 Systemic anaphylaxis is also associated with significant mucosal mast cell degranulation in this model

Blood was collected one hour after intraperitoneal challenge and used to measure the mucosal mast cell degranulation response (MMCR) in mice. The elevation of MMCP-1 in the blood demonstrates a true IgE antibody-mediated type-1 hypersensitivity reaction to food proteins in mouse models as described before (Khodoun et al., 2011). As seen in Figure 5.5, significant MMCR is seen in mice undergoing anaphylaxis; no such MMCR was noted in control mice.

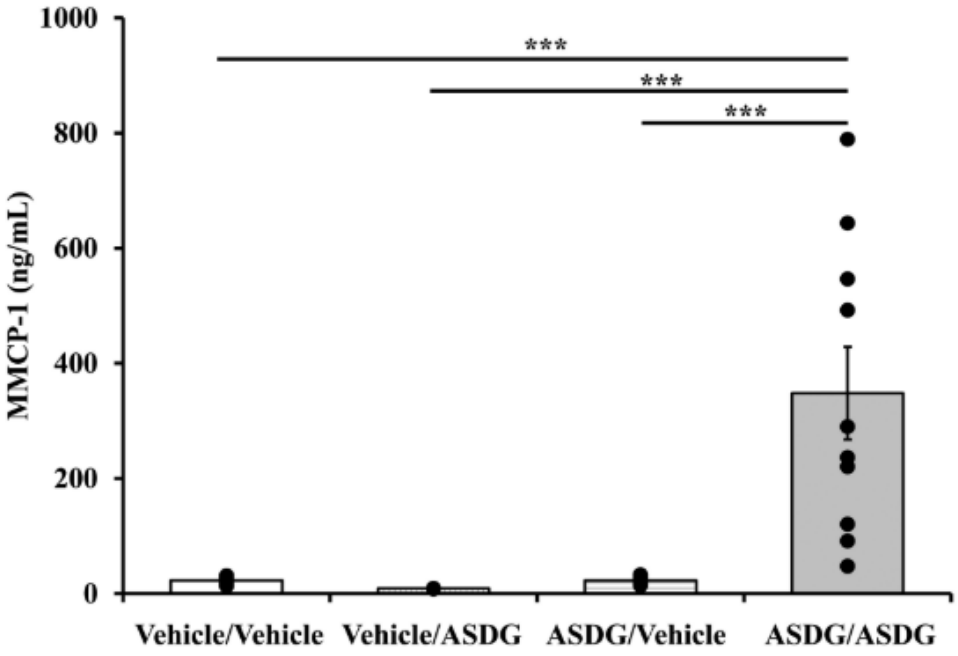

Figure 5.5 Systemic anaphylaxis induced by alcohol soluble durum gluten (ASDG) is associated with degranulation of mucosal mast cells in this model.

Mice were treated as described in Materials and Methods. Their plasma mucosal mast cell protease-1 (MMCP-1) levels (ng/ml) were measured using an ELISA-based method described in the text. Vehicle/Vehicle = skin exposure with vehicle followed by vehicle challenge; Vehicle/ASDG = skin exposure with vehicle followed by ASDG challenge; ASDG/Vehicle = skin exposure with ASDG followed by vehicle challenge; ASDG/ASDG = skin exposure with ASDG followed by ASDG challenge. *** $p < 0.005$, one-way ANOVA and Tukey's post hoc tests.

5.4.7 Correlation analysis between MMCR and HSR

Pearson correlation coefficient analysis was conducted using individual mouse data for absolute change in rectal temperatures and for MMCP-1. A significant positive correlation was observed between the two readouts that sustained from 10 to 20 minutes time points (Figure 5.6A-B).

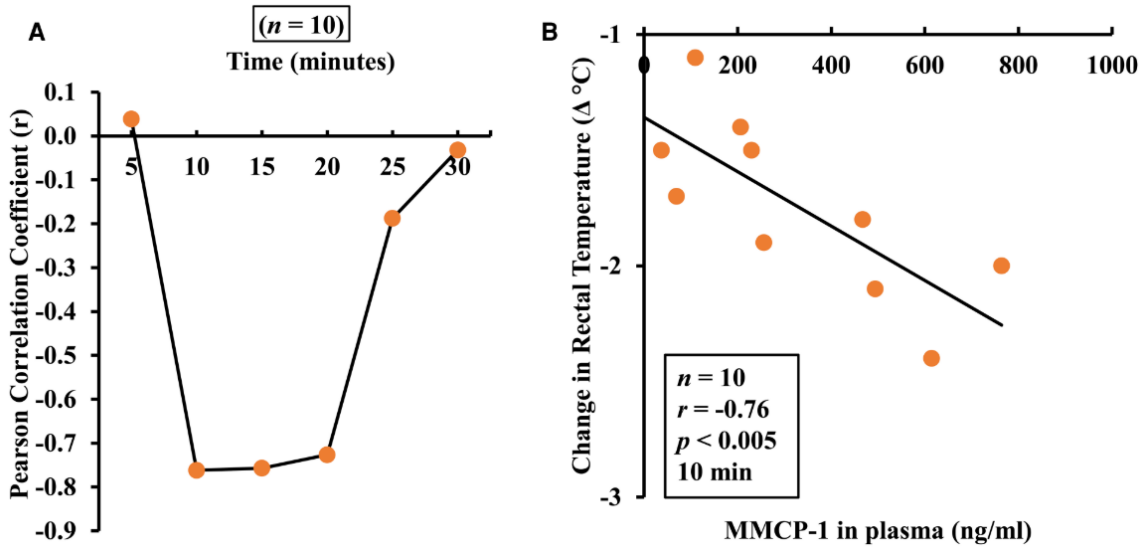

Figure 5.6 Pearson correlation analysis between hypothermic shock response and mucosal mast cell degranulation in this model.

Mice were exposed to alcohol soluble durum gluten (ASDG) or vehicle and challenged as described in Materials and Methods. Pearson correlation coefficient (*r*) between mucosal mast cell degranulation (MMCP-1) and change in rectal temperature (Δ°C) in mice sensitized and systemically challenged with ASDG (0.5 mg/mouse). (A) Pearson correlation coefficient (*r*) at indicated time points. (B) Pearson correlation analysis between mucosal mast cell degranulation and Δ°C at 10 min post challenge

5.4.8 Correlation analysis between MMCR and IgE levels

Pearson correlation coefficient analysis was conducted using individual mouse data for IgE and MMCP-1. A significant positive correlation was noted between the two readouts (Figure 5.7A-B).

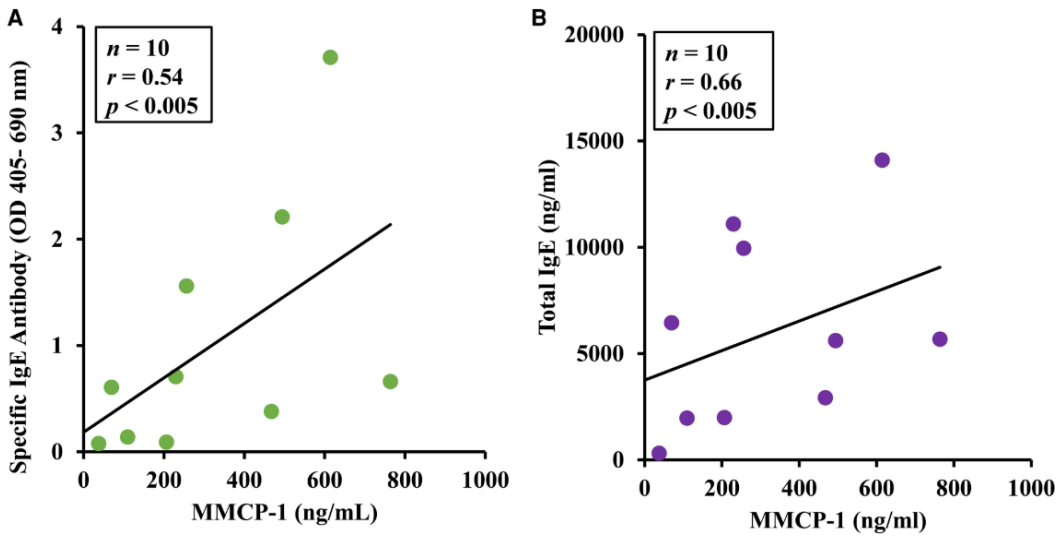

Figure 5.7 Pearson correlation analysis of IgE responses and mucosal mast cell degranulation responses in this model.

Mice were treated as described in Materials and Methods. Pearson correlation analysis was used to test the relationship between mucosal mast cell protease-1 (MMCP-1) levels (ng/ml) and IgE responses (specific IgE antibody or total IgE) in the plasma after 6th transdermal exposure to alcohol soluble durum gluten (ASDG). (A) Pearson correlation analysis between ASDG-specific IgE antibody levels and MMCP-1 levels in systemically challenged mice. (B) Pearson correlation analysis between total IgE antibody levels and MMCP-1 levels in systemically challenged mice.

5.4.9 Characterization of systemic Th1/Th2, and Th17 cytokine responses in the spleen

Spleen biomarkers of Th1, and Th2 immune responses were analyzed

Fold change was determined using mean values. As evident, Th2 cytokines IL-4, IL-5, IL-6, and TSLP were substantially elevated in mice undergoing anaphylaxis (Figure 5.8A-B). Among Th1 markers, IFN-γ and TNF-α were significantly down-regulated; other markers were not substantially altered (Figure 5.8C). Among Th17 cytokines, IL-17A and IL-23 were significantly increased (Figure 5.8D-E). A panel of chemokines, and adhesion molecules were also analyzed, and the results are shown in Tables 5.1. As evident, a number of these markers were substantially elevated (ranging from 2.5-fold to 43-fold) in the mice that had systemic anaphylaxis (Table 5.1).

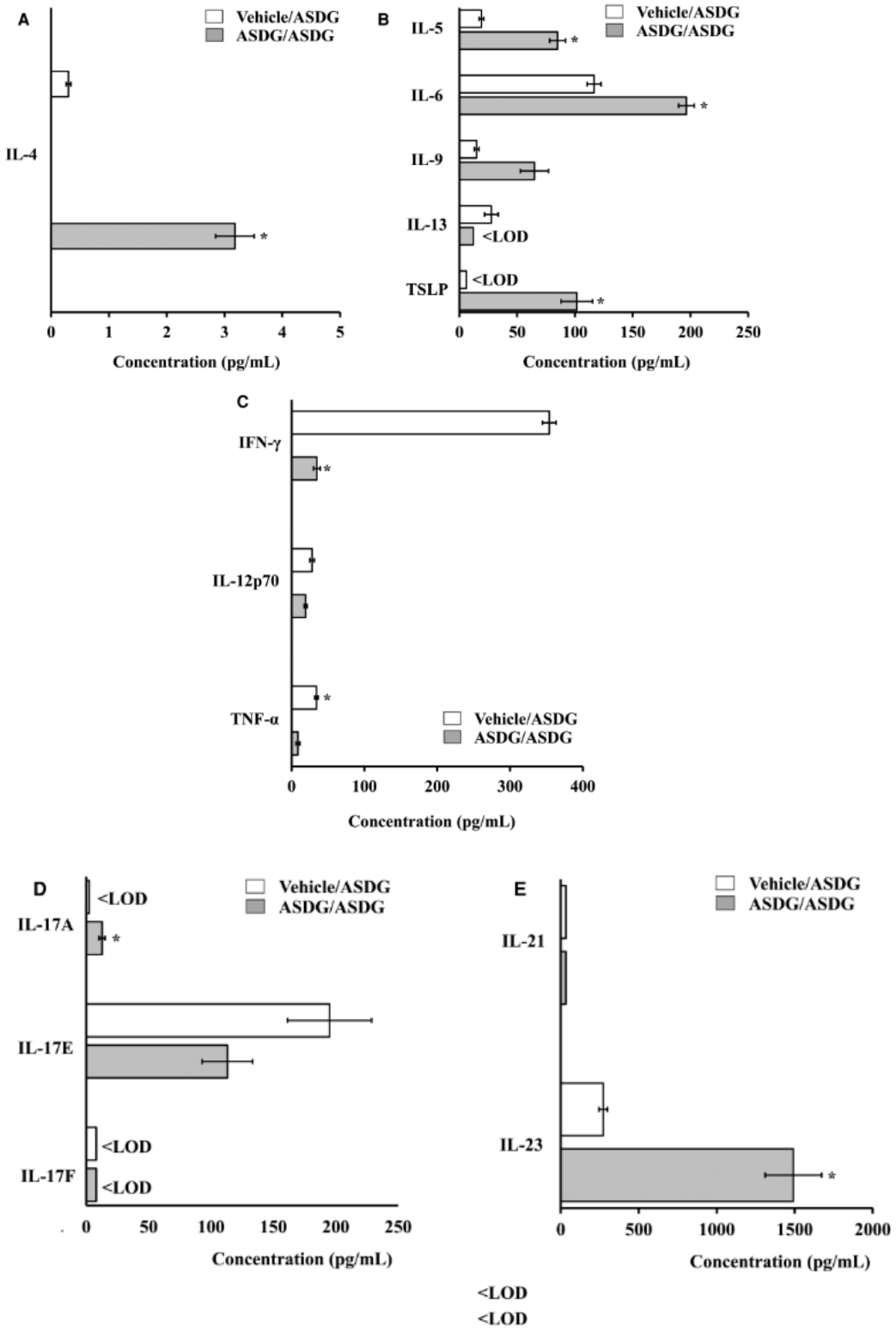

Figure 5.8 Effect of systemic challenge with alcohol-soluble durum gluten (ASDG) on spleen cytokine responses in vivo in vehicle-sensitized control mice vs. ASDG- sensitized allergic mice.

Spleen tissues were collected from the experimental groups at 1-h post challenge with ASDG and used in cytokine protein analysis (A–E) as described in the methods section. Absolute concentrations of various cytokines are shown (pg/mL).

Table 5.1 Chemokines and adhesion molecule in the spleen associated with systemic anaphylaxis induced by alcohol-soluble durum gluten (ASDG) in skin-sensitized mice.

Biomarker[a]	Vehicle-sensitized/ ASDG-challenged mice ($n = 5$)	ASDG-sensitized/ ASDG-challenged mice ($n = 5$)	Fold-change
Chemokines			
I-TAC (CXCL11)	<2[b]	86.19 ± 38.3	43.094
KC (CXCL1)	32.79 ± 3.03	92.74 ± 3.89	2.828
MCP-1 (CCL2)	18.8 ± 4.9	214.24 ± 25.05	11.393
MIG (CXCL9)	508.27 ± 11.2	1,409.06 ± 114.65	2.772
MIP-3a (CCL20)	<2[a]	22.35 ± 4.65	11.176
MIP-3b (CCL19)	27.03 ± 1.18	97.31 ± 12.63	3.599
Adhesion molecule			
MadCAM-1	778.18 ± 46.16	1,976.45 ± 164.17	2.540

[a]Spleen immune marker protein contents are shown as pg/ml, mean ± SE.
[b]Below limit of detection.

5.4.10 Induction of systemic anaphylaxis upon oral challenge with ASDG in this model

Oral challenge with ASDG to ASDG-sensitized mice lead to a significant drop in rectal temperature as shown (Supplemental Figure 5.2).

5.4.11 Chronic application of alcohol-soluble durum gluten (ASDG) onto undamaged skin elicits robust specific IgG1 antibody response in Balb/c mice

Specific IgG1 levels were measured in blood collected before (pre) exposure and after 6 skin exposures using an ELISA method pre-optimized for this purpose. As shown (Supplemental Figure 5.3), skin exposure to ASDG elicited robust IgG1 antibody response with 1 in 1,024,000 dilution of plasma showing positive reaction in the assay.

5.4.12 Chronic application of vehicle or alcohol-soluble durum gluten (ASDG) caused no major damage to the skin

Skin tissues from the site of application of ASDG and vehicle were stained with H and E. Microscopic examination of skin samples from the site of application of vehicle or ASDG was conducted. As shown, neither application caused any marked skin damage as evidence by intact stratum corneum (Supplemental Figure 5.4).

5.4.13 Systemic anaphylaxis upon intraperitoneal challenge with ASDG, but not vehicle, is associated with marked elevation of plasma histamine levels

Histamine elevation in the blood upon systemic challenge with ASDG or vehicle was measured using an ELISA method. As shown, dramatic elevation of histamine was noted only in ASDG-sensitized mice challenged with ASDG (Supplemental Figure 5.5).

5.5 Discussion

Wheat allergies are potentially deadly, and the mechanisms underlying the development of clinical sensitization for life-threatening systemic anaphylaxis are incompletely understood at

present. Both gluten and non-gluten wheat allergens have been implicated in wheat allergies. This study evaluated whether skin exposure to wheat gluten acts as a potential route for deadly immune sensitization. Here we tested the hypothesis that chronic exposure to alcohol-soluble durum wheat gluten (ASDG) extract via skin without artificial skin damage and without co-exposure to adjuvant will clinically sensitize mice for life-threatening anaphylaxis. Our data collectively supports this hypothesis.

There are 8 novel findings from this study: i) chronic skin exposure to ASDG extract results in progressive increase in systemic levels of anti-ASDG-specific IgE antibody; ii) chronic skin exposure to ASDG extract also elevates total (t)IgE levels in the blood that significantly correlates with sIgE levels; iii) mice with chronic skin exposure to ASDG, but not to vehicle, exhibit life-threatening clinical symptoms of anaphylaxis upon systemic exposure to ASDG via intraperitoneal injection; iv) clinical symptoms of anaphylaxis are associated with a dramatic hypothermia shock response (HSR); v) anaphylaxis is associated with a significant gut mucosal mast cell degranulation response (MMCR); vi) single mouse data analysis demonstrates significant correlations among sIgE, HSR and MMCR; vii) clinical symptoms of anaphylaxis are associated with significant elevations of prototypic Th2 cytokines in the spleen; and viii) several other novel immune biomarkers associated with gluten-induced anaphylaxis are identified in this model.

To qualify as an allergen, a protein should exhibit binding affinity to serum IgE (Goodman & Breiteneder, 2019). The protein must also possess two distinct types of epitopes: those that interact with IgE antibodies and those that bind to Th2 cells (Huby et al., 2000). The generation of antigen-specific IgE antibodies involves the activation of antigen presentation by professional antigen-presenting cells (APCs) like dendritic cells, macrophages/monocytes, and B

cells via the T helper (Th)-2 immune response (Jin et al. 2019). In instances of skin sensitization, the protein must also have the ability to activate local Langerhans cells upon breaching the skin barrier. Notably, alcohol could potentially facilitate this process by aiding in skin penetration. Similarly, within the gastrointestinal tract, alcohol might play a role in aiding the breach of the epithelial barrier by substances like gluten, enabling their interaction with immune system APCs in the gut, as gluten epitopes have been known to trigger celiac disease upon consumption of commercial beers (Hofstadt et al., 2023). After the production of the antigen-specific IgE antibodies, and the subsequent fixation of IgE to FcεR1 receptors on mast cells and basophils, re-exposure to wheat allergens can initiate and propagate life-threatening anaphylaxis upon degranulation of mast cells and basophils (Jin et al., 2019).

Routes of exposure to wheat gluten as possible mechanisms of sensitization of individuals to gluten resulting in hypersensitivity have been explored for many decades (Platts-Mills, 2015; Renz et al., 2018; Sicherer & Sampson, 2018). Thus, chronic oral and nasal exposures to wheat gluten are generally thought to lead to gluten food allergy and allergic airways disease, including bakers' asthma in genetically susceptible subjects (Baur et al., 1998; Jeebhay & Baatjies, 2020; Salcedo et al., 2011; Matsumura et al., 1994). However, potential clinical consequences of chronic skin exposure to wheat gluten have been understudied and underappreciated. There is a strong rationale to evaluate this possibility. Human exposure to wheat gluten via skin is expected in the baking industry, and in families. Furthermore, gluten is also used as an ingredient in several cosmetic and skin health products. There are also several reports of life-threatening human clinical sensitizations to gluten via cosmetic such as shampoos and facial soaps (Chinuki & Morita, 2012; Fukutomi et al., 2011; Laurière et al., 2006; Pelkonen et al., 2011). Thus, it is very critical to evaluate and characterize the immune and clinical

consequences of repeated skin exposures to gluten as there is significant recent evidence supporting the general hypothesis that skin exposures to environmental agents have health consequences (Kourosh et al., 2018; Lee-Sarwar et al., 2018; Marshall et al., 2017; Rockwell et al., 2012; Tobar, Tordesillas, and Berin, 2016; Walters et al., 2017).

Specific structural and functional properties required to determine protein allergenicity is a topic of intense research (Huby et al., 2000). Accordingly, it is important to determine the structural and functional properties of ASDG that makes it a powerful allergen. We speculate the following to explain why ASDG is potently allergenic: i) ASDG possess highly potent multiple IgE binding epitope structures; for instance previous studies demonstrated presence of several IgE epitopes in the N-terminal domains of α- and γ-gliadins, although both N and C-terminal domains contain IgE epitopes, and the disulfide bonds appear to be of high importance for IgE binding to the epitopes (Mameri et al., 2015). Interestingly IgE epitope structure of gluten for human wheat allergenicity appear to be very similar to that in Balb/c mice model (Denery-Papini et al., 2011); ii) ASDG also possesses very potent T cell epitopes that drive Th2 immune responses: previous study have shown that gluten elicits powerful Th2 cytokine responses in Balb/c model as evidenced by robust recall IL-4 and IL-5 responses of spleen cells from gliadin sensitized mice upon short-term culture with gliadin allergen ex vivo (Adachi et al., 2012); iii) in the context of skin exposure method we have developed here, we speculate that alcohol-solubility of gluten is a critical factor required for enabling skin penetration by gliadin and making it accessible to the Langerhan's dendritic cells of the skin to activate systemic Th2 response; our date support this hypothesis. Similarly, within the gastrointestinal tract also, alcohol-solubility of gluten might promote easy access of ASDG to the gut dendritic cells triggering Th2 responses.

We used alcohol-solubilized gluten in this study for the development of an experimental model of skin sensitization followed by anaphylaxis disease elicitation. We hypothesize that alcohol solubility of gluten is an important contributor to make immunogenic and allergenic gluten epitopes available to the immune system to generate allergic reactions. It is unclear at present whether exposure to ASDG occurs physiologically in human situation. However, there are few hypothetical situations where exposures to ASDG are possible in humans: i) some of the alcoholic beverages contain gluten naturally (lagers, stouts, ales, and wheat beer). Therefore, in principle, such beverages have the potential to elicit sensitization via the gut as well as systemic reactions if ASDG were to enter the immune system via the gut; ii) in the present model, skin sensitization was used as an experimental system to bypass the gastrointestinal immune tolerance that is innate in mice; in this model, ASDG was used without causing any damage to the skin; there are a number of cosmetic skin products (shampoo, moisturizer, soap, hand sanitizers, conditioners, etc.) that contain industrially processed gluten, and such gluten has been known to elicit wheat allergy (Gao et al., 2021; Hiragun et al., 2013; Kobayashi et al., 2015). In addition, there are also after-shave lotions, and conditioners, containing both alcohol and gluten in the same product, thus making skin exposure to alcohol soluble gluten a possibility; and iii) with the widespread use of alcohol sanitizers due to COVID-19 pandemic, whether concurrent use of excessive alcohol sanitizers along with gluten-containing skin lotions/cosmetics, makes it possible for alcohol-soluble gluten to enter the human skin remains to be tested. These are clearly speculated hypothetical situations that need further study.

Animal models used to study gluten allergenicity have been reviewed recently (Gao et al., 2021; Jin et al., 2019). Typically, in animal models such as mice, sensitization is done by repeated IP injections of the gluten along with alum adjuvant; and the challenges are done by IP

injections with the gluten protein alone. Our goal in this study was to test the utility of a non-invasive skin exposure method to induce sensitization to gluten. Therefore, we used skin application of gluten protein without tape-stripping of the stratum corneum. In order to evaluate whether skin-sensitization results in clinical sensitization for systemic anaphylaxis, we challenged the skin-sensitized mice with gluten using the systemic route of challenge (that is IP injection). Our results demonstrate that this approach can provide us the data to confirm clinical sensitization of skin-sensitized mice for systemic anaphylaxis.

One previous study in Balb/c mice evaluated the consequence of 4 weekly exposures to gluten via deliberately damaged skin by tape-stripping the stratum corneum (Adachi et al., 2012). They found that gluten alone was unable to elicit IgE after 4 exposures, and unable to clinically sensitize mice and cause anaphylaxis to be triggered. However, application of gluten in the presence of a detergent (sodium dodecyl sulfate) did elicit an IgE response by 4 exposures, and produce clinical sensitization. There are two major differences between our study and this previous report that may explain the discrepancy in IgE responses: i) we tested chronic exposures (9 exposures vs. 4 exposures); and we detected IgE even after 4 exposures itself; ii) we did not deliberately create the skin wound before exposing to gluten vs. previous researchers removed stratum corneum using tape-stripping method and then applied gluten over the damaged skin; whether deliberate damage to the skin via tape-stripping prevents IgE response to four applications of gluten in the previous study remains to be tested.

In preliminary studies we determined the IgE responses on a bi-weekly basis because of the requirement to have at least 10 days between two bleedings per animal guidelines. Our analysis showed progressive increase in IgE responses over time. A primary goal of this study was to determine the consequences of chronic exposure to gluten on IgE responses. Therefore,

we tested the 9-time skin exposures as a model of chronic exposure to gluten. Furthermore, we also sought to saturate the system with as high IgE antibodies as possible, so that robust anaphylaxis readout would be established. As evident from HSR data, dramatic anaphylactic reactions were achieved. It is noteworthy that most previous studies of gluten allergenicity had typically used 4-time IP exposures with alum to elicit sensitization (Jin et al., 2019). Here our goal was to establish robust IgE and anaphylactic responses to chronic skin exposure to gluten without adjuvant and without injections. It is noteworthy that because significant sensitizations are elicited as early as after 4-time skin exposures, other investigators interested in short-term exposure studies may also be able to use this model for their specific needs.

Fujii et al. (2009) reported the effects of intradermal administration of 20 μl/site of 0.01%, 0.1%, and 1% ethanol solution on non-specific scratching responses in a dermatitis prone mice strain fed with a special diet that causes dermatitis in that model (Fujii et al., 2009). They report that all doses of ethanol administered intradermally failed to induce a scratching response in mice fed with both normal diet- and the HR-AD-fed mice that develop dermatitis. Thus, they show that alcohol application in fact protects mice from scratching response in HR-AD-fed that normally develop scratching. Notably, in that model dermatitis develops upon feeding this diet, and not by allergen application. There is no involvement of IgE antibody responses to allergens in this model. In contrast, in our model, Balb/c mice are used and IgE antibody responses to ASDG allergen application over the skin is studied. Both are different types of studies and therefore side-by-side comparison is not feasible.

In our study, ASDG was dissolved in 70% ethanol and applied over a small area (2 cm^2) of rump skin to elicit specific-IgE antibody responses because gliadin protein requires alcohol for solubilization. Because of this, we have used the term ‘alcohol-soluble durum gluten’

(ASDG) in this paper. It is important to note that we are not studying the intrinsic allergenicity of gliadin per se because it is an insoluble protein in aqueous solutions. Rather, here we are reporting the intrinsic allergenicity of 'alcohol-solubilized gliadin protein'. Therefore, whether 70% ethanol, that is required for solubilizing the gliadin protein, might act as an adjuvant for specific IgE antibody responses to gliadin remains unknown. Technically, it is not possible to apply ASDG over the skin in aqueous solutions such as PBS in a consistently dose-controlled manner as it becomes insoluble and forms a viscous suspension. Also, we are not aware of any previous published studies suggesting that 70% ethyl alcohol has any adjuvant activity on allergic responses in mouse models. Since typical allergens such as ovalbumin are not soluble in 70% ethanol it is not possible to test whether ethanol might act as adjuvant for ovalbumin or any other aqueous soluble protein allergens in skin sensitization model since applied insoluble material does not stay in place and measurement of responses in a dose-controlled manner are technically not feasible. There is one interesting study that tests the effect of dodecyl alcohol ethoxylate (a nonionic surfactant derived from alcohol) on specific IgE response to ovalbumin in Balb/cA mice (Clausen et al., 2000). They reported that there was no significant impact of dodecyl alcohol on IgE responses in general except that at one dose there was inhibitory effect (Clausen et al., 2000).

There is some evidence in humans that sensitization to gluten on to undamaged skin can occur via usage of cosmetics such as facial soap (Hiragun et al., 2013; Kobayashi et al., 2015). In these reports, authors demonstrate that facial soaps containing Glupearl 19S (an acid hydrolyzed wheat protein) caused sensitization of several Japanese subjects that subsequentially developed anaphylaxis or urticaria upon consumption of wheat-containing food products. Thus, our results

in mouse model further support the hypothesis that exposure to gluten via undamaged skin may have the potential to sensitize humans.

In this study we measured the systemic in vivo levels of cytokines in the spleen extracts (which is a crude way of measurement) because our goal was to identify the cytokine biomarkers associated with systemic anaphylaxis that are elevated by 1 hour-after systemic ASDG challenge. The rationale for this objective was that such cytokines might as biomarkers of life-threatening systemic anaphylaxis. We have identified that a few cytokines are significantly elevated during the systemic anaphylaxis reaction in this model. Our goal was not to study recall cytokine responses in this model as cytokine responses to gliadin in Balb/c mice has been previously reported (Bodinier et al., 2009).

In this study, we report cytokine productions in vivo in the spleen of mice undergoing systemic anaphylaxis. However, the cellular source of various cytokines remains to be determined. Due to limited resources, and focus on the current grant funding, we have not been able to conduct flow cytometry studies at this point. However, it is important to conduct such studies. We plan to do detailed flow cytometry analysis to identify the cellular contributors of cytokine production observed in the spleen in future studies.

Multiple parameters were evaluated during the course of the current study, providing detailed characterization of the mouse model for gluten sensitization and anaphylaxis, aspects that previous animal model studies have not reported on. These include: i) systemic anaphylaxis to wheat gluten is associated with mucosal mast cell degranulation responses (MMCRs), quantifiable by measuring MMCP-1 levels in the blood which is a specific biological marker of IgE antibody-mediated systemic anaphylaxis in mice (Khodoun et al., 2011); ii) using single mouse data we conducted analyses and demonstrated correlations among the 3 quantifiable and

objective readouts of anaphylaxis (IgE, HSR and MMCR); iii) using spleen cells, we characterized the Th1, and Th2 cytokine profile of mice undergoing life-threatening systemic anaphylaxis; and iv) we also analyzed a large panel of other immune markers (chemokines, and adhesion molecules) and identified those that are acutely and substantially elevated (2.5 to 43-fold) during the clinical symptoms of systemic anaphylaxis. It is noteworthy that most of these immune markers have been previously linked to allergic and Th2 immune responses (Bondar et al., 2014; Gu et al., 2000; Li et al., 2007; Ogawa et al., 2004; Pathogenesis et al., 2019). Thus, our study has significantly advanced the mouse model development and characterization efforts to improve the current models of gluten allergy, particularly using an adjuvant-free approach to elicit gluten hypersensitivity. Furthermore, our approach of conducting exposures to gluten on intact murine skin, without deliberately causing skin-damage by tape-stripping, has also advanced the humane use of animals for model development.

Hypothermic shock responses (HSRs) were measured by rectal thermometry before challenge and at every 5 minutes up to 30 minutes after challenge. At one hour after challenge, mice were bled, and plasma was used to measure MMCP-1 levels. Measurement of MMCP-1 in the blood at 1 hour after challenge reflects anaphylaxis caused by IgE antibody mediated mucosal mast cell degranulation response in mice (Khodoun et al., 2011). The HSR represents the consequence of anaphylaxis on neurological and cardiovascular functions involved in thermoregulation. Our goal was to determine whether mucosal mast cell response was associated with HSR noted upon systemic challenge. Our data show that there is a significant co-relationship between the HSR and MMCP-1 responses--the two objective and quantitative markers of anaphylaxis. This explains that the underlying mechanism of gluten-induced

anaphylaxis as quantified by HSR involves participation of IgE antibody mediated activation and degranulation of mucosal mast cells in this model.

The gastrointestinal tract (gut) contains mucosal mast cells that are filled with MMCP-1 in their granules, which is a characteristic granule protein of only mucosal mast cells, and it is absent in connective tissue mast cells. The gut mucosal mast cells have receptors for IgE antibodies. Upon sensitization to gluten, IgE antibodies bind to mucosal mast cells via the high affinity IgE antibody receptors (FcεRI). At this point, blood has very little background levels of MMCP-1 as reflected in pre-challenge samples because the mucosal mast cells are not activated. Upon challenge, gluten binds to gut mast cells associated IgE antibody leading to activation and degranulation of mast cells, thus releasing MMCP1 into the systemic circulation. Elevation of MMCP1 at one-hour timepoint after challenge indicates IgE antibody mediated anaphylaxis in the gut as reported previously (Khodoun et al., 2011). Therefore, demonstrating significant elevation of MMCP-1 in the blood at 1-hour after challenge represents IgE antibody mediated mucosal mast cell degranulation response associated with systemic anaphylaxis in this model.

The most common quantitative parameters used to study allergic response in mouse models food allergy are food specific IgE antibody levels in the blood, elevation of MMCP-1 upon allergen challenge in the blood, and significant drop in the rectal temperature upon allergen challenge (Gao et al., 2021; Jin et al., 2019). One of the aims of the current study was to test the relationships among quantitative parameters associated with systemic anaphylaxis in this model and validate them for testing changes to gluten allergenicity by processing methods and genetic modifications in the future. The rationale underlying this idea is that it will inform the mechanisms leading to life-threatening anaphylaxis. As we found, there is a significant correlation among the three quantitative parameters, which suggests that the underlying

mechanism of systemic anaphylaxis as well as hypothermic shock response observed in this model involves specific IgE antibody mediated activation of mucosal mast cells resulting in degranulation of mediators of anaphylaxis. Therefore, these data validate the future use of all the three readouts for testing the impact of modification of gluten allergenicity by processing methods and genetic approaches in future research studies.

Gluten hypersensitivity has been studied in a number of animal models previously, including dog, rat, guinea pig and mice (Ballegaard et al., 2019; Buchanan et al., 1997; Frick et al., 2005; Kohno et al., 2016; Kroghsboet al., 2014; Yamada et al., 2019). In dog models, a genetic breed variant was identified to develop wheat allergy along with allergy to other foods when wheat flour was administered by injections along with adjuvants followed by repeated oral challenges to elicit diarrhea (Buchanan et al., 1997). They demonstrated that not only gluten, but also non-gluten proteins elicited IgE responses leading to skin sensitization as measured by skin prick testing with respective proteins. Based on their results, we made some improvements in indicators of wheat allergenicity, such as life-threatening wheat anaphylaxis, correlations among allergy readouts, and characterized cytokine and other immune response markers (Gao et al., 2023).

In rat models, consequences of both the oral route of exposure as well as the skin route of exposure to gluten have been reported (Ballegaard et al., 2019; Kroghsboet al. 2014). Oral exposure to gluten was shown to enhance allergic sensitization (IgE) capacity only with enzyme-hydrolyzed gluten. They also found that acid hydrolysis of gluten results in the generation of novel IgG binding epitopes (Kroghsbo et al. 2014). This study had focused on specific IgE, IgG, and rat basophilic leukemia cell degranulation in vitro as the readouts of disease. In the skin exposure rat model, researchers used sandpaper to cause skin injury like the use of tape-stripping

to remove stratum corneum in the previous mouse model discussed above (Adachi et al., 2012). In mouse model of systemic anaphylaxis induced by intraperitoneal injections, specific IgG1 antibodies participate in reactions (Miyajima et al., 1997). Therefore, we measured ASDG-specific IgG1 antibodies in this model and our data demonstrate robust IgG1 responses in this model.

Several studies have reported on mouse models used to study gluten hypersensitivity (Abe et al., 2014; Bodinier et al., 2009; Denery-Papini et al., 2011; Gourbeyre et al., 2012; Kozai et al., 2006; Tanaka et al., 2011); all except one used alum adjuvant to elicit IgE responses to gluten upon intraperitoneal injections, the exception used detergent as adjuvant to elicit sensitization upon application over damaged skin by tape-stripping of stratum corneum. Key endpoints used in these studies were as follows: specific IgE and total IgE, HSR, and symptom scoring. None of the studies determined MMCP-1 responses. It should be noted that neither correlation studies among allergenicity markers nor systemic immune marker analysis associated with anaphylaxis have been reported before in gluten hypersensitivity mouse models.

Notably, of the previous animal models of gluten hypersensitivity investigated, none have reported systemic immune markers associated with clinical symptoms of systemic anaphylaxis. We found that selected Th2 markers are positively associated with anaphylaxis and selected Th1 markers are negatively associated with anaphylaxis. Furthermore, the large panel of other novel immune markers found to be associated with anaphylaxis were also identified in this study. Therefore, these data suggest the complexity of the acute adverse immune response elicited by gluten in the ASDG-sensitized murine host. Thus, these markers' relevance can be tested in human gluten anaphylaxis and their suitability for monitoring the severity of the disease can be determined in future. Furthermore, they may also provide leads to developing novel therapeutics

targeting the common pathways leading to their activation during the life-threatening anaphylactic response to gluten.

Previous elegant studies have identified specific gluten allergens in humans as follows: α-, γ-, ω-2, and ω-5 gliadins and low molecular weight glutenin subunit (Balakireva & Zamyatnin, 2016; Cianferoni, 2016; Denery-Papini et al., 2011). Furthermore, epitope mapping demonstrated that IgE epitopes on these allergens are very similar between wheat allergic humans and Balb/c mice that are sensitized to wheat (Denery-Papini et al., 2011). Previously, Jorgensen et al. demonstrated that non-gluten salt-soluble allergens in sensitized Balb/c mice are also reported as major allergens in humans allergic to wheat (Jorgensen et al., 2022). Thus, Balb/c mouse model represents a very valuable experimental system to study human wheat allergenicity.

Therefore, the mouse model we report here is not only significantly different from previous reported models, but it also makes further improvements to the existing models so that the following novel and critical questions can be addressed effectively in the future which was not possible previously: i) study the intrinsic allergenicity of glutens from wheat lines that are genetically different in ploidy, as well as compare the intrinsic allergenicity of various wheat lines within diploid, tetraploid and hexaploid wheats; this approach has the potential to identify hypo/non/hyper-allergenic wheats for future consumer and medical uses; ii) determine the intrinsic allergenicity potential of genetically engineered (GE) wheats to establish substantial equivalence with the native wheat line as part of evaluating the food safety of GE foods as recommended by the Food and Agriculture Organization/World Health Organization in their flow chart model for this purpose (FAO, 2001); iii) determine the impact of processing on gluten allergenicity so that effects of food and industrial processing on gluten allergenicity can be

quantitatively assessed to protect consumers of products from both the wheat food industry and the cosmetic skin health care industry (Gao et al., 2021); iv) the pre-clinical adjuvant-free mouse model reported here will also be useful in developing novel dietary intervention and therapeutic and vaccine approaches for gluten hypersensitivity.

This study collectively demonstrates that ASDG is intrinsically allergenic; and chronic exposure to ASDG via undamaged skin may clinically sensitize mice for life-threatening anaphylaxis via activating the systemic Th2 immune response. In conclusion, we report the development and characterization of a novel mouse model of gluten hypersensitivity that has significantly advanced the animal model research on gluten allergenicity.

Supplementary Materials: The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2023. 1214051/full#supplementary-material

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CHAPTER 6 IS WHEAT GLUTENIN INTRINSICALLY ALLERGENIC? EVALUATION USING A NOVEL ADJUVANT-FREE MOUSE MODEL OF SYSTEMIC ANAPHYLAXIS

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6.1 Abstract

Wheat is a prominent allergenic food that can trigger life-threatening anaphylaxis. Presently, it remains unclear whether wheat glutenin (WG) extract possesses inherent sensitization potential independently, without the use of adjuvants, and whether it can sensitize mice to the extent of inducing life-threatening systemic anaphylaxis. Here we tested the hypothesis that repeated skin exposures to WG extract without adjuvant will sensitize mice with the resultant anaphylactic reaction upon systemic WG challenge. Balb/c mice were bred and maintained on a strict plant protein-free diet and were repeatedly exposed to WG extract or vehicle once a week for 9-weeks. WG-specific (s)IgE and total (t)IgE levels were quantified. Mice were challenged with WG extract to induce anaphylactic reactions as measured by hypothermic shock response (HSR) and mucosal mast cell degranulation response (MMCR). We have also conducted proteomic analysis of 120 spleen immune markers was conducted. These skin-sensitized mice exhibited exposure-dependent IgE responses and near-fatal anaphylaxis upon challenge. Proteomic analysis identified 7 dramatically elevated immune biomarkers in

anaphylactic mice. These data reveal that WG is intrinsically allergenic; and chronic skin exposure to WG extract can prime the mice for potentially fatal anaphylaxis.

6.2 Introduction

Wheat hypersensitivity is estimated to affect 0.4-3% in the United States (Venter et al., 2008; Venter et al., 2006; Vierk et al., 2007). Symptoms of wheat allergy can manifest as urticaria/angioedema, asthma, allergic rhinitis, abdominal pain, vomiting, acute dermatitis, anaphylaxis, and WDEIA (Cianferoni, 2016; Cianferoni et al., 2013; Cianferoni & Muraro 2012; Salcedo, Quirce, and Diaz-Perales, 2011). Currently there is no cure for wheat allergy (Sicherer and Sampson 2018). Affected individuals must maintain a gluten-free/wheat-free diet as the primary method of treatment, which can reduce quality of life and cause a serious social burden (Gupta et al., 2019).

Wheat allergens are divided into two groups: gluten proteins and non-gluten proteins. Non-gluten proteins are further divided into albumins (water-soluble) and globulins (salt-soluble) which have metabolic and structural functionalities (de Sousa et al., 2021). Gluten proteins are seed storage proteins that are constituted by gliadins and glutenins. Gliadins are prolamin proteins that are ethanol-soluble, and glutenins are glutelin proteins that are soluble in weak acid (acetic acid) solution (Cabanillas, 2020). Both non-gluten and gluten proteins are linked to wheat hypersensitivity (or allergy) in humans (Pastorello et al., 2007; Ricci et al., 2019).

The development of wheat allergy comprises two consecutive stages: 1) the production of IgE antibodies against specific wheat allergens upon initial encounters with the allergen causing sensitization; 2) Triggering of allergic response upon subsequent exposure to wheat allergens eliciting potentially life-threatening anaphylaxis. It is generally accepted that oral consumption

of dietary gluten may cause sensitization (Cabanillas, 2020). There is also suggestion that skin, airways, and eyes exposure may be involved in sensitization also (Gao et al., 2019; Platts-Mills, 2015; Renz et al. 2018; Sicherer & Sampson 2018). Currently, it is unknown whether wheat glutenin (WG) has intrinsic sensitization capacity, and whether skin exposure to WG can cause clinical sensitization for life-threatening systemic anaphylaxis.

There are several animal models (dog, rat, mice) reported to study wheat allergenicity using gluten and non-gluten proteins (Adachi et al., 2012; Bodinier et al., 2009; Buchanan et al., 1997; Gao et al., 2023; Jorgensen et al., 2023; Kozai et al., 2006; Kroghsbo et al., 2014). Wheat gliadin has been used in most gluten allergy mouse model studies (Bodinier et al., 2009; Gourbeyre et al., 2012; Jorgensen et al., 2023; Tanaka et al., 2011). There are two mouse models reported for wheat glutenin hypersensitivity (Kozai et al., 2006; Wang et al., 2020). While these models are very useful with their own strengths and novelty, there are few major challenges that limit their applications such as: i) use of adjuvants to elicit sensitization to glutenin; ii) exposure to glutenin via injections to elicit sensitization; and iii) lack of robust quantitative readouts of systemic anaphylaxis (e.g., hypothermia shock response, and mediators). Here we sought to address these limitations, and thereby further refine and improve the animal model of glutenin hypersensitivity.

We used a transdermal sensitization method to develop this mouse model of wheat glutenin allergy because: i) there is evidence that humans can be sensitized to food allergens including glutenin allergens via skin exposure (Sampson et al., 2018; Warnberg et al., 2022; Brough et al., 2020); for example there are reports of developing wheat glutenin sensitization upon using facial soaps in Japan (Fukutomi et al., 2011; Chinuki et al., 2012; Lauriere et al., 2006); and ii) in mouse models, oral exposure to dietary proteins results in immune tolerance

unless adjuvants such as cholera toxin etc., are co-administered; our intent was to develop an adjuvant-free mouse model of systemic anaphylaxis, therefore to bypass oral immune tolerance, we used transdermal route of exposure without an adjuvant to induce sensitization.

We used glutenin to develop this mouse model because: i) glutenin proteins are associated with sensitization as well as life-threatening anaphylaxis in humans, therefore a mouse model to study wheat glutenin-induced sensitization and anaphylaxis would be useful to understand mechanisms as well as develop novel preventative and therapeutics; ii) facial soaps containing glutenin have been reported to induce sensitization for systemic anaphylaxis in humans (Hiragun et al., 2013; Kobayashi et al., 2015); iii) the gluten family of proteins are classified into two distinct groups: gliadin (30-40% of total wheat protein) and glutenin (45-50% total wheat proteins) (Cabanillas, 2016; Jin et al., 2019); although there are several mouse models of anaphylaxis reported for gliadins (Adachi et al., 2012; Jorgensen et al., 2023; Birmingham et al., 2007), there are only two mouse models (adjuvant and injection-based) reported for glutenin sensitization (Koazi et al., 2006; Wang et al., 2020); however, an adjuvant-free mouse model simulating skin exposure to glutenin leading to sensitization and anaphylaxis is unavailable at present and such a model would be very useful in basic and applied research on wheat glutenin allergy.

In this study, we tested the hypothesis that repeated skin exposures to WG extract without adjuvant will sensitize mice with the resultant anaphylactic reaction upon intraperitoneal WG challenge. There were six objectives for this research: 1) establish a colony of plant protein-free Balb/c mice colony; 2) Asses WG's inherent sensitization potential (i.e., IgE response) via repeated skin application; 3) investigate anaphylactic clinical symptoms upon systemic WG challenge; 4) Quantify the anaphylaxis using hypothermic shock responses (HSR); 5) Measure

mucosal mast cell degranulation responses (MMCR) by quantifying blood levels of MMCP-1; and 6) Identify spleen biomarkers associated with life-threatening systemic anaphylaxis in this model. Overall, these data reveal for the first time that WG is intrinsically allergenic; and chronic skin exposure to WG can prime the mice for potentially fatal anaphylaxis. Life-threatening anaphylaxis is associated with differential expression of immune biomarkers involved in vascular permeability, and allergic immune regulation.

6.3 Results

6.3.1 Chronic application of wheat glutenin (WG) onto undamaged skin elicits robust specific IgE antibody response in Balb/c mice

The potential of WG to induce sensitization when applied repeatedly to the skin was conducted as follows: Female adult Balb/c mice were divided into groups (n = 10/group) and subjected to topical application of either WG or control vehicle once per week for nine weeks as described in the methods section. Blood samples were collected before the initial exposure (pre), and after six skin exposures using a pre-optimized ELISA method to quantify specific (s) IgE levels. As depicted in Figure 6.1A-B, skin exposure to WG resulted in significant sIgE response, whereas the vehicle displayed no such elevation.

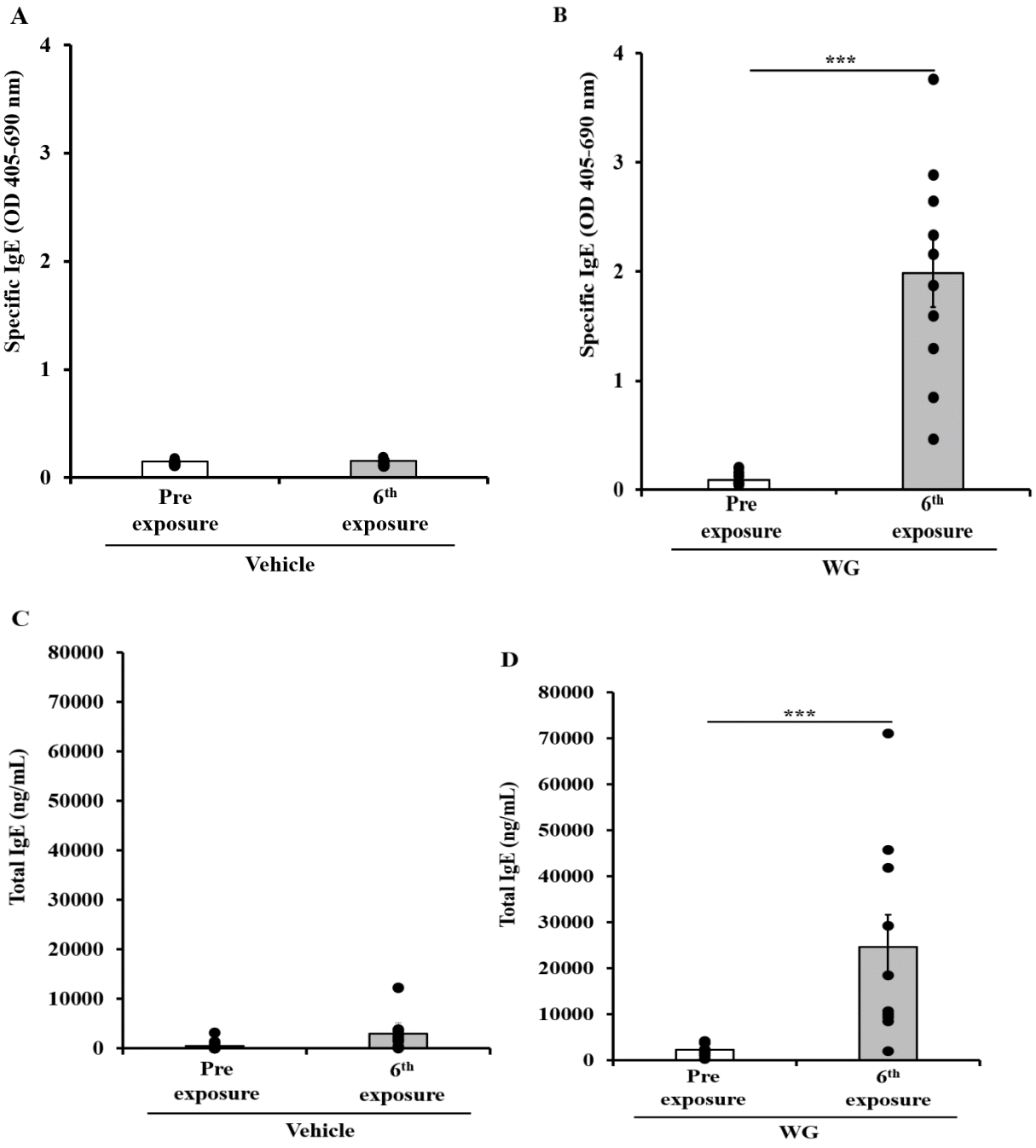


Figure 6.1 Chronic skin exposure to wheat glutenin (WG) extract elicited specific IgE antibody responses and elevation of total IgE in Balb/c mice.

Mice were exposed to WG or to vehicle as described in Materials and Methods. Blood was collected before 1st exposure (Pre) and after 6th exposure. Plasma was used in measurement of WG-specific IgE levels (OD 405-690 nm) using an ELISA method described previously. (**A**)

WG-specific IgE antibody levels in control mice. (**B**) WG-specific IgE antibody levels in sensitized mice. (C) total IgE levels in vehicle sensitized control mice. (D) total IgE levels in WG-sensitized mice. Student's two-tailed t-test: ***$p < 0.001$.

6.3.2 Chronic application of wheat glutenin (WG) onto undamaged skin also elevates total IgE levels, which correlate with sIgE levels

Total (t)IgE levels in the blood were assessed using an ELISA method before (pre) and after six exposures to either WG or to vehicle. As evident, prolonged exposure to WG significantly elevated tIgE levels, which was not observed in mice exposed to the vehicle (Figure 6.1C-D). To examine the relationship between individual mouse data for sIgE and tIgE, Pearson correlation coefficient analysis was performed. A significant positive correlation between the two measurements was observed (Figure 6.2).

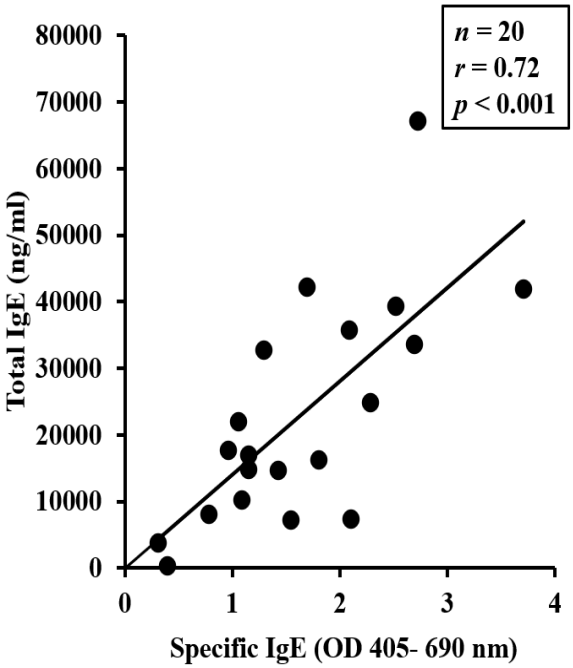


Figure 6.2 Pearson correlation analysis between wheat glutenin (WG)-specific IgE antibody levels and total IgE levels.

Mice were treated as described in Materials and Methods. Pearson correlation analysis was used to test the relationship between WG-specific IgE antibody and total IgE levels in the plasma after 6th transdermal exposure to WG.

6.3.3 WG-sensitized, but not vehicle-sensitized mice, exhibit life-threatening symptoms of anaphylaxis upon systemic challenge with WG

Two separate sets of mice, one sensitized with WG and the other sensitized with vehicle, were challenged with intraperitoneal WG to assess the presence of systemic anaphylaxis. Clinical symptoms were assessed using established methods (Birmingham et al., 2007) Notably, life-threatening clinical symptoms were observed exclusively in the WG-sensitized mice, but not in the vehicle-exposed groups (Figure 6.3). The most prevalent symptoms included altered

respiration, scratching, rubbing of the nose, face, and/or head, and lack of activity upon prodding.

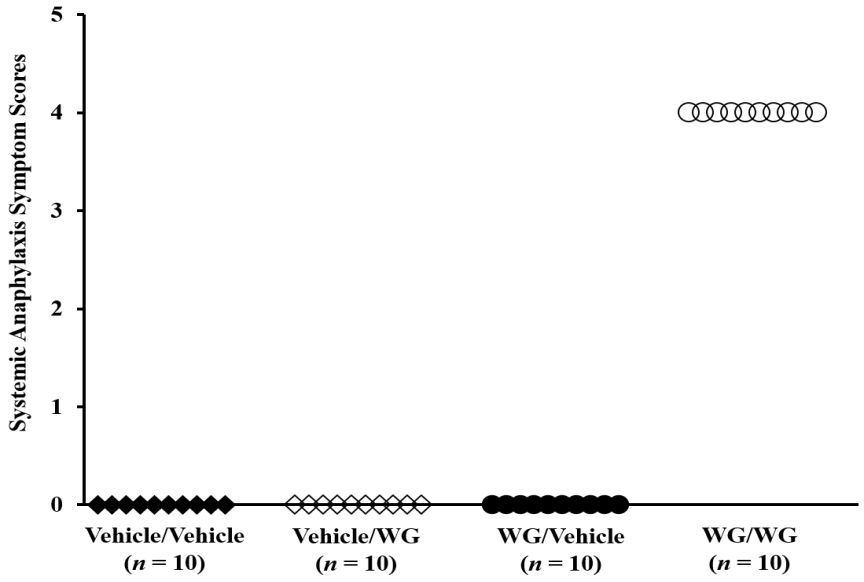


Figure 6.3 Chronic skin exposure to wheat glutenin (WG) is sufficient to clinically sensitize Balb/c mice for systemic anaphylaxis.

Groups of Balb/c mice were sensitized with either vehicle or WG as described in Methods. After 9 transdermal exposures mice were challenged with either vehicle or WG (0.5 mg/mouse) by intraperitoneal injection. Each symbol represents an individual mouse.

6.3.4 Mice experiencing systemic anaphylaxis symptoms following a systemic challenge with WG displayed pronounced hypothermic shock responses (HSR)

Anaphylactic reactions were further assessed using rectal thermometry to examine hypothermic shock responses (HSR). It is evident that systemic challenge with WG led to life-threatening HSR in WG-sensitized mice, while no such response was observed in the vehicle control mice (Figures 6.4A-D). Actual temperature changes are depicted in Figures 6.4A, and 6.4C. Absolute temperature changes are shown in 6.4B and 6.4D. Notably, no HSR was

observed in mice challenged with vehicle, or in mice sensitized with vehicle and subsequently challenged with WG.

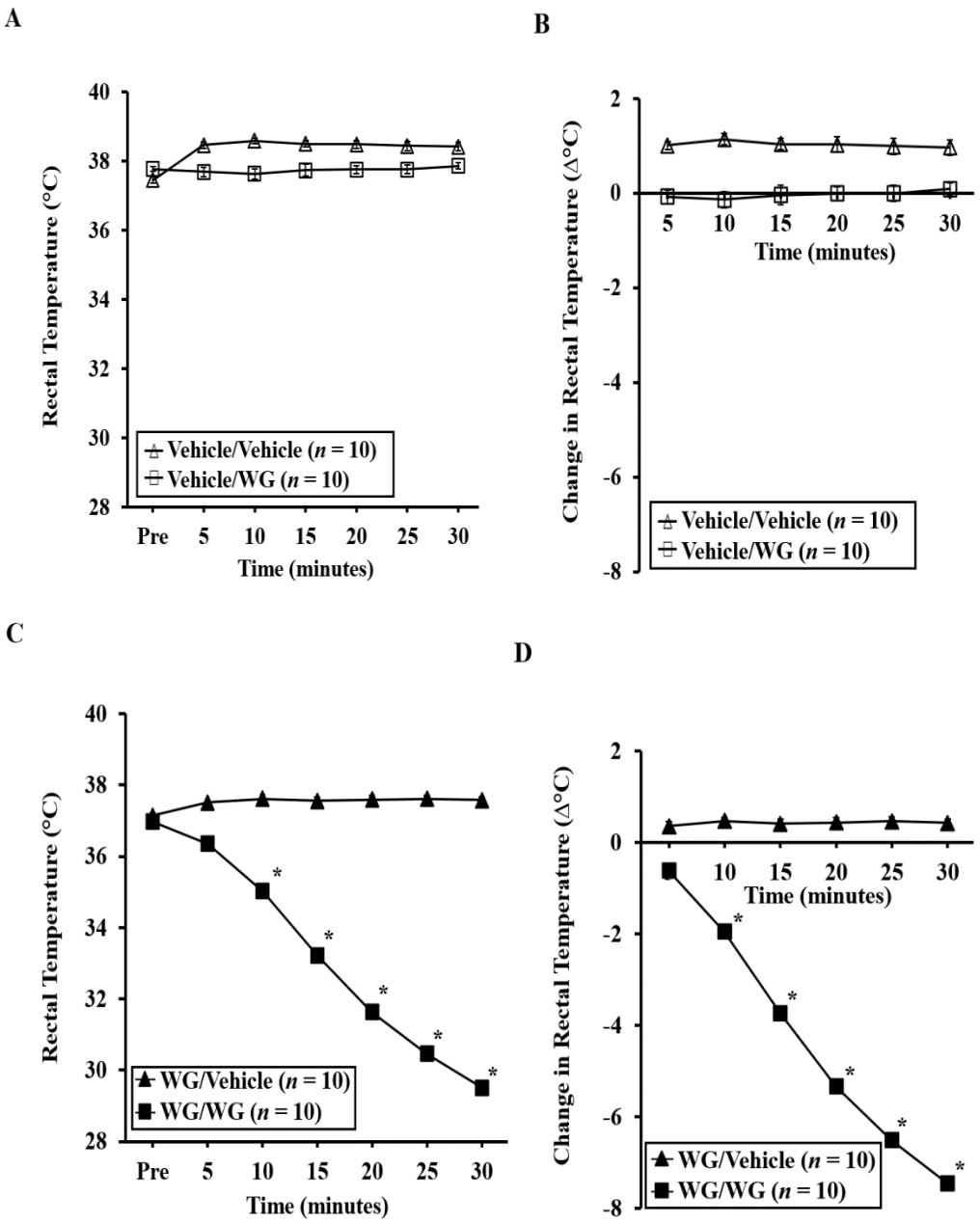


Figure 6.4 Induction of hypothermic shock responses upon systemic challenge with wheat glutenin (WG).

Mice exposed to WG or to vehicle were systemically challenged by intraperitoneal injection as described in Materials and Methods. (A) Rectal temperatures (°C) at indicated time points in

vehicle-sensitized mice challenged with WG or vehicle. (B) Change in rectal temperature (Δ°C) at indicated time points in vehicle-sensitized mice challenged with WG or vehicle. (C) Rectal temperatures (°C) at indicated time points in WG-sensitized mice challenged with WG or vehicle. (D) Change in rectal temperature (Δ°C) at indicated time points in WG-sensitized mice challenged with WG or vehicle.

6.3.5 Systemic anaphylaxis is also linked to substantial mucosal mast cell degranulation in this model

Blood samples were obtained one hour after intraperitoneal challenge and utilized to assess the mucosal mast cell degranulation response (MMCR) in mice. The increase in mucosal mast cell protease-1 (MMCP-1) serves as evidence of a genuine IgE antibody-mediated type-1 hypersensitivity reaction to food proteins in mouse models, as described previously (Khodoun et al., 2015). Figure 6.5A-D clearly demonstrates a significant MMCR in mice undergoing anaphylaxis, while no such MMCR was observed in the control mic

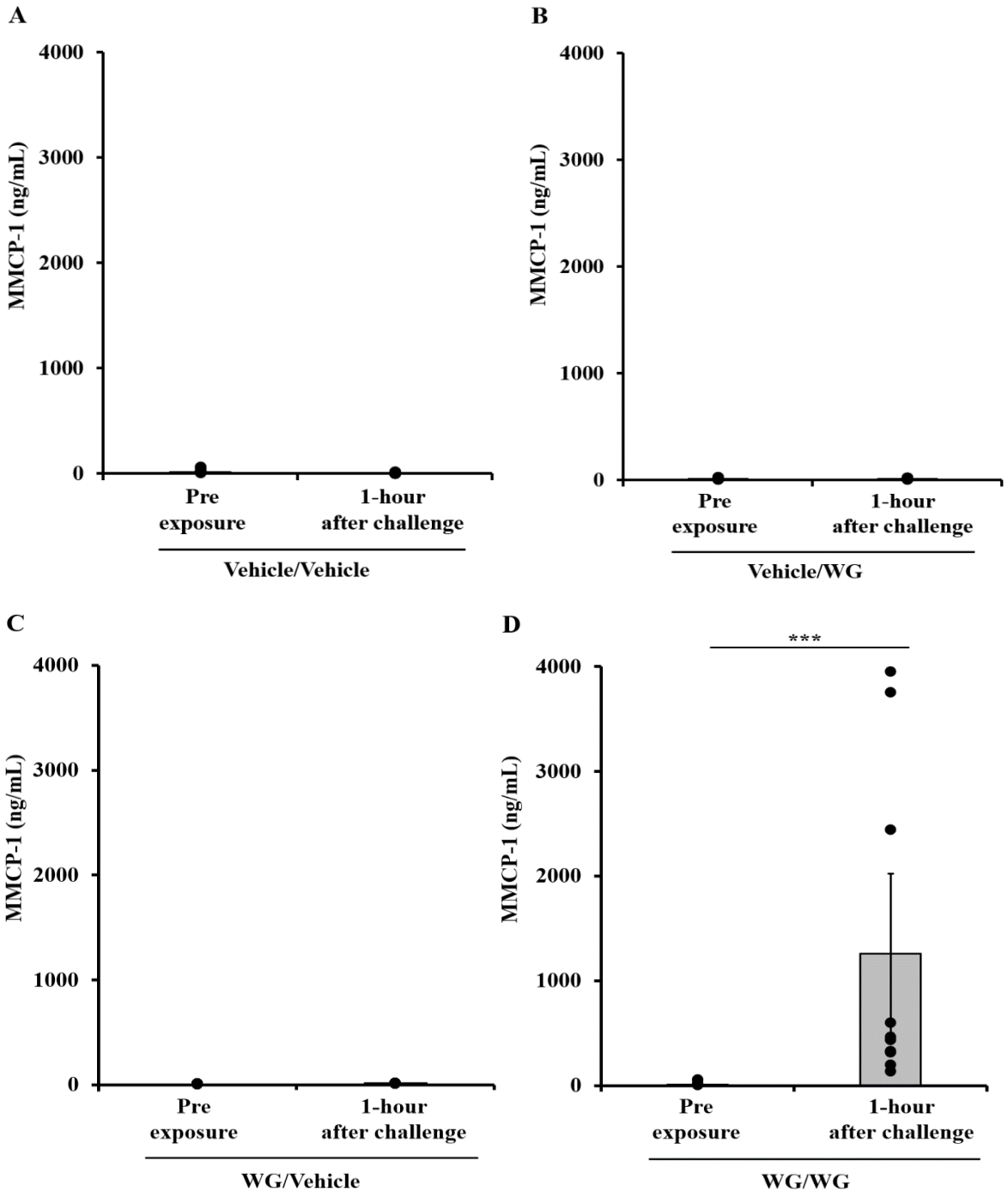


Figure 6.5 Systemic anaphylaxis induced by wheat glutenin (WG) is associated with degranulation of mucosal mast cells in this model.

Mice were treated as described in Materials and Methods. Their plasma mucosal mast cell protease-1 (MMCP) levels (ng/mL) were measured using an ELISA-based method described in the texts. (A) MMCP-1 levels in control mice challenged with vehicle. (B) MMCP-1 levels in

vehicle-sensitized control mice challenged with WG. (C) MMCP-1 levels in WG-sensitized mice challenged with vehicle. (D) MMCP-1 levels in WG-sensitized mice challenged with WG. Student's two-tailed t-test: ***$p < 0.001$

6.3.6 Proteomic analysis and identification of differentially expressed immune biomarkers in the spleen of mice undergoing systemic anaphylaxis

We conducted a heat map analysis of the expression of a panel of 120 proteomic immune biomarkers in mice undergoing anaphylaxis versus the control mice as described in methods (Figure 6.6A-C). Among the differentially expressed immune biomarkers, 27 markers were significantly elevated and 37 were significantly reduced (student's t-test, two-tailed, $p < 0.05$) in anaphylactic mice (Tables 6.1 and 6.2).

Then we classified these makers into four categories based on fold-change in protein expression as follows: low importance (up to 1.9-fold change), medium importance (2-3.9-fold change), high importance (4-5.9-fold change), and critical importance (6 and above-fold change). The following 7 immune biomarkers were substantially elevated in anaphylaxis: IL-6, IL-9, IL-17E, and MIP-3a (high importance), Resistin, VEGF-D, and VEGF-R3 (critical importance) (Figure 6.7A). The following 4 immune biomarkers were markedly reduced in anaphylaxis: IL-1b (high importance), MIP-3b, Pentraxin 3, and TWEAK R (critical importance) (Figure 6.7B).

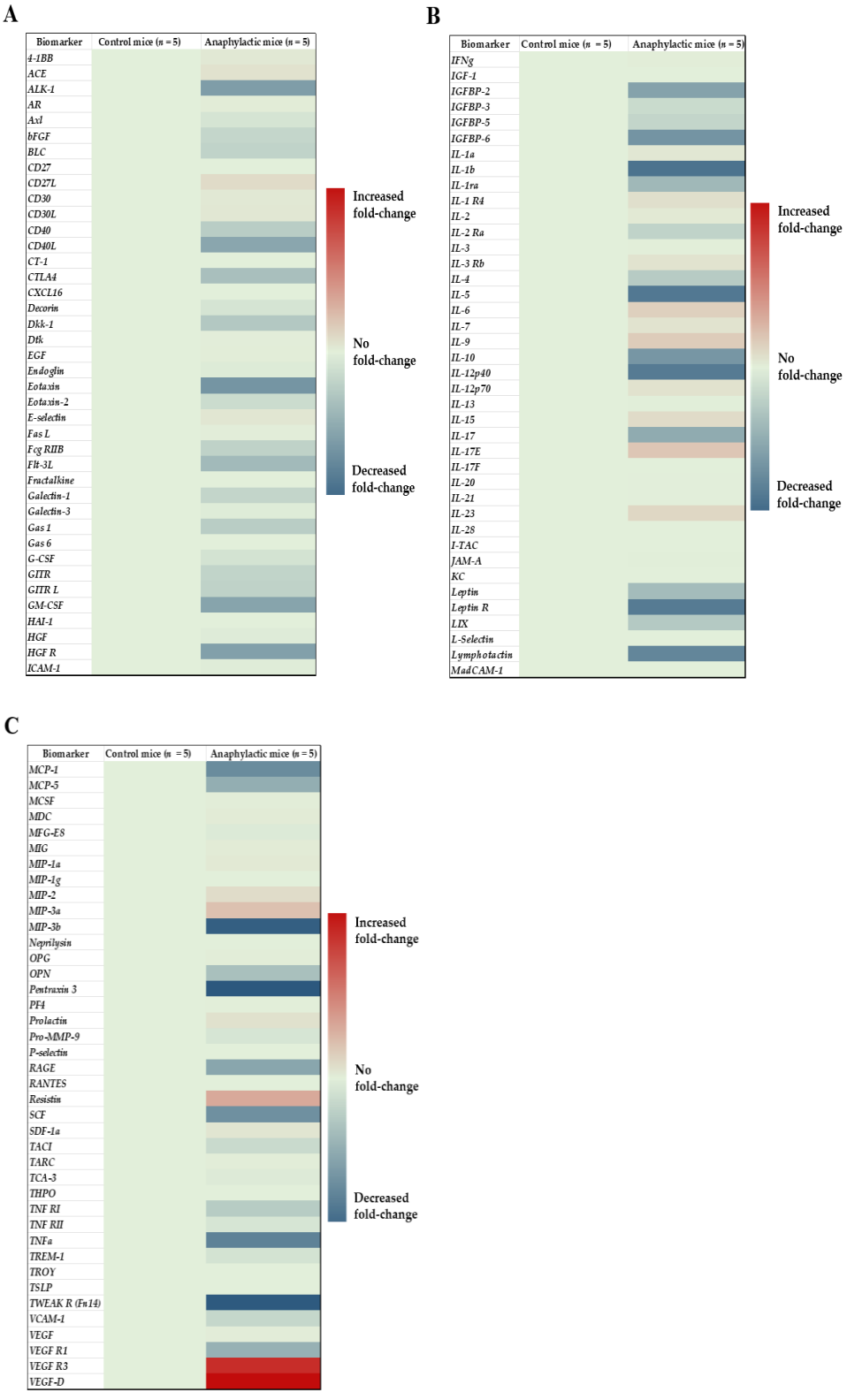


Figure 6.6 Heat map analysis of 120 spleen immune biomarkers in glutenin-induced systemic anaphylaxis.

Using spleen extracts from control mice and anaphylactic mice, a proteomic microarray analysis was conducted using Ray-Biotech system cytokine panels, **(A)** CYT-4, **(B)** CYT-5 and **(C)** CYT-6 as described in the methods. Background levels of immune biomarkers are shown in green color. Upregulated biomarkers are shown in red color and down-regulated biomarkers are shown in blue color.

Table 6.1 Identification of immune biomarkers that are significantly increased in the spleen during systemic anaphylaxis.

Biomarker	Control mice (n = 5)	Anaphylactic mice (n = 5)	*Student's t-test p* <
ACE	38316.3 ± 1853.51	91252.75 ± 2471.8	0.001
CD27L	<40 (LOD)	121.18 ± 18.33	0.005
CD30L	7.57 ± 0.44	13.68 ± 0.67	0.001
Dtk	1117.62 ± 26.36	1348.25 ± 46.15	0.005
IL-1a	14.09 ± 2.34	25.88 ± 2.26	0.05
IL-1 R4	391.66 ± 114.88	1022.84 ± 20.13	0.005
IL-2	45.09 ± 1.4	75.5 ± 9.72	0.05
IL-6	59.98 ± 8.13	247.23 ± 26.76	0.001
IL-9	27.2 ± 4.86	124.92 ± 14.87	0.001
IL-12p70	33.78 ± 9.04	78.5 ± 11.13	0.05
IL-15	8185.98 ± 1532.53	25061.06 ± 3151.03	0.005
IL-17E	44.87 ± 19.9	191.72 ± 89.92	0.05
IL-23	273.89 ± 96.98	939.23 ± 164.11	0.05
MCSF	80.09 ± 2.31	102.92 ± 3.43	0.001
MDC	132.25 ± 5.29	198.63 ± 8.4	0.001
MIG	280.49 ± 12.63	419.55 ± 6.13	0.001
MIP-1a	78.61 ± 7.59	132.23 ± 2.6	0.001
MIP-1g	943.39 ± 9.41	1001.9 ± 10.58	0.01
MIP-2	1.56 ± 0.76	4.65 ± 0.29	0.01
OPG	299.86 ± 5.99	373.37 ± 22.5	0.05
PF4	27562.44 ± 363.93	31638.78 ± 629.49	0.001
Prolactin	6.8 ± 1.27	16.57 ± 3.65	0.05
Resistin	152.79 ± 11.04	1237.69 ± 38.04	0.001
SDF-1a	161.28 ± 5.99	325.65 ± 14.87	0.001
VEGF	253.17 ± 6.92	332.56 ± 6.53	0.001
VEGF R3	<20 (LOD)	410.16 ± 105.46	0.01
VEGF-D	1.67 ± 0.57	39.8 ± 3.04	0.001

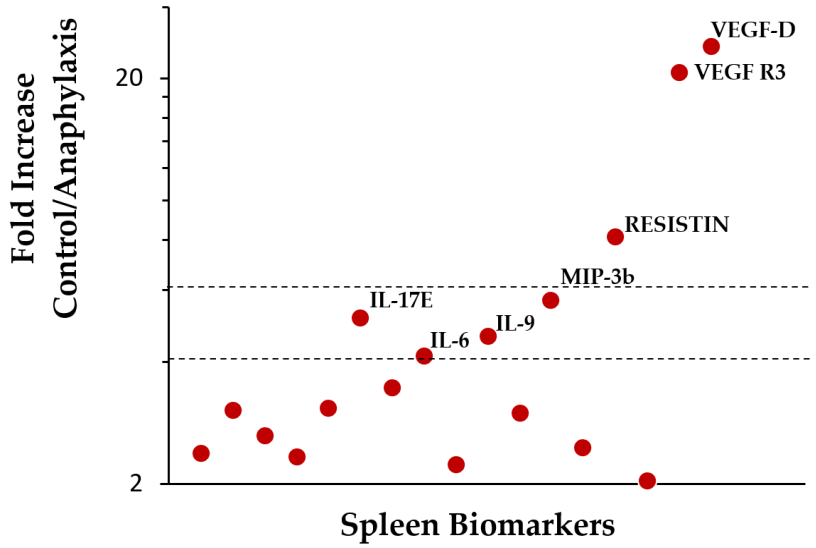
Statistical significance was determined using students' two-tailed testing.

Table 6.2 Identification of immune biomarkers that are significantly decreased in the spleen during systemic anaphylaxis.

Biomarker	Control mice (n = 5)	Anaphylactic mice (n = 5)	*Student's t-test* $p <$
ALK-1	270.55 ± 10.48	134.18 ± 9.6	0.001
bFGF	3828.16 ± 43.65	3255.51 ± 24.39	0.001
BLC	5639.31 ± 124.83	4678.57 ± 44.06	0.001
CD40	4686.52 ± 208.06	3714.48 ± 242.08	0.05
CD40L	3709.09 ± 69.08	2050.96 ± 74.63	0.001
CTLA4	887.48 ± 10.02	628.25 ± 9.38	0.001
Decorin	14941.56 ± 302.52	13997.32 ± 180.47	0.05
Dkk-1	684.12 ± 27.13	521.43 ± 47.91	0.05
Eotaxin	417.45 ± 3.96	184.63 ± 3.05	0.001
Fcg RIIB	6800.55 ± 101.37	5598.51 ± 122.2	0.001
Flt-3L	828.49 ± 8.84	559.04 ± 7.96	0.001
Galectin-1	6404.2 ± 235.85	5393.21 ± 164.45	0.05
Gas 1	1232.06 ± 66.89	974.73 ± 30.9	0.05
GITR	9266.56 ± 301.11	7734.18 ± 336.51	0.05
HGF R	768.41 ± 125.47	394.09 ± 80.25	0.05
IGFBP-2	408.43 ± 23.57	215.84 ± 36.66	0.005
IGFBP-3	2167.55 ± 64.32	1898.07 ± 38.67	0.05
IGFBP-6	1228.83 ± 77.13	541.13 ± 23.11	0.001
IL-1b	14.15 ± 0.7	3.29 ± 0.63	0.001
IL-1ra	618.95 ± 24.81	410.55 ± 14.05	0.001
IL-2 Ra	810.79 ± 36.47	672 ± 26.15	0.05
IL-5	25.43 ± 3.36	<6.8 (LOD)	0.001
IL-12p40	12.32 ± 1.19	3.51 ± 0.91	0.001
IL-17	4.14 ± 0.67	<2.4 (LOD)	0.05
Leptin	911.32 ± 76.19	626.32 ± 34.19	0.05
Leptin R	136.2 ± 28.35	38.75 ± 6.82	0.05
LIX	193.96 ± 2.38	149.09 ± 4.5	0.001

Statistical significance was determined using students' two-tailed testing.

A



B

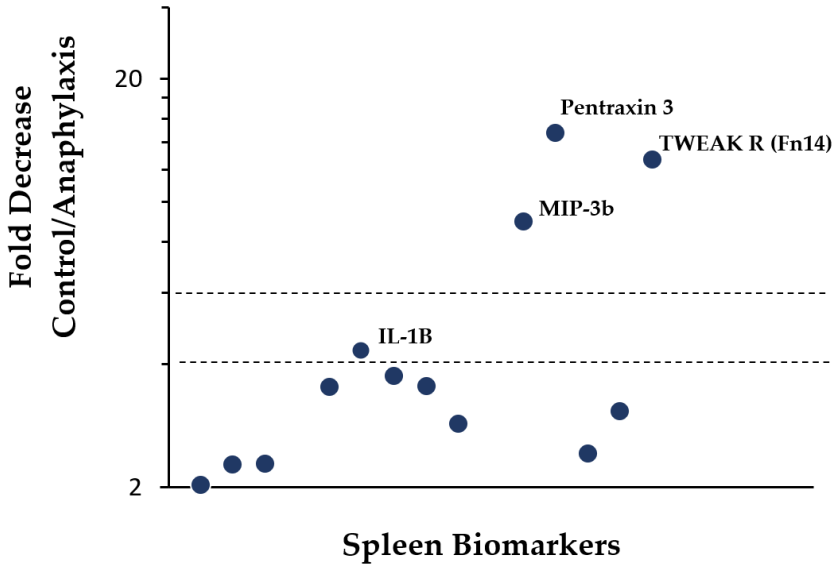


Figure 6.7 Identification of differentially expressed immune biomarkers in the spleen of control vs. anaphylactic mice.

(A) Immune biomarkers that are significantly elevated (2-fold or higher, $p < 0.05$) in anaphylactic mice are shown. (B) Immune biomarkers that are significantly decreased (2-fold or lower, $p < 0.05$) in anaphylactic mice are shown. The dotted lines indicate a 4-fold and 6-fold changes in protein expression. Immune biomarkers that show 4-fold or higher changes are identified with names.

6.4 Discussion

The primary objective of this study was to determine whether wheat glutenin is intrinsically allergenic in mice. It is currently unknown whether wheat glutenin by itself in the absence of adjuvants such as alum, or complete Freund's adjuvant, etc., is capable of sensitizing animals for clinical elicitation of systemic anaphylaxis. Therefore, here we tested the hypothesis that wheat glutenin will clinically sensitize mice upon transdermal application to glutenin without any external adjuvants. Our data collectively support this hypothesis.

There are eight novel findings: i) Chronic application of glutenin onto undamaged skin elicits robust specific IgE antibody response in Balb/c mice in an exposure dependent fashion; ii) chronic application of glutenin onto undamaged skin also elevates total IgE levels, which correlate with sIgE levels; iii) adjuvant-free glutenin skin-sensitized, but not vehicle-sensitized mice, exhibit life-threatening symptoms of anaphylaxis upon systemic challenge with glutenin, but not with vehicle; iv) mice with systemic anaphylaxis symptoms upon intraperitoneal challenge with glutenin exhibit dramatic and life-threatening hypothermic shock responses (HSR); v) HSR was associated with significantly elevated mucosal mast cell response as quantified by MMCP-1 levels in the plasma; vi) identification of differentially expressed immune biomarkers by heat map analysis in the spleen of anaphylactic vs. control mice; vii)

identification of biomarkers positively and negatively associated with anaphylaxis compared to healthy mice based on significant fold-change in protein expression; and viii) identification of biomarkers of high and critical im-portance that are substantially altered in mice during life-threatening anaphylactic reaction compared to healthy control mice in this model.

In this study we chose to establish a mouse model of glutenin because of the following reasons: i) It is unknown at present whether wheat glutenin has intrinsic capacity to elicit IgE antibody responses and whether it can cause systemic anaphylaxis in the absence of exercise as a cofactor; ii) it is unknown at present whether wheat glutenin skin exposure can clinically sensitize mice for systemic anaphylaxis; iii) most of the previous mouse models of wheat hypersensitivity have used wheat gliadins for developing the models; iv) there are only two previous mouse model studies, both of which used adjuvants to elicit IgE responses to glutenin (Kozai et al., 2006; Wang et al., 2020).

Kozai et al., 2006, reported the first mouse model to elicit IgE antibody responses to wheat glutenin (Kozai et al., 2006). Strengths of their model include: i) This was the first model of glutenin-dependent exercise-induced exhaustion reported in the literature; ii) these authors elegantly demonstrated that sensitization to glutenin by IP injections with alum followed by oral glutenin challenge (20 mg/mouse) results in significantly reduced time to exhaustion upon subjecting to exercise using a treadmill. The limitations of the study include: i) Indicators of anaphylaxis such as clinical symptom scores, hypothermic shock responses, histamine responses, or mucosal mast cell mediator proteins were not reported; therefore, whether IgE antibody response resulted in clinical sensitization for systemic anaphylaxis was not studied; ii) this was a complete Freund's adjuvant-based model to elicit sensitization; therefore, intrinsic sensitization capacity of wheat glutenin was not studied.

Wang et al., 2020, reported a mouse model of glutenin sensitization using alum adjuvant-based method (Wang et al., 2020). Strengths of their model include: i) Demonstration of IgE antibody responses to glutenin injection with alum; ii) demonstration of clinical symptoms of anaphylaxis by one hour after intragastric challenge with glutenin (20 mg/mouse). Limitations of this study include: i) Use of alum adjuvant to elicit sensitization to wheat glutenin; therefore, intrinsic sensitization capacity of glutenin was not studied; ii) hypothermic shock responses which are widely used as a quantitative indicator of systemic anaphylaxis was not studied; iii) immediate hypersensitivity mediators were not studied; responses were studied at 24 hours post intragastric challenge, which does not reflect immediate hypersensitivity response that is well established to occur within one hour post challenge (Khodoun et al., 2011) .

Other animal models have been reported for gluten allergy such as Buchanon et al., 1997, which utilized a dog model of food allergy to multiple foods including wheat (Buchanan et al., 1997). In this model, genetically selected dogs were used for sensitization to wheat flour along with alum adjuvant. In addition, distemper and hepatitis vaccination was also administered. Dogs developed immediate hypersensitivity reactions to both gluten allergens (gliadin and glutenin) as well as non-gluten allergens (albumin and globulin) as evidenced by positive skin prick test reactions. Strengths of this model include: i) this was the first animal model demonstrating sensitization to wheat glutenin when injected with alum adjuvant; ii) oral challenge with wheat flour gruel elicited diarrhea indicating oral food allergic reaction. Limitations of this study include: i) Use of alum adjuvant does not allow for the investigation of the intrinsic allergenicity of the wheat glutenins; ii) it is a model of oral wheat induced diarrhea and dogs did not develop life-threatening anaphylactic reactions; iii) they did not report characterization of IgE antibody responses to glutenin.

We conducted a proteomic analysis of spleen immune biomarkers and identified differentially expressed immune biomarkers in mice undergoing near-fatal systemic anaphylaxis versus healthy control mice using a large panel (120) of biomarkers implicated in immune and inflammatory responses. Among them, 27 biomarkers were significantly upregulated, and 37 biomarkers were significantly downregulated during anaphylaxis. These markers have been linked to inflammation, immune regulation, and airways allergic responses in mouse models and in humans (Feng et al., 2022; Gilles et al., 2012; Harris et al., 2010; Herberth et al., 2010; Hosoki et al., 2016; Lee & Park 2022; Li et al., 2007; Shik et al., 2017). However, we demonstrate the differential expression of immune biomarkers associated positively, and negatively with glutenin-induced life-threatening anaphylaxis compared to healthy control mice for the first time.

Based on fold-change analyses, we identified 4 immune markers (IL-6, IL-9, IL-17E, MIP-3a) that were of high importance (4-5.9-fold or higher) during anaphylaxis. Consistent with our findings here, both IL-17E and MIP-3a have been implicated in the pathogenesis of allergic immune responses previously, although linkage to glutenin-induced anaphylaxis is a novel finding (Khodoun et al., 2018; Li et al., 2007). Previously, IL-9 has been found to be associated with anaphylaxis induction (Osterfeld et al., 2010). Three biomarkers that were determined to be of critical importance (6-fold or higher) during ana-phylactic reactions (resistin, VEGF R3, and VEGF-D). Previous studies have reported conflicting role of serum resistin levels in mouse-models of airways allergies (Ramalho et al., 2013). Here we demonstrate a potential role for resistin in glutenin-induced systemic anaphylaxis for the first time. Two cytokines important in vascular permeability (VEGF R3, VEGF-D) were also dramatically elevated consistent with the concept of life-threatening uncontrolled vascular leakage of fluid during anaphylactic shock

(Heinolainen et al., 2017; Rissanen et al., 2003). Therefore, these cytokines may represent potential targets for modulating vascular permeability during anaphylaxis.

Interestingly, there were 4 biomarkers whose expression was markedly reduced during anaphylaxis. Of these, we identified one biomarker that is of high importance (IL-1B), and three others (MIP-3b, TWEAK R, and Pentraxin 3) that were of critical im-portance during anaphylaxis. There are previous studies that propose a protective role for Pentraxin 3 and MIP-3b in airways allergies (Koussih et al., 2021; Yamashita et al., 2006). Our findings of their negative association with anaphylaxis expands their potential protective roles beyond airways allergic reactions to glutenin-induced life-threatening systemic anaphylaxis also. Notedly, TWEAK R (Fn14) axis has been positively linked to anaphylaxis in previous mouse models of passive and active sensitization with adjuvant (Galli, 2020; Mendez-Barbero et al., 2020). Here we demonstrate for the first time TWEAK R is negatively linked to glutenin-induced active anaphylaxis in an adjuvant-free mouse model of food allergy. This discrepancy is discussed below.

In our adjuvant-free mouse model of glutenin-induced systemic anaphylaxis, we found that TWEAK R protein levels in the spleen tissue was critically reduced by in ana-phylactic mice. This finding contrasts with a previous report that TWEAK R expression was significantly elevated in the lung tissue in an adjuvant-based mouse model of anaphylaxis and in an anti-hapten IgE antibody sensitized passive systemic anaphylaxis model (Tsujimura et al., 2008). This discrepancy may be due to differences between our model and their models as follows: i) they used C57/BL 6 mice sensitized passively with anti-hapten (DNP) IgE antibodies followed by intravenous injection with hapten-carrier complex to elicit passive systemic anaphylaxis. They also used C57/BL 6 mice sensitized with BSA (bovine serum albumin) plus pertussis toxin

adjuvant via intraperitoneal injection followed by active systemic anaphylaxis induced by intravenous injection with BSA; in contrast we used Balb/c mice sensitized with WG without adjuvant via skin application, followed by intraperitoneal injection with WG to elicit active systemic anaphylaxis; ii) they studied TWEAK R expression and reported differences in staining intensity of lung tissue section by immunohistochemistry; in contrast, we quantified TWEAK R protein absolute concentration in the spleen tissue extract using a quantitative method in our model. These discrepancies suggest that molecular characteristics of anaphylaxis may be different in different models and tissues.

In this report spleen immune biomarkers were studied in healthy control mice and in anaphylactic mice. Therefore, at least three follow-up studies are needed to establish anaphylaxis specificity of the differentially expressed biomarkers reported in this study: i) to determine the contribution of sensitization (in the absence of anaphylaxis) to observed changes in expression of biomarkers in the spleen; ii) to determine whether WG injection to unsensitized healthy mice impact expression of immune biomarkers in the spleen; and iii) to determine tissue-specific changes in the expression of these immune biomarkers during anaphylaxis in this mouse model.

Here we have significantly advanced the animal model development for glutenin hypersensitivity compared to previously existing models as discussed above. In particular, several major limitations of previous models were addressed in our model as follows: i) we report a novel adjuvant-free animal model of glutenin hypersensitivity that can be used to assess intrinsic allergenicity potential of wheat glutenin; ii) we demonstrate that chronic skin exposures to glutenin without causing deliberate damage (for example tape-stripping of stratum corneum, an approach commonly used to develop skin sensitization rodent models); iii) we not only characterized clinical symptom scores of systemic anaphylaxis, but advanced it further by

developing a robust quantifiable method such as hypothermic shock responses; iv) we report a robust immediate (at one-hour) mucosal mast cell degranulation response upon systemic challenge with glutenin; v) using single mouse data analysis, we demonstrate a significant correlations between two common clinical indicators of sensitization (specific IgE, total IgE); and vi) we also identified several systemic immune markers associated with life-threatening anaphylaxis compared to healthy mice.

Systemic anaphylaxis upon allergen injection (intraperitoneal, intravenous) in mouse models can be mediated by allergen specific IgG1 antibodies (Miyajima et al., 1997; Ning et al., 2022). Therefore, we measured WG-specific IgG1 antibody responses. Results show that WG elicits a robust IgG1 antibody response in this model. Thus, WG-specific IgG1 antibodies may also contribute to systemic anaphylaxis upon intraperitoneal injection in this model, and this may represent a limitation of this study.

This study was conducted using glutenin obtained from hexaploidy wheat (*Triticum aestivum*, ambassador variety, genome AABBDD). Therefore, this model can be used to compare intrinsic allergenicity of glutenin from other genetically distinct wheats available on AABBDD genome as well as other wheats with different ploidy such as AA (*Triticum monococcum*), AABB (*Triticum durum*), and DD (*Aegilops tauschii*). Any novel wheat developed on these genomes as background can also be preemptively tested for their intrinsic allergenicity of glutenins.

Currently, genetically engineered (GM) wheat is not commercially available. However, there are efforts to develop them. For example, US FDA approved a GM wheat developed by Argentina (Ning et al., 2022). There were previous incidents of GM wheat contamination of US and Canadian farms that were investigated (Boehm, 2018). A critical question for GM wheat

would be to address their potential allergenicity concerns. International scientific organizations (WHO/FAO) have provided a decision tree approach to evaluate allergenicity hazard of novel GM wheats (FAO, 2001). They have suggested using validated animal models for testing. The model that we have described here would be very useful for preclinical evaluation of intrinsic allergenicity of glutenins obtained from such GM wheats.

This model can be used for developing novel immunotherapies to wheat glutenin allergy. For example, wheat glutenin sensitized mice can be used to test a novel protocol such as repeated low-dose oral administration of native or modified wheat glutenin to desensitize mice from wheat glutenin allergy. In the same way, novel drugs can be developed for glutenin-induced life-threatening systemic anaphylaxis using this model for pre-clinical testing.

Food processing methods have been shown to influence (increase/decrease/eliminate) wheat allergenicity in in vitro methods (Gao et al., 2021). This model provides an opportunity to determine the effects of various physical, chemical, microbiological processing methods on glutenin allergenicity, and aid in development of potentially hypo/non-allergenic glutenin proteins. Similarly, inadvertent creation of hyper-allergenic and dangerous glutenins can be prevented by preemptive testing using this model.

In summary, this study collectively reveals wheat glutenin's intrinsic allergenic nature for the first time in an animal model. This model also provides robust quantifiable readouts of life-threatening systemic anaphylaxis. Therefore, this improved model of glutenin allergenicity can be utilized to develop novel methods to prevent and treat life-threatening anaphylactic reactions to glutenin in humans.

6.5 Materials and Methods

6.5.1 Chemicals and reagents

Biotin-conjugated rat anti-mouse IgE-paired antibodies were procured from BD Bio-Sciences (San Jose, CA, USA). The p-nitro-phenyl phosphate compound was sourced from Sigma (St. Louis, MO, USA). Streptavidin alkaline phosphatase was acquired from Jackson ImmunoResearch (West Grove, PA, USA). Folin reagent was obtained from BioRad (Hercules, CA). The following reagents were secured as specified: IgE Mouse Uncoated ELISA Kit with Plates, Streptavidin-HRP, TMB substrate, MCPT-1 (mMCP-1) Mouse Un-coated ELISA Kit with Plates, Avidin-HRP, TMB substrate, all of which were procured from Invitrogen (MA, USA). The Tissue Protein Extraction Reagent (T-PERTM), a proprietary detergent with a composition of 25mM bicine and 150mM sodium chloride at pH 7.6, was obtained from ThermoFisher Scientific (MA, USA). For protease inhibition, a cocktail of serine, cysteine, and acid proteases, along with aminopeptidases, was acquired from Sigma-Aldrich (MO, USA).

6.5.2 Mice breeding and establishment of a plant-protein-free mouse colony

Adult Balb/c breeder pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, the mice were introduced to a rigorous plant-protein-free diet (AIN-93G, Envigo, Madison, MI). Following a one-week acclimation period, breeding was initiated using conventional methods. For this study, adult female mice aged 6-8 weeks from the litter were selected. Throughout the entire duration of the study, all mice were consistently maintained on the strict plant-protein-free diet (AIN-93G). All animal procedures adhered to the guidelines outlined by Michigan State University.

6.5.3 Preparation of acid-soluble protein extract from wheat flour

Hexaploid wheat flour was used for protein extraction purposes. The acid-soluble wheat glutenin was obtained through an Osborne sequential extraction method (Chen & Bushuk, 1969). In brief, a mixture of flour and filter-sterilized 0.5 M NaCl at a ratio of 1:10 (m/v) was continuously agitated for 2 hours and then subjected to centrifugation at 20000 x g for 30 minutes. The resultant pellets were preserved and utilized for alcohol extraction. The salt-insoluble pellets were subsequently mixed in a 1:10 ratio with 70% ethanol for 2 hours and then centrifuged at 20000 g for 15 minutes. The resultant pellets (alcohol-insoluble) were preserved to be used in acid extraction. The alcohol-insoluble pellets were combined in a 1:4 ratio with 0.05 M acetic acid for two hours and then centrifuged at 20000 x g for 15 minutes. The resulting supernatant was frozen at -70°C overnight and then subjected to freeze-drying the following day. The lyophilized acid-soluble wheat glutenin (WG) was reconstituted using 0.05 M acetic acid to achieve a concentration of 1 mg protein per 100 µL, intended for topical application. For challenges involving intraperitoneal (IP) injections, the WG was reconstituted with phosphate buffered saline (PBS) to attain concentrations of 0.5 mg/mouse. The protein content was quantified using the LECO total combustion method from LECO (St. Joseph, MI). To assess protein quality, SDS-PAGE testing was performed.

6.5.4 Skin sensitization, bleeding, and plasma sample preparation

Female adult Balb/c mice were employed for experimental purposes. To facilitate the procedures, the hair on the mice's rumps was removed bilaterally using a Philips hair clipper (Amsterdam, Netherlands). The acid-soluble wheat glutenin (WG) was administered onto the rump at a dosage of 1 mg per mouse in 100 µL, alternatively using a vehicle solution of 0.05 M

acetic acid. Following application, the treated area was covered with a non-latex bandage sourced from Johnson & Johnson (New Brunswick, New Jersey), and left in place for one day. This process was reiterated on a weekly basis, occurring nine times over a span of nine weeks. Blood samples were collected from the saphenous vein prior to the initial exposure and after the sixth exposure. The blood was drawn into tubes coated with the anticoagulant lithium heparin (Sarstedt Inc MicrovetteCB 300 LH, Germany). The collected blood was subsequently subjected to centrifugation to isolate plasma, which was then stored individually at -70°C until required for subsequent testing of (s)IgE and (t)IgE.

6.5.5 Elicitation of systemic anaphylaxis and clinical symptom scoring

Two weeks after the final cutaneous exposure to acid-soluble wheat glutenin (WG) or the vehicle, the mice were subjected to an intraperitoneal (IP) injection. This injection consisted of either 0.5 mg of WG or the vehicle (phosphate-buffered saline, PBS). Following the injection, the mice were closely monitored for signs of systemic anaphylaxis over a 30-minute period, in accordance with previously outlined protocols (Birmingham et al., 2007; Jorgensen et al., 2023). Assessment scores were assigned based on the ensuing criteria: 0 indicated an absence of symptoms; 1 denoted behaviors like nose and head scratching, along with rubbing; a score of 2 encompassed observations such as swelling around the eyes and mouth, diarrhea, erection of hair (pilar erecti), reduced activity, and/or lowered activity coupled with an elevated respiratory rate; 3 was attributed to manifestations like wheezing, labored breathing, and bluish discoloration near the tail and mouth; 4 marked a lack of activity even after stimulation, accompanied by tremors and convulsions; and, ultimately, 5 indicated mortality.

6.5.6 Determination of hypothermic shock responses

Rectal temperature (°C) measurements were taken both prior to the challenge and at 5-minute intervals following the challenge, up to a 30-minute duration. These measurements were conducted using a rectal thermometer (DIGI-SENSE, MA, USA). The recorded values included the specific temperatures and the corresponding differences (Δ°C) in comparison to the pre-challenge temperatures for each individual mouse. These recorded data points were then employed for subsequent analyses.

6.5.7 Measurement of specific IgE antibody levels

WG-specific (s) IgE antibody levels were quantified using a highly sensitive ELISA method, as previously detailed with certain modifications (Gao et al., 2019; Jin et al., 2020; Jin et al., 2017; Jorgensen et al., 2023). Initially, 96-well Corning 3369 plates were coated with WG, and subsequently blocked using a 5% gelatin solution. After a thorough washing step, plasma samples were introduced onto the plate. Further washing ensued, followed by the addition of a biotin-conjugated anti-mouse IgE antibody. Subsequent washes were performed before introducing streptavidin alkaline phosphatase, and eventually, p-nitro-phenyl phosphate was added to enable quantification, mirroring established methodologies (Gao et al., 2019; Jin et al., 2017; Jorgensen et al., 2023).

6.5.8 Measurement of total plasma IgE concentration

Total IgE concentrations were determined utilizing an Invitrogen ELISA kit (Waltham, MA). Briefly, 96-well Corning Costar 9018 plates were coated with anti-mouse IgE capture antibodies with the subsequent addition of standards and plasma samples (recombinant mouse

IgE). Anti-mouse IgE was utilized as a secondary antibody followed by a detection system of streptavidin-HRP and TMB substrate as described by previous studies (Gao et al., 2019; Jin et al., 2017). The assay limit of detection is 4 ng/mL. 4-250 ng/mL was the standard range used for analysis.

6.5.9 Quantification of mucosal mast cell protease-1 (MMCP-1) level

Blood samples were collected one hour after the challenge and utilized for quantifying mucosal mast cell protease-1 (MMCP-1) levels in the plasma. This measurement was conducted using an ELISA-based approach developed by Invitrogen, consistent with previously outlined procedures (Gao et al., 2019; Jin et al., 2017). To elaborate, 96-well Corning Costar 9018 plates were initially coated with a capture antibody (anti-mouse MMCP-1). Subsequently, samples and standards (recombinant mouse MMCP-1) were introduced onto the plate. Biotin-conjugated anti-mouse MMCP-1 antibody was then added as the secondary antibody. Detection was accomplished through the utilization of an avidin-HRP/TMB substrate system. Notably, the assay possesses a limit of detection set at 120 pg/mL, and the range of standards spanned from 120 to 15000 pg/mL. Each individual mouse's plasma was subjected to testing in quadruplicate.

6.5.10 Spleen extract preparation and proteomic analysis of immune biomarkers

An hour following the challenge, mice were humanely euthanized, and their spleens were procured. The harvested spleens were promptly frozen in liquid nitrogen and preserved at -70°C. The tissue extraction process followed previously established procedures (Jin et al., 2020; Jorgensen et al., 2023). To elaborate, the spleen tissue was immersed in tissue protein extraction reagent (T-PER) buffer, containing protease inhibitor. For every 100 mg of tissue, a proportion

of 10 µL of protease inhibitor per 1 mL of T-PER buffer was utilized. Homogenization of the spleen tissue was achieved through ultrasonication, performed over two 30-second cycles with an intervening rest period of 5 minutes. After the second homogenization step, the samples were allowed to rest for 15 minutes and then subjected to centrifugation at 13500 x g at 4°C for 10 minutes. The resulting supernatant was meticulously collected and divided into aliquots for storage at -70°C. For quantification of immune markers, Quantibody microarray (CYT-4, 5, and 6, 120 marker panel in-volved in inflammation, immune regulation, and hypersensitivity) was employed (RayBiotech, Atlanta, GA), accessible at this link: https://www.raybiotech.com/mouse-cytokine-array-q2000/. This array allowed for the assessment of immune markers. The analysis was conducted in quadruplicate for each sample.

6.5.11 IgG1

WG-specific IgG1 antibody levels were measured using a modified ultrasensitive ELISA method as previously reported (Birmingham et al., 2003; Jorgensen et al., 2023). 96-well Corning 3369 plates were coated with WG and blocked with bovine serum albumin. The plate was then washed, and plasma samples were added. After a subsequent washing step, biotin-conjugated anti-mouse IgG antibodies were added, followed by additional washes. Streptavidin alkaline phosphatase was then added, and p-nitro-phenyl phosphate was used for quantification, as described previously (Birmingham et al., 2003; Jorgensen et al., 2023).

6.5.12 IgE cross-reactivity

Allergen-specific (s) IgE antibody levels were quantified using a highly sensitive ELISA method, as outlined previously with specific modifications (Gao et al. 2023; Jorgensen et al.

2023). Initially, 96-well Corning 3369 plates were coated with WG, bovine serum albumin, ovalbumin, peanut, or hazelnut, then subsequently blocked using a 5% gelatin solution. Following a thorough washing step, plasma samples were applied to the plates. Subsequent washes were performed before introducing a biotin-conjugated anti-mouse IgE antibody. After additional washes, streptavidin alkaline phosphatase, and eventually, p-nitro-phenyl phosphate was added to enable quantification, following established methodologies (Gao et al., 2023; Jorgensen et al., 2023).

6.5.13 Statistics

Pearson correlation coefficient calculation excel built-in program was used. The following formula was used to calculate *r*-scores:

$$r = \frac{\sum_i (x_i - \overline{x})(y_i - \overline{y})}{\sqrt{\sum_i (x_i - \overline{x})^2}\sqrt{\sum_i (y_i - \overline{y})^2}}$$

Using the *r* scores, and *n*-values significance was calculated with $p < 0.05$. An online software service was used in these analyses (https://www.socscistatistics.com/tests/) accessed on multiple days during July 1st 2023 to October 1st 2023). A student's t-test was used to compare two groups. The statistical significance level was set at $p < 0.05$.

6.6 Conclusions

This data reveals that WG is intrinsically allergenic, and that chronic skin exposure to WG can prime mice for potentially fatal anaphylaxis. Life-threatening anaphylaxis is associated

with differential expression of immune biomarkers involved in vascular permeability and allergic immune regulation compared to healthy control mice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms242417247/s1.

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CHAPTER 7 CREATION OF THE FIRST GLUTEN ALLERGENICITY MAP: INTRINSIC ALLERGENICITY OF DIPLOID, TETRAPLOID, AND HEXAPLOID WHEATS IN A MOUSE MODEL

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7.1 Abstract

Gluten allergy is a growing global disease affecting individuals with high risk of potentially deadly anaphylactic reactions. The relative intrinsic allergenicity of glutens from the three commercially grown wheat species (Diploid *Triticum monococcum*, tetraploid *Triticum durum*, hexaploid *Triticum aestivum*) is unknown. A gluten allergenicity map of these three species will enable the identification of potentially hyper/hypo/iso-allergenic species/varieties of wheat as well as in determination of substantial equivalence of genetically engineered novel wheat lines. Here, using a recently described adjuvant-free mouse model, we tested the hypothesis that the three different wheat species will exhibit natural variation in their gluten allergenicity. Groups of Balb/c mice were transdermally sensitized to alcohol-soluble or acid-soluble gluten extracts followed by elicitation of systemic anaphylaxis. Initial studies were performed to validate the model for glutens from the three wheat species. Both glutens from all three wheat species elicited robust sIgE responses, as well as systemic anaphylaxis. However, comparative mapping analysis revealed differences in capacity to elicit sIgE among the three wheat species with *T. aestivum* being the most potent in both gluten extracts. Hypothermic shock

response (HSR) analysis revealed that the three species elicited similar kinetics and intensity of anaphylaxis. Nevertheless, when analyzing Mucosal Mast Cell Response (MMCR), it was revealed that the glutens from *T. aestivum* (hexaploid) emerged as the most potent elicitor. Collectively, these results yield a comparative map of the natural variation in intrinsic sensitization and disease elicitation potencies of the three consumed wheat glutens for the first time. This map may be utilized for quantitative comparison the allergenic potential of glutens from novel wheats and processed wheat products against existing wheat glutens.

7.2 Introduction

The recognition of wheat as a common trigger for immune-mediated food allergies has become a significant global health concern, impacting both public health and food safety. Given the potential for life-threatening anaphylaxis, this presents a substantial health and financial burden (Cianferoni, 2016). A recent worldwide meta-analysis on wheat allergy revealed that 0.22% to 1.93% of individuals are affected by this condition (Liu et al., 2023). The prevalence varies across different regions, relying on self-reported physician-diagnosed wheat allergy (Liu et al., 2023). With no current cure for wheat allergy, individuals must adhere to strict elimination diets, leading to a compromised quality of life due to an increased risk of severe allergic reactions at any given time (Gupta et al., 2018).

Gluten constitutes a group of proteins present in cereal grains like wheat, barley, and rye. Traditionally, these glutens are categorized based on their solubility properties into two main groups: ethanol-soluble prolamin proteins (gliadins, comprising 30%–40% of total proteins) and weak acid-soluble proteins (glutenins, making up 45%–50% of total protein). The remaining soluble proteins in aqueous solutions are non-gluten proteins, specifically albumins and globulins

(Cabanillas, 2020; Jin et al. 2019). Gliadins function as individual proteins interacting through hydrogen bonds, predominantly featuring intramolecular disulfide bonds. In contrast, glutenins are polymeric proteins forming connections through both intermolecular and intramolecular disulfide bonds. Additionally, gliadins can be linked to the glutenin network through intermolecular disulfide bonds (Gao et al., 2021). Among the ethanol-soluble proteins, ω−1, 2, 5 gliadin, and α/β/γ-gliadins have undergone extensive characterization and are recognized for inducing allergic reactions in susceptible individuals (Cianferoni, 2016; Juhász et al., 2018). Within the acid-soluble fraction, both high-molecular weight (HMW) and low-molecular weight (LMW) glutenin subunits are able to bind IgE antibodies causing severe allergic reactions (Cianferoni, 2016; Juhász et al., 2018; Pastorello et al., 2007).

There is substantial evidence indicating that wheat gluten has the potential to trigger various immune-mediated diseases, including gluten hypersensitivity, celiac disease (CD), and non-celiac gluten sensitivity (NCGS) (Cabanillas, 2020). Among these, gluten hyper-sensitivity, also known as gluten food allergy or wheat food allergy, poses a threat to life (Cianferoni, 2016; Quirce, Boyano-Martínez, and Diáz-Perales, 2016; Ricci et al., 2019). In-appropriate activation of the immune system by wheat proteins, encompassing both gluten and non-gluten proteins, is responsible for hypersensitivity reactions to wheat (Cianferoni, 2016; Leonard & Vasagar, 2014). The immune mechanisms and clinical manifestations of gluten hypersensitivity differ significantly from those of celiac disease (CD) and non-celiac gluten sensitivities (NCGS). Gluten hypersensitivity is primarily attributed to the production of IgE antibodies against gluten during initial exposures, sensitizing mast cells. Subsequent encounters with gluten lead to mast cell and basophil degranulation, resulting in potentially life-threatening anaphylactic reactions (Gao et al., 2021; Rubin & Crowe, 2020). In contrast, gluten-induced CD is an autoimmune

chronic inflammatory disease that predominantly affects the small intestine; in some instances, gluten can also trigger celiac disease associated with dermatitis (referred to as dermatitis herpetiformis) and brain dysfunction (referred to as “hyper-excitable celiac brain”) (Croall et al., 2020; Kaunisto et al., 2022; Pennisi et al., 2017). NCGS manifests clinically as a chronic digestive disorder with unknown mechanisms, although activation of the innate immune system is implicated (Cárdenas-Torres et al., 2021).

Genetically, modern wheats trace their origins to three genomes - A, B, and D (Juhász et al., 2018). The commonly consumed wheats fall into three categories: diploid (AA; for instance, Einkorn), tetraploid (AABB; such as durum), or hexaploid (AABBDD; exemplified by bread wheat) (Appels et al., 2018; Juhász et al., 2018; Shewry, 2018). Hexaploid and tetraploid wheat encompass numerous species, each hosting hundreds and thousands of varieties and accessions (Gao et al., 2021). Given that allergenic protein content and structure are genetically determined, variations in wheat allergenicity can be anticipated at the ploidy level, species level, and even at the variety/accession levels (Gao et al., 2019; Nakamura et al., 2005). Currently, it is assumed that gluten from all wheat species have equal allergenic potential, however there is no data to justify this assumption. This was the rationale for undertaking this study.

There is extensive evidence in the literature that industrial and food processing of glutens has potential to alter their allergenicity (Gao et al., 2021). A standardized comparative map of intrinsic allergenicity of native glutens from various species will be valuable to evaluate the impact of processing methods on gluten allergenicity. Such a map is unavailable at present - this was the focus of the present study.

Currently, genetically modified (GM) or genetically engineered (GE) wheat is not commercially available. However, efforts are underway to develop these and get approval from

regulatory bodies such as USFDA (Ning et al., 2022). A major concern regarding the safety of such GM/GE wheats would be clear elucidation of the allergenicity potential of glutens from such wheats. In aiding the safety assessment of GM foods, international regulatory and health agencies, such as FAO/WHO, have introduced the 'substantial equivalence' concept as a general guideline (FAO, 2001; Selgrade et al., 2009). This concept serves as a tool to evaluate the allergenicity of GM foods, including GM/GE wheats, by comparing them with their conventional non-GM counterparts (Domingo, 2016; Hollingworth et al., 2003; Selgrade et al., 2009). Although FAO/WHO recommends the use of animal models in assessing *in vivo* allergenicity of GM foods, however there is currently no validated model available for testing allergenic potential of glutens from GM/GE wheat (FAO, 2001).

The literature describes various animal models for gluten hypersensitivity, encompassing, dogs, rats, guinea pigs, and mice (Adachi et al., 2012; Ballegaard, Madsen, and Bøgh, 2019; Buchanan et al., 1997; Frick et al., 2005; Kohno et al., 2016; Kroghsbo et al., 2014; Yamada et al., 2019). In general, these models can be classified into two types: i) adjuvant-based models; and ii) adjuvant-free models. Adjuvant-based models have been more prominent because historically, they were the first models to be developed and they provide robust readouts of immune and allergenicity markers. However, they do not have the power to decipher inherent allergenicity potential of glutens due to enhancement of the immune response by the adjuvants that are co-administered with gluten. In contrast, the recently reported adjuvant-free models provide a unique opportunity to evaluate the intrinsic allergenicity potential of glutens from various wheat species.

In this study, we tested the hypothesis that the three different commonly cultivated wheat species (*Triticum monococcum* AA genome, *Triticum aestivum* AABBDD genome, *Triticum*

durum AABB genome) will exhibit natural variations in their gluten allergenicity. The objectives of this study were as follows: i) to validate the transdermal sensitization and systemic elicitation of disease model using alcohol-soluble gluten extract from *Triticum monococcum,* and *Triticum aestivum*; ii) to develop comparative maps of intrinsic sensitization and disease elicitation maps of alcohol-soluble gluten extracts from three wheat species *Triticum durum*, *Triticum monococcum* and *Triticum aestivum*; iii) to validate the trans-dermal sensitization and systemic elicitation of disease model using acid-soluble gluten extract from *Triticum monococcum,* and *Triticum durum*; and iv) to develop comparative maps of intrinsic sensitization and disease elicitation maps of acid-soluble gluten extracts from three wheat species *Triticum durum*, *Triticum monococcum* and *Triticum aestivum.*

Collectively, these results yield a comparative map of the natural variation in intrinsic sensitization and disease elicitation potencies of the two types of glutens from three commonly consumed wheat species for the first time. This comparative map may be utilized for the assessment of the allergenic potential of glutens from novel wheats such as GM/GE wheats, and differently processed wheat products.

7.3 Results

7.3.1 Validation of the Transdermal Sensitization and Systemic Elicitation of Disease Mouse Model for *Triticum monococcum* Using Alcohol-Soluble Gluten Extract

7.3.1.1 Transdermal Exposure to Alcohol-Soluble Gluten Extract from *T. monococcum* Elicits Robust Specific-IgE Antibody Response in Balb/c Mice

Groups of Balb/c female mice were allocated and exposed to alcohol-soluble gluten extract from *Triticum monococcum* (genome AA) or vehicle via transdermal application, following a repeated weekly exposure regimen as per the Methods section. Blood samples collected before the first exposure (pre) and after the sixth exposure (6R) were subjected to analysis for sIgE levels. Notably, a significant rise in sIgE antibody levels were observed following transdermal exposure to alcohol-soluble gluten extract from *T. monococcum* in allergic mice compared to the vehicle control mice, as illustrated in Figure 7.1A and B.

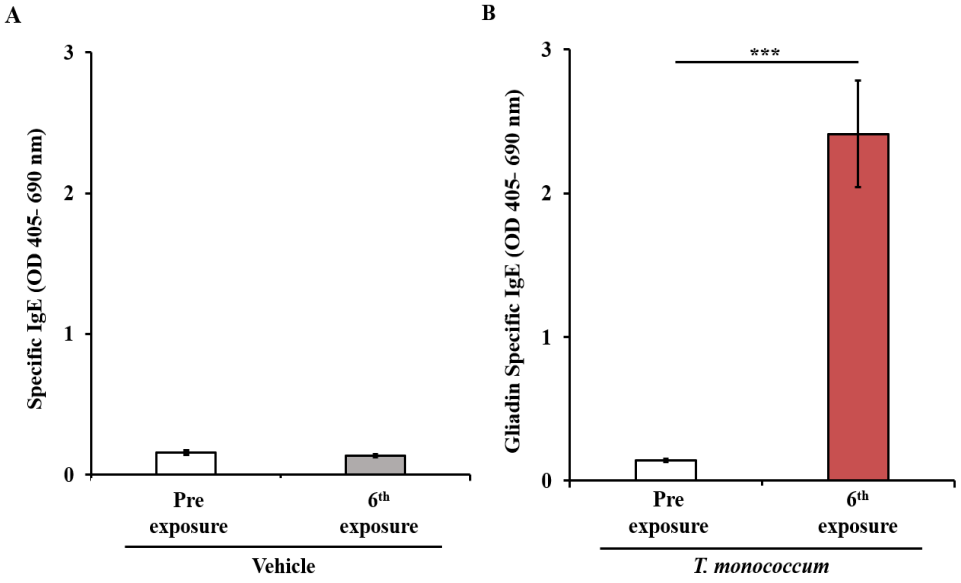

Figure 7.1 Transdermal exposure of Balb/c mice to alcohol-soluble gluten extract from *Triticum monococcum* (genome AA) elicited robust specific (s) IgE antibody responses.

Mice were exposed to alcohol-soluble gluten extract from *T. monococcum*, or vehicle as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 6th expo-sure

(6R) was used in the measurement of sIgE levels (OD 405–690 nm), with a sample size of n = 10/group. (A) sIgE levels in control mice. (B) sIgE levels in sensitized mice. *** $p < 0.001$, student's t-test. Ab: antibody; n: number of mice; OD: optical density.

7.3.1.2 Systemic Challenge with *T. monococcum* Alcohol-soluble Gluten Extract Elicits Hypothermic Shock Responses in Skin-Sensitized Mice

Parallel groups of skin-sensitized mice were employed to induce anaphylaxis through systemic challenge with *Triticum monococcum* alcohol-soluble gluten extract (0.5 mg/mouse) or vehicle. The quantification of anaphylactic reactions was performed by assessing hypothermic shock reactions (HSR) using rectal thermometry, as detailed in the methods. No HSR was observed upon vehicle (i.e., zero allergen) or alcohol-soluble gluten extract challenge in control mice (Figure 7.2 A-B). In contrast, acute HSRs were evident upon systemic allergen challenge in sensitized mice (Figure 7.2 C-D). Significant HSRs were noted from 5 to 30 minutes post-systemic allergen challenge, as indicated by ANOVA analysis ($p < 0.05$).

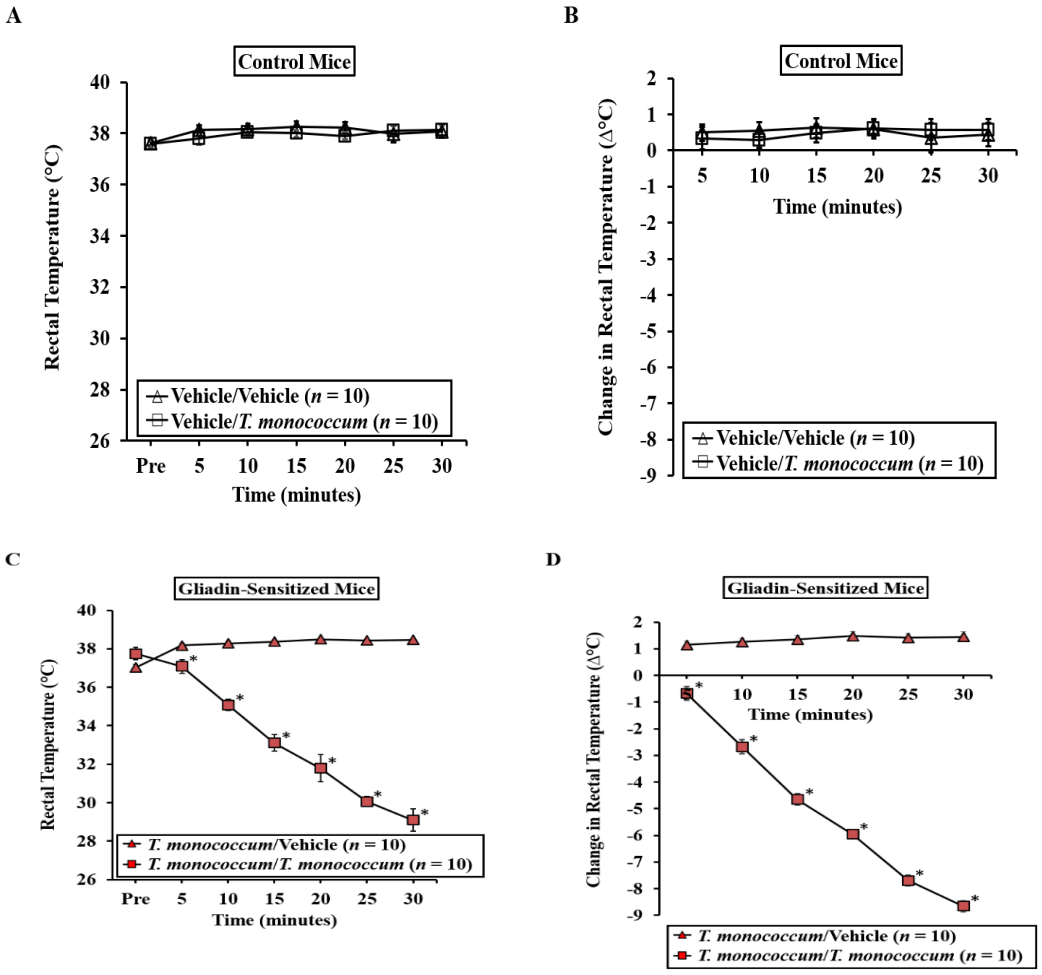

Figure 7.2 Transdermal sensitization with alcohol-soluble gluten extract from *Triticum monococcum* is sufficient for eliciting systemic anaphylaxis in Balb/c mice.

Mice were sensitized and systemically challenged with alcohol-soluble gluten extract from *T. monococcum* or vehicle, as described in Materials and Methods. *n* = 10/group. (**A**) Actual rectal temperature at indicated time points in control mice challenged with alcohol-soluble gluten extract from *T. monococcum* or vehicle. (**B**) Change in rectal temperature at indicated time points in control mice challenged with alcohol-soluble gluten extract from *T. monococcum* or vehicle. (**C**) Actual rectal temperature at indicated time points in alcohol soluble *T. monococcum* protein extract-sensitized mice challenged with alcohol-soluble *T. monococcum* protein extract or vehicle. (**D**) Change in rectal temperature at indicated time points in alcohol-soluble gluten

extract from *T. monococcum*-sensitized mice challenged with alcohol-soluble gluten extract from *T. monococcum* or vehicle. *n*: number of mice.

7.3.1.3 Balb/c Mice Exhibit Strong Mucosal Mast Cell Response (MMCR) in Response to alcohol- soluble *T. monococcum* induced systemic anaphylaxis

Figure 7.3A and B depict the MMCP-1 response in both control mice and allergic mice. The systemic challenge with *T. monococcum* alcohol-soluble gluten extract, as opposed to the vehicle, leads to a significant elevation in MMCP-1 levels.

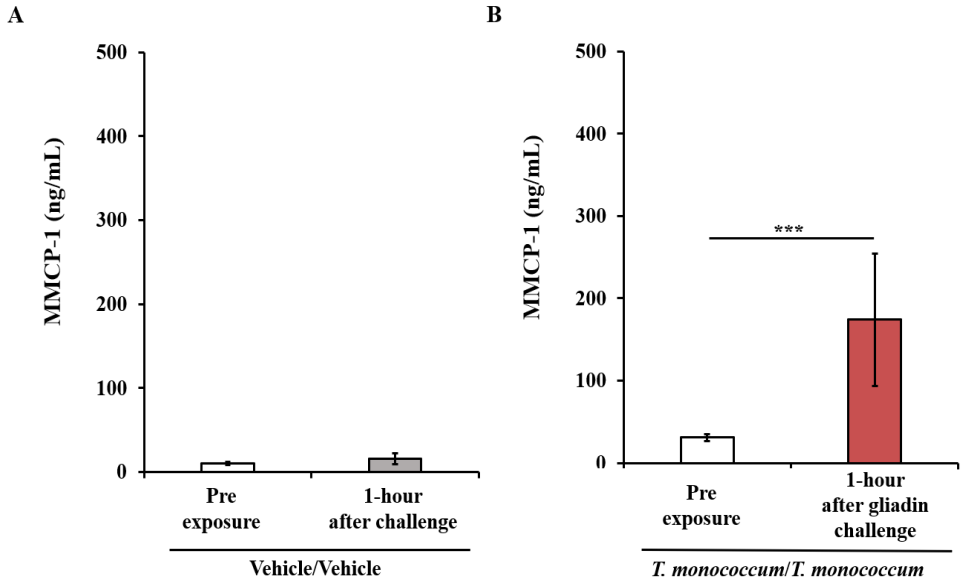

Figure 7.3 Systemic challenge with alcohol-soluble gluten extract from *T. monococcum* elicits a robust mucosal mast cell response (MMCR) in Balb/c mice.

Mice were sensitized and systemically challenged with alcohol-soluble gluten extract from *T. monococcum* or vehicle, as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 (ng/mL) in pre and one-hour after challenge were measured via ELISA. *n* =10/group. (A). MMCP-1 levels in control mice challenged with vehicle. (B) MMCP-1 levels in allergic mice challenged with alcohol-soluble gluten extract from *T. monococcum*. *** $p < 0.001$, student's t-test; *n*: number of mice.

7.3.2 Validation of the Transdermal Sensitization and Systemic Elicitation of Disease Mouse Model for *Triticum aestivum* Using Alcohol-soluble Gluten Extract

7.3.2.1 Transdermal Exposure to Alcohol-Soluble Gluten Extract from *T. aestivum* Elicits Robust Specific-IgE Antibody Response in Balb/c Mice

Balb/c female mice were grouped and subjected to transdermal exposure to alcohol-soluble gluten extract from *Triticum aestivum* (genome AABBDD) or vehicle following repeated weekly exposure regime outline in the Methods section. Blood samples obtained before the initial exposure (pre) and after the sixth skin exposure (6R) were analyzed for sIgE levels. Remarkably, a significant elevation in sIgE antibody levels were noted after transdermal exposure to alcohol-soluble gluten extract from *T. aestivum*, indicating an increase in allergic mice compared to the vehicle control mice, as depicted in Figure 7.4A and B.

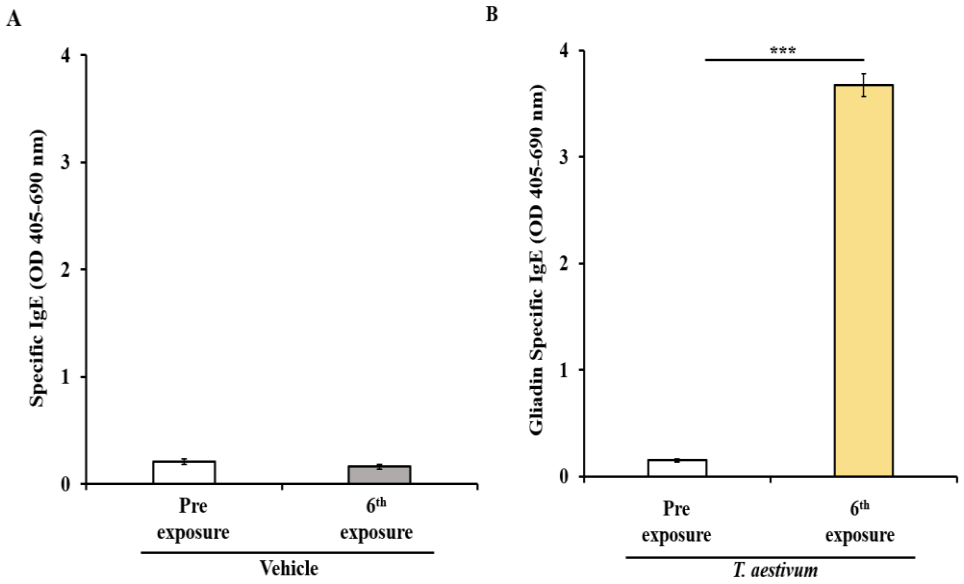

Figure 7.4 Transdermal exposure of Balb/c mice to alcohol-soluble gluten extract from *Triticum aestivum* (genome AABBDD) elicited robust specific (s) IgE antibody responses.

Mice were exposed to alcohol-soluble gluten extract from *Triticum aestivum* or vehicle as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 6th exposure (6R) was used in the measurement of sIgE levels (OD 405–690 nm), with a sample size of *n* =

10/group. (**A**) sIgE levels in control mice. (**B**) sIgE levels in sensitized mice. *** $p < 0.001$, student's t-test. Ab: antibody; n: number of mice; OD: optical density.

7.3.2.2 Systemic Challenge with *T. aestivum* Alcohol-soluble Gluten Extract Elicits Hypothermic Shock Responses in Skin-Sensitized Mice

Groups of skin-sensitized mice were utilized to induce anaphylaxis through systemic challenge with *T. aestivum* alcohol-soluble gluten extract (0.5 mg/mouse) or vehicle. The quantification of anaphylactic reactions was conducted by assessing hypothermic shock reactions (HSR) using rectal thermometry, as outlined in the methods. No HSR was observed in control mice upon vehicle (i.e., zero allergen) or alcohol-soluble gluten extract challenge (Figure 7.5 A-B). Conversely, acute HSRs were evident upon systemic allergen challenge in sensitized mice (Figure 7.5 C-D). Significant HSRs were noted from 5 to 30 minutes post-systemic allergen challenge, as indicated by ANOVA analysis ($p < 0.05$).

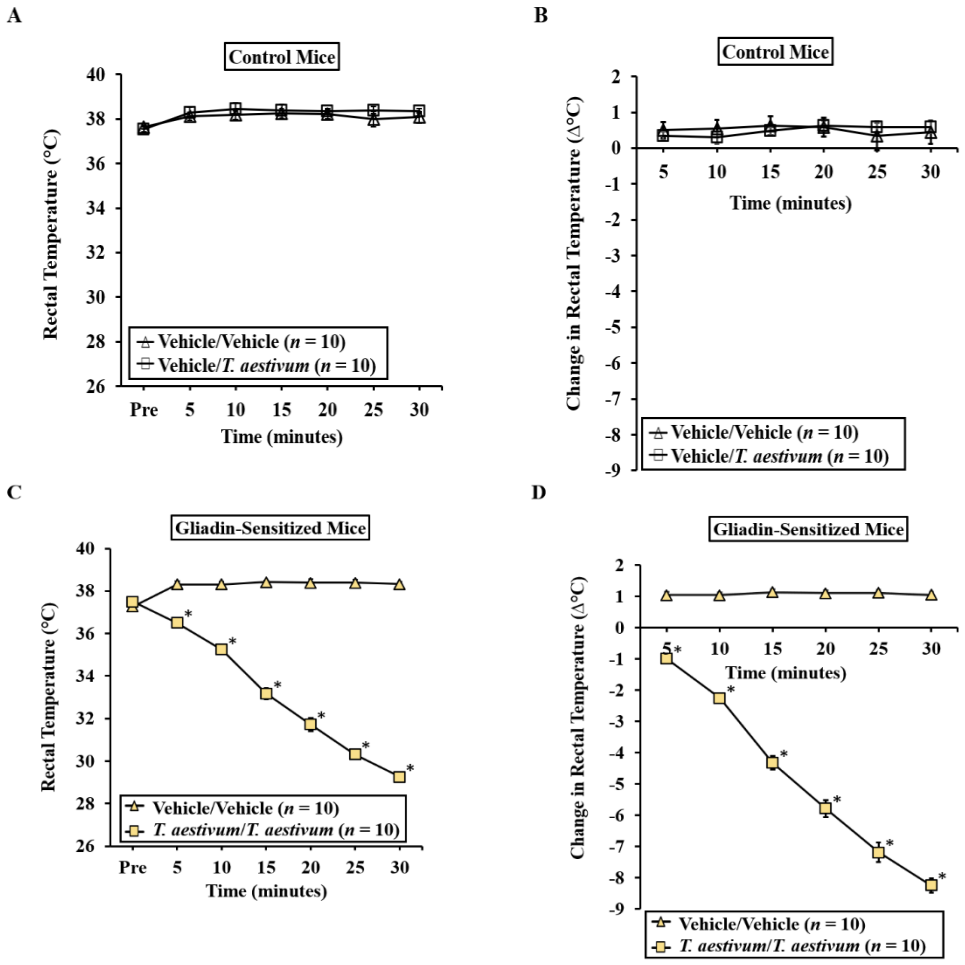

Figure 7.5 Transdermal sensitization with alcohol-soluble gluten extract from *Triticum aestivum* is sufficient for eliciting systemic anaphylaxis in Balb/c mice.

Mice were sensitized and systemically challenged with alcohol-soluble gluten extract from *Triticum aestivum* or vehicle, as described in Materials and Methods. *n* = 10/group. (**A**) Actual rectal temperature at indicated time points in control mice challenged with alcohol-soluble gluten extract from *Triticum aestivum* or vehicle. (**B**) Change in rectal temperature at indicated time points in control mice challenged with alcohol-soluble gluten extract from *Triticum aestivum* or vehicle. (**C**) Actual rectal temperature at indicated time points in alcohol-soluble gluten extract from *Triticum aestivum* sensitized mice challenged with alcohol-soluble gluten extract from *Triticum aestivum* or vehicle. (D) Change in rectal temperature at indicated

time points in alcohol-soluble gluten extract from *Triticum aestivum* sensitized mice challenged with alcohol-soluble gluten extract from *Triticum aestivum* or vehicle. *n*: number of mice.

7.3.2.3 Balb/c Mice Exhibit Strong Mucosal Mast Cell Response (MMCR) in Response to Alcohol-Soluble Gluten Extract from *Triticum aestivum* Induced Systemic Anaphylaxis

Figures 7.6A and B illustrate the MMCP-1 response in both control mice and allergic mice. It is evident that systemic challenge with *T. aestivum* alcohol-soluble gluten extract, in contrast to vehicle, results in a significant increase in MMCR in the blood.

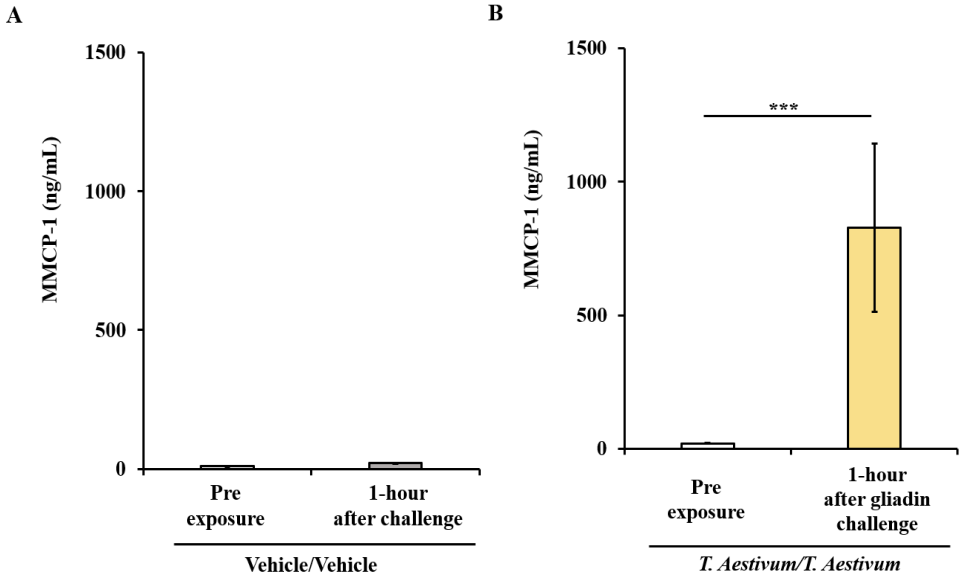

Figure 7.6 Systemic challenge with alcohol-soluble gluten extract from *Triticum aestivum* elicits a robust mucosal mast cell response (MMCR) in Balb/c mice.

Mice were sensitized and systemically challenged with alcohol-soluble gluten extract from *Triticum aestivum* or vehicle, as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 (ng/mL) in pre and one-hour after challenge were measured via ELISA. *n* = 10/group. (**A**) MMCP-1 levels in control mice challenged with vehicle. (**B**) MMCP-1 levels in allergic mice were challenged with alcohol-soluble gluten extract from *Triticum aestivum*. ***$p < 0.001$, student's t-test; *n* = number of mice.

7.3.3.1 Mapping the Inherent Allergenicity and Sensitization Potential of Alcohol-Soluble Gluten Extracts Across Diploid, Tetraploid, and Hexaploid Wheat Species

Utilizing the sIgE data from the validation studies mentioned earlier, and our previously reported durum wheat study (Jorgensen et al., 2023) we constructed a comparative sensitization map. The levels of sIgE antibodies induced by each wheat species were calculated by subtracting the baseline (pre) sIgE levels from the sIgE levels observed after the 6th response (6R). The resulting map illustrates the intrinsic allergenicity and sensitization potential of the three genetically distinct wheat, as depicted in Figure 7.7. Notably, *T. durum* and *T. monococcum* alcohol-soluble gluten extracts yielded nearly identical sIgE levels, while *T. aestivum* elicited significantly higher sIgE levels.

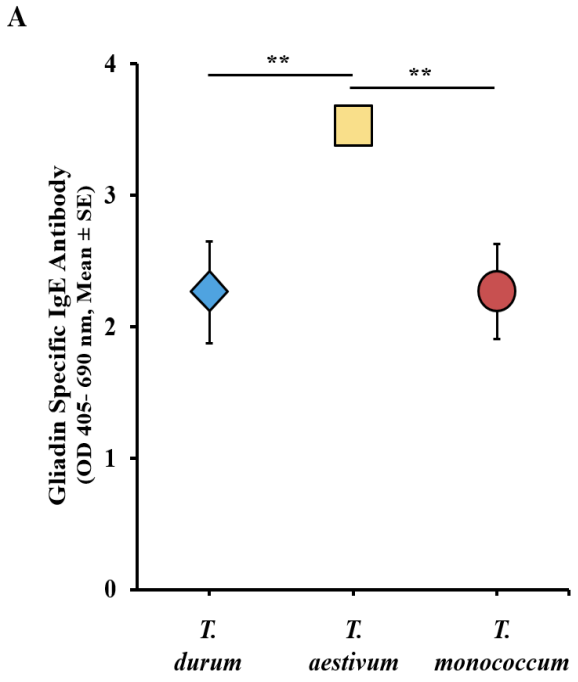

Figure 7.7 Comparative map of the intrinsic sensitization potentials of wheats from diploid (*Triticum monococcum*), tetraploid (*Triticum durum*), and hexaploid wheats (*Triticum aestivum*).

The change in gliadin-specific IgE antibody levels after the 6th transdermal exposure to alcohol-soluble gluten extracts from the respective depicted wheat species. ** $p < 0.01$, one-way ANOVA and Tukey's post hoc test. $n = 10$/group. *n*: number of mice.

7.3.3.2 Comparative Map of the Intrinsic Allergenicity Disease Elicitation Potential of Alcohol-Soluble Protein Extracts from the Diploid, Tetraploid, and Hexaploid Wheats

We utilized the absolute changes in rectal temperature data resulting from systemic challenges in the validation studies of *T. durum*, *T. aestivum*, and *T. monococcum*, alongside our previously reported durum wheat study (Jorgensen et al., 2023) to create a comparative disease elicitation map. Figures 7.8A through D display the disease elicitation potential map of the three species after 15, 20, 25, and 30 minutes respectively. As evident, the three wheat species have similar reaction kinetics until the 30-minute mark, where *T. monococcum* is significantly decreased compared to *T. durum* (Figure 7.8D).

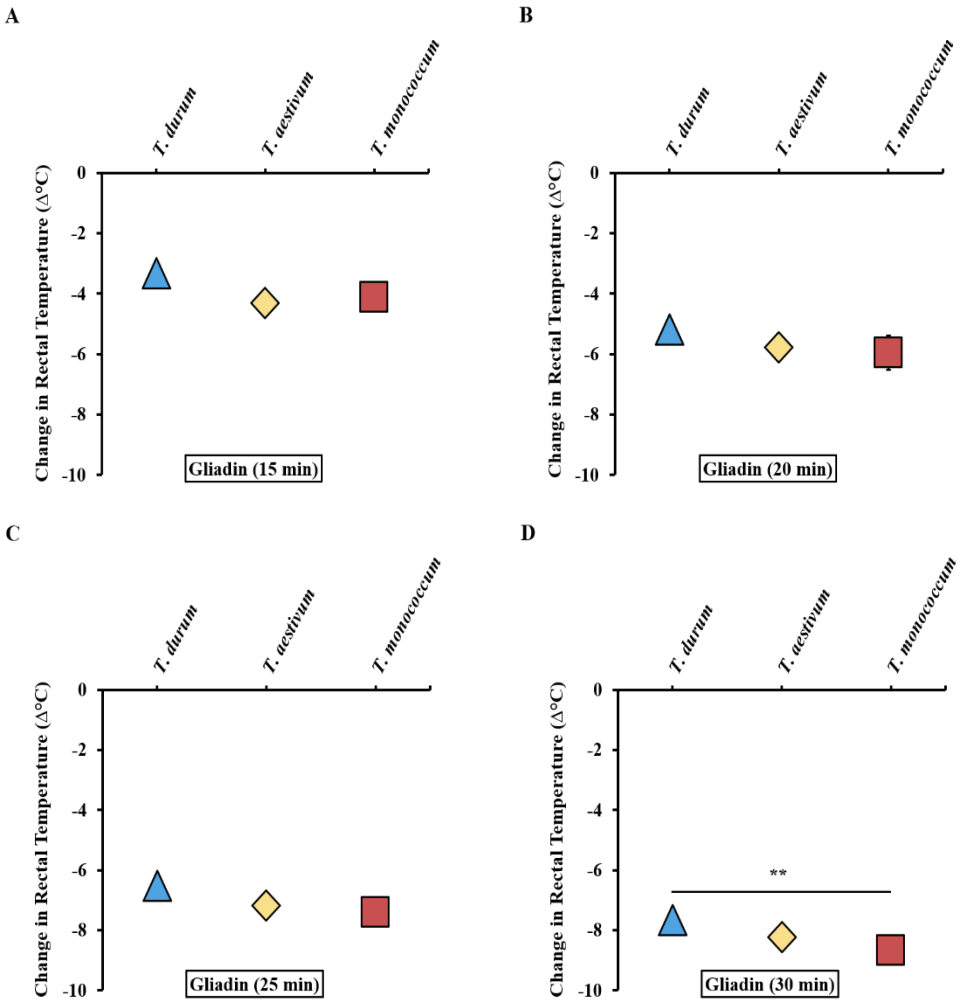

Figure 7.8 Comparative map of the intrinsic allergenicity disease elicitation potential of alcohol-soluble gluten extracts from tetraploid, hexaploid, and diploid wheat species.

(**A,B**) HSRs at 15 and 20 min after systemic challenge with 0.5 mg of *Triticum durum*, *Triticum aestivum*, and *Triticum monococcum* respectively. (**C,D**) HSRs at 25 and 30 min after systemic challenge with 0.5 mg of *Triticum durum*, *Triticum aestivum*, and *Triticum monococcum* respectively. ** $p < 0.05$, one-way ANOVA and Tukey's post hoc test. *n* = 10/group. *n*: number of mice.

7.3.3.3 Comparative Map of the Mucosal Mast Cell Response (MMCR) Elicitation Potential of Alcohol-Soluble Gluten Extracts from the Diploid, Tetraploid, and Hexaploid Wheats

We utilized the MMCP-1 data obtain from systemic allergen challenges in the validation studies of *T. durum*, *T. monococcum*, and *T. aestivum* wheats, along with previously reported durum wheat studies (Jorgensen et al., 2023) to construct a comparative Mast Cell Degranulation (MMCR) elicitation potential map. Figure 7.9 presents the MMCR elicitation potential map at 0.5 mg systemic allergen challenge. It is apparent that *T. aestivum* induced the highest MMCR followed by *T. durum* and *T. monococcum*.

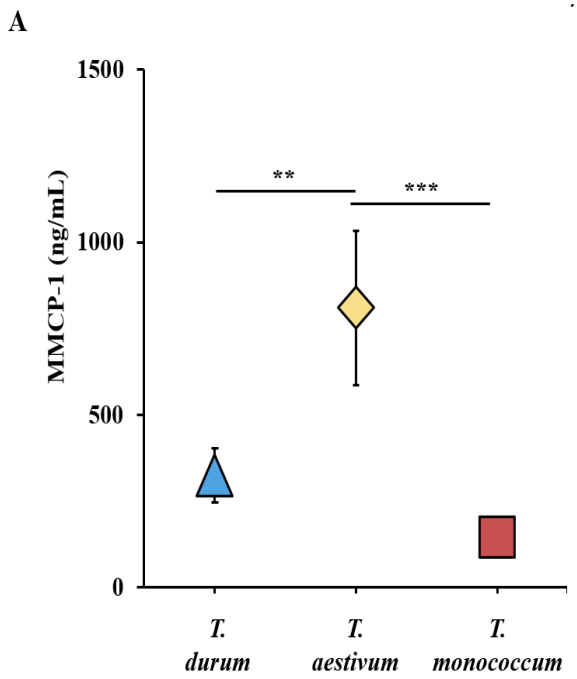

Figure 7.9 Comparative map of the mucosal mast cell response (MMCR) elicitation potential of alcohol-soluble gluten extracts from diploid, tetraploid, and hexaploid wheats.

Average MMCP-1 blood level after 0.5 mg systemic allergen challenge dose. ** $p < 0.05$, *** $p < 0.005$, one-way ANOVA and Tukey's post hoc tests. $n = 10$/group. MMCP-1: mucosal mast cell

7.3.4 Validation of the Transdermal Sensitization and Systemic Elicitation of Disease Mouse Model for *Triticum durum* Using Acid-soluble Gluten Extract

7.3.4.1 Transdermal Exposure to Acid-soluble gluten extract from *T. durum* Elicits Robust Specific-IgE Antibody Response in Balb/c Mice

Balb/c female mice were grouped and exposed to transdermal application of acid-soluble gluten extract from *Triticum durum* (genome AABB) or vehicle, following a repeated weekly exposure regimen as outlined in the specified methods. Blood samples collected before the initial exposure (pre) and after the sixth skin exposure (6R) were analyzed for specific (s) IgE levels. Significantly, there was a substantial elevation in sIgE antibody levels after transdermal exposure to acid-soluble gluten extract from *T. durum*, indicating an increase in allergic mice compared to the vehicle control mice, as depicted in Figure 7.10A and B.

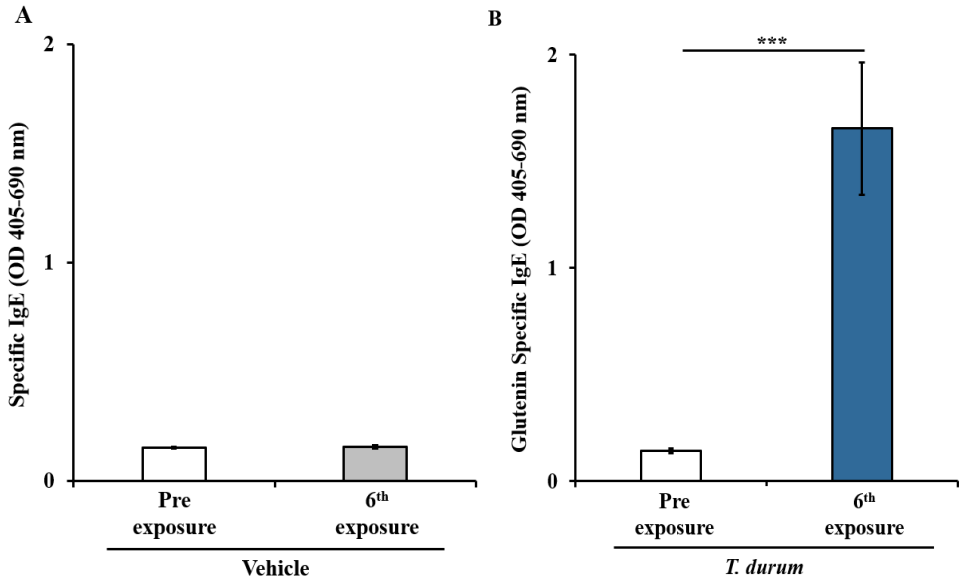

Figure 7.10 Transdermal exposure of Balb/c mice to acid-soluble gluten extract from *T. durum* (genome AABB) elicited robust specific (s) IgE antibody responses.

Mice were exposed to acid-soluble gluten extract from *Triticum durum* or vehicle as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 6th exposure (6R) was used in the measurement of sIgE levels (OD 405–690 nm), with a sample size of n = 10/group.

(**A**) sIgE levels in control mice. (**B**) sIgE levels in sensitized mice. *** $p < 0.001$, student's t-test. Ab: antibody; *n*: number of mice; OD: optical density.

7.3.4.2 Systemic Challenge with *T. durum* Acid-soluble gluten extract Elicits Hypothermic Shock Responses in Skin-Sensitized Mice

Groups of skin-sensitized mice were employed to induce anaphylaxis through systemic challenge with *T. durum* acid-soluble gluten extract (0.5 mg/mouse) or vehicle. Anaphylactic reactions were quantified using hypothermic shock reactions (HSR) and monitored with rectal thermometry, as detailed in the methods. No HSR was observed in control mice upon vehicle (i.e., zero allergen) or acid-soluble gluten extract challenge (Figure 7.11 A-B). In contrast, acute HSRs were evident upon systemic allergen challenge in sensitized mice (Figure 7.11 C-D). Significant HSRs were noted from 5 to 30 minutes post-systemic allergen challenge, as indicated by ANOVA analysis ($p < 0.05$).

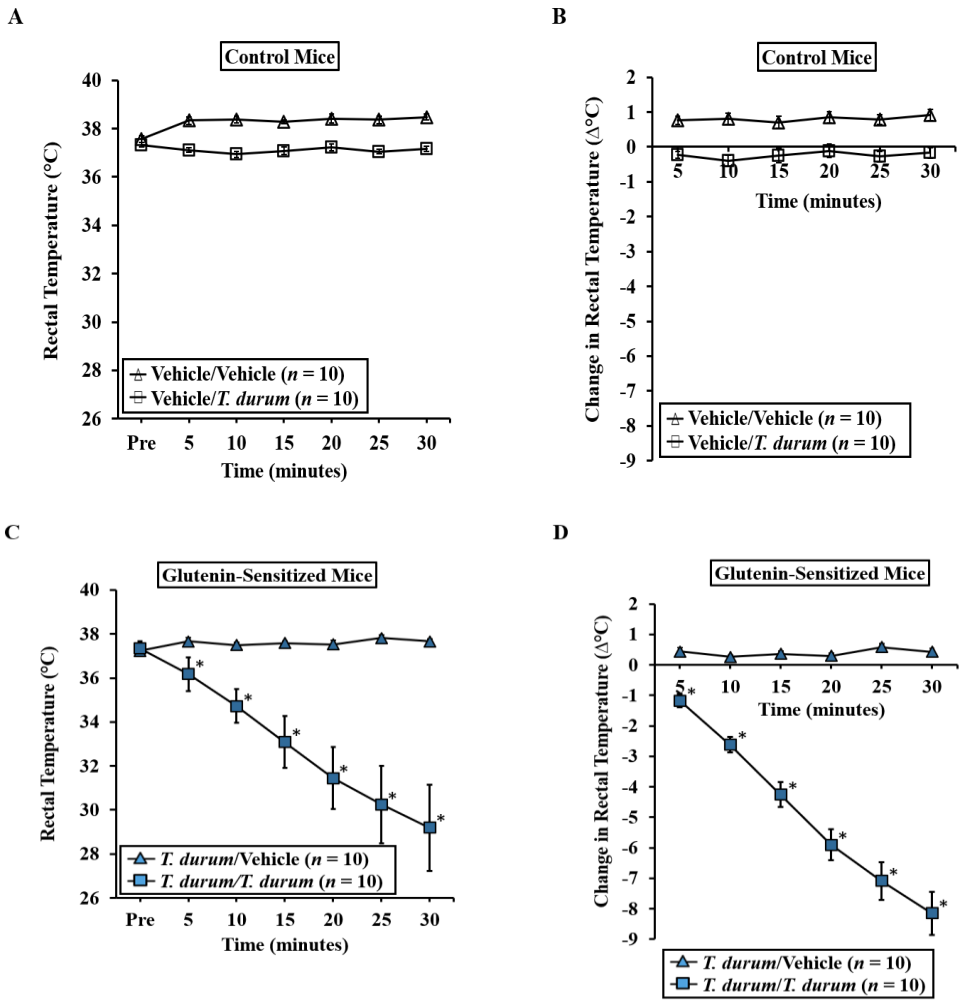

Figure 7.11 Transdermal sensitization with acid-soluble gluten extract from *Triticum durum* is sufficient for eliciting systemic anaphylaxis in Balb/c mice.

Mice were sensitized and systemically challenged with acid-soluble gluten extract from *Triticum durum* or vehicle, as described in Materials and Methods. *n* = 10/group. (**A**) Actual rectal temperature at indicated time points in control mice challenged with acid-soluble gluten extract from *Triticum durum* or vehicle. (**B**) Change in rectal temperature at indicated time points in control mice challenged with acid-soluble gluten extract from *Triticum durum* or vehicle. (**C**) Actual rectal temperature at indicated time points in acid-soluble gluten extract from *Triticum durum* sensitized mice challenged with acid-soluble gluten extract from *Triticum durum* or vehicle. (**D**) Change in rectal temperature at indicated time points in acid-soluble *Triticum durum*

protein extract sensitized mice challenged with acid-soluble *Triticum durum* protein extract or vehicle. *n*: number of mice.

7.3.4.3 Balb/c Mice Exhibit Strong Mucosal Mast Cell Response (MMCR) in Response to Acid-soluble *T. durum induced* systemic anaphylaxis

Figure 7.12A and B illustrate the MMCP-1 responses in both control mice and allergic mice. It is evident that the systemic challenge with *T. durum* acid-soluble gluten extract, as opposed to vehicle, results in a significant increase in MMCP-1 levels in the blood, as depicted in Figure 7.12A and B.

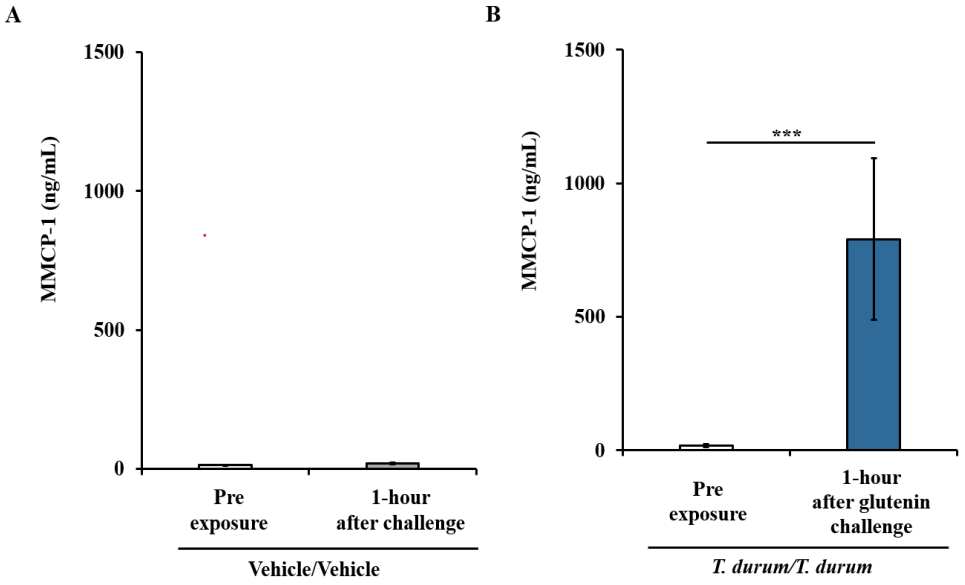

Figure 7.12 Systemic challenge with *Triticum durum* acid-soluble gluten extract elicits a robust mucosal mast cell response (MMCR) in Balb/c mice.

Mice were sensitized and systemically challenged with acid-soluble *T. durum* protein extract or vehicle, as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 (ng/mL) in pre and one-hour after challenge were measured via ELISA. *n* = 10/group. (A). MMCP-1 levels in control mice challenged with vehicle. (B) MMCP-1 levels in

allergic mice challenged with acid-soluble *T. durum* protein extract. ***$p < 0.05$, student's t-test; *n*: number of mice.

7.3.5 Validation of the Transdermal Sensitization and Systemic Elicitation of Disease Mouse Model for *Triticum monococcum* Using Acid-soluble gluten extract

7.3.5.1 Transdermal Exposure to Acid-soluble Gluten Extract from *T. monococcum* Elicits Robust Specific-IgE Antibody Response in Balb/c Mice

Balb/c female mice were assigned to groups and subjected to transdermal exposure to acid-soluble gluten extract from *Triticum monococcum* (genome AA) or vehicle, following a repeated weekly exposure regimen as outlined in the Methods section. Blood samples obtained before the initial exposure (pre) and after the sixth exposure (6R) were analyzed for specific (s) IgE levels. A significant elevation in sIgE antibody levels was observed following transdermal exposure to acid-soluble gluten extract from *Triticum monococcum*, indicating an increase in allergic mice compared to the vehicle control mice, as illustrated in Figure 7.13A and B.

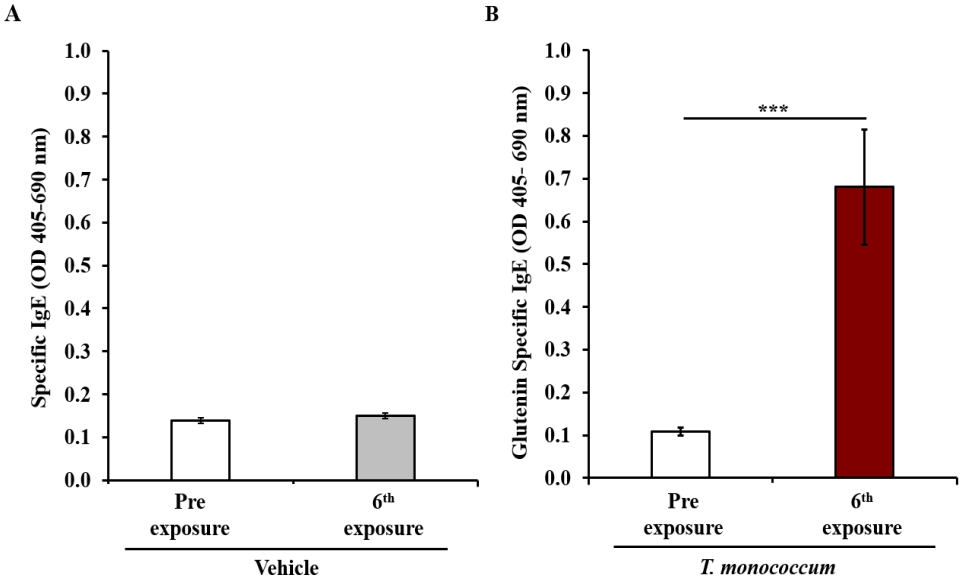

Figure 7.13 Transdermal exposure of Balb/c mice to acid-soluble gluten extract from *Triticum monococcum* (genome AA) elicited robust specific (s) IgE antibody responses.

Mice were exposed to acid-soluble gluten extract from *Triticum monococcum* or vehicle as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 6th exposure (6R) was used in the measurement of sIgE levels (OD 405–690 nm). n = 10/group. (**A**) sIgE levels in control mice. (**B**) sIgE levels in sensitized mice. *** $p < 0.001$, student's t-test. Ab: antibody; *n*: number of mice; OD: optical density.

7.3.5.2 Systemic Challenge with *T. monococcum* Acid-soluble Gluten Extract Elicits Hypothermic Shock Responses in Skin-Sensitized Mice

Parallel groups of skin-sensitized mice were utilized to induce anaphylaxis through systemic challenge with *Triticum monococcum* acid-soluble gluten extract (0.5 mg/mouse) or vehicle. The quantification of anaphylactic reactions was conducted by assessing hypothermic shock reactions (HSR) using rectal thermometry, as detailed in the methods. No HSR was observed upon vehicle (i.e., zero allergen) or acid-soluble gluten extract challenge in control mice (Figure 7.14 A-B). In contrast, acute HSRs were evident upon systemic allergen challenge in sensitized mice (Figure 7.14 C-D). Significant HSRs were noted from 10 to 30 minutes post-systemic allergen challenge, as indicated by ANOVA analysis ($p < 0.05$).

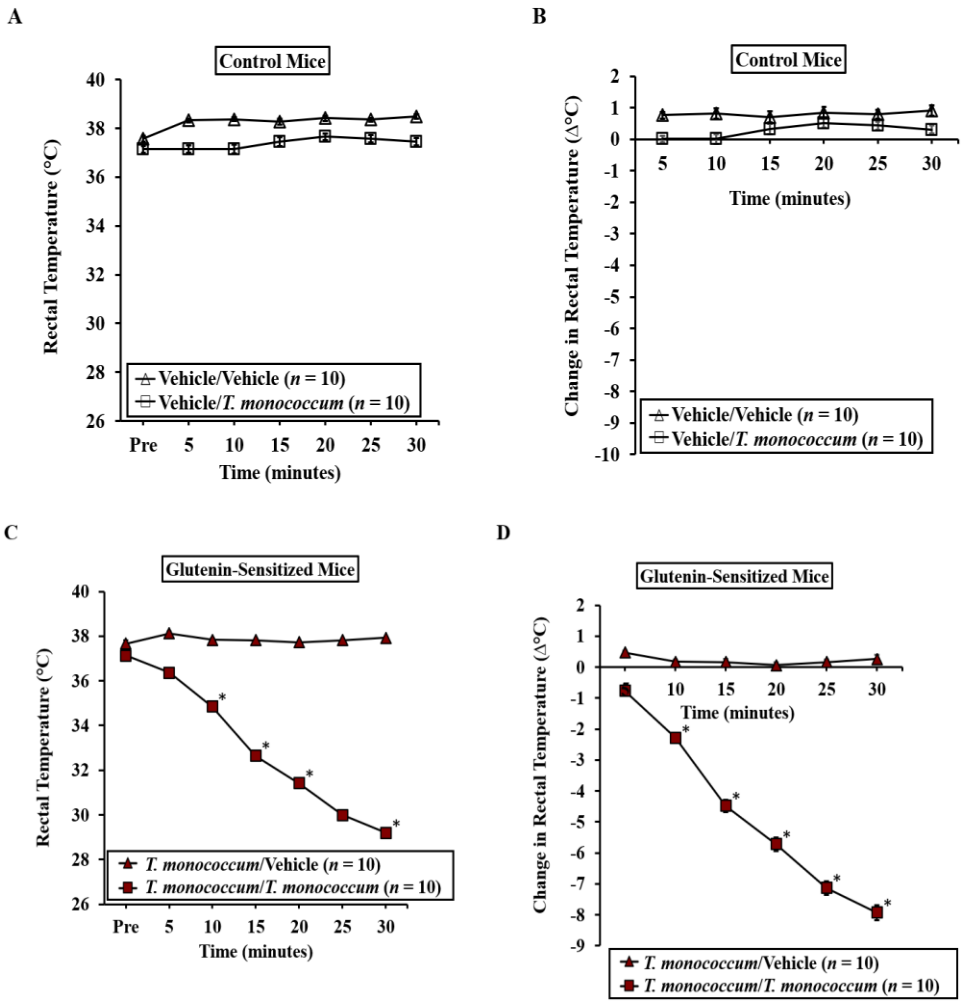

Figure 7.14 Transdermal sensitization with acid-soluble gluten extract from *Triticum monococcum* is sufficient for eliciting systemic anaphylaxis in Balb/c mice.

Mice were sensitized and systemically challenged acid-soluble gluten extract from *Triticum monococcum* or vehicle, as described in Materials and Methods. *n* = 10/group. (**A**) Actual rectal temperature at indicated time points in control mice challenged with acid-soluble gluten extract from *Triticum monococcum* or vehicle. (**B**) Change in rectal temperature at indicated time points in control mice challenged acid-soluble gluten extract from *Triticum monococcum* or vehicle. (**C**) Actual rectal temperature at indicated time points in acid-soluble gluten extract from *Triticum monococcum* sensitized mice challenged with acid-soluble gluten extract from *Triticum monococcum* or vehicle. (**D**) Change in rectal temperature at indicated time points in acid-soluble

gluten extract from *Triticum monococcum* sensitized mice challenged with acid-soluble gluten extract from *Triticum monococcum* or vehicle. *n*: number of mice.

7.3.5.3 Balb/c Mice Exhibit Strong Mucosal Mast Cell Response (MMCR) in Response to Acid-soluble *T. monococcum* Induced Systemic Anaphylaxis

Figures 7.15A and 15B illustrate the MMCP-1 responses in both control mice and allergic mice. Upon systemic challenge with *T. monococcum* acid-soluble gluten extract, there is a notable increase in MMCP-1 levels compared to the vehicle, indicating a significant immune response.

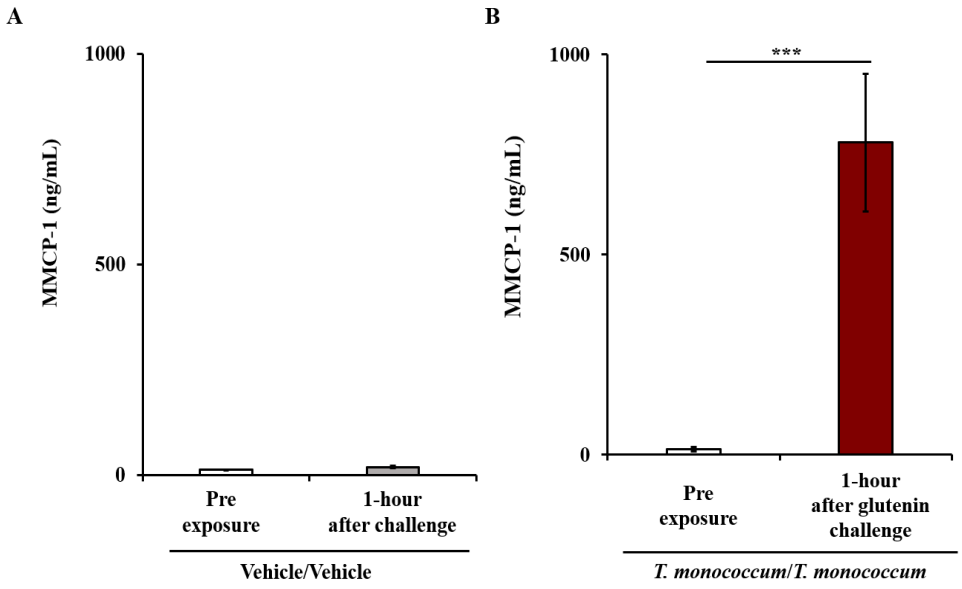

Figure 7.15 Systemic challenge with acid-soluble gluten extract from *Triticum monococcum* elicits a robust mucosal mast cell response (MMCR) in Balb/c mice.

Mice were sensitized and systemically challenged with acid-soluble gluten extract from *Triticum monococcum* or vehicle, as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 (ng/mL) in pre and one-hour after challenge were measured via ELISA. *n* = 10/group. (**A**). MMCP-1 levels in control mice challenged with vehicle. (**B**) MMCP-1 levels

in allergic mice challenged with acid-soluble gluten extract from *Triticum monococcum*. ***p < 0.05, student's t-test; *n*: number of mice.

7.3.6 Mapping the Inherent Allergenicity and Sensitization Potential of Acid-Soluble Gluten Extracts Across Diploid, Tetraploid, and Hexaploid Wheat Species

Utilizing the sIgE data from the validation studies mentioned earlier, and our previously reported ambassador wheat study (Jorgensen et al., 2023), we constructed a comparative sensitization map. The levels of sIgE antibodies induced by each wheat species were calculated by subtracting the baseline (pre) sIgE levels from the sIgE levels observed after the 6th response (6R). The resulting map illustrates the intrinsic allergenicity and sensitization potential of the three genetically distinct wheat, as depicted in Figure 7.16. Notably, *T. aestivum* and *T. durum* were significantly elevated when compared to *T. monococcum* acid-soluble gluten extract.

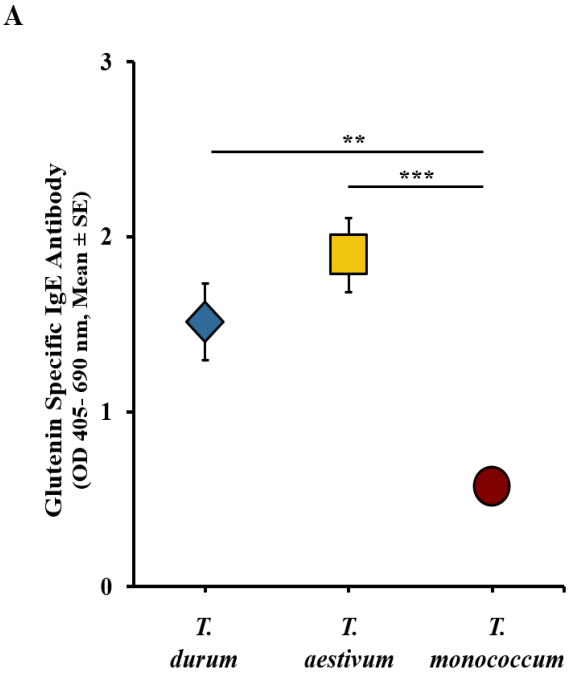

Figure 7.16 A comparative map of the intrinsic allergenicity sensitization potential of acid-soluble gluten extracts from *Triticum durum*, *Triticum aestivum*, and *Triticum monococcum*.

The changes in acid-soluble gluten extract-specific IgE antibody (Ab) levels after the 6th skin exposure to acid-soluble gluten extract from *T. durum*, *T. monococcum*, *T. aestivum*. ** $p < 0.05$, *** $p < 0.005$ one-way ANOVA and Tukey's post hoc tests. *n*: number of mice.

7.3.6.1 Comparative Map of the Intrinsic Allergenicity Disease Elicitation Potential of Acid-soluble gluten extracts from the Diploid, Tetraploid, and Hexaploid Wheats

We utilized the absolute changes in rectal temperature data resulting from systemic challenges in the validation studies of *T. durum*, *T. aestivum*, and *T. monococcum*, alongside our previously reported hexaploid wheat study (Jorgensen et al., 2023) to create a comparative disease elicitation map. Figures 7.17A through D display the disease elicitation potential map of the three species after 15, 20, 25, and 30 minutes respectively. As evident, the three wheat species have similar reaction kinetics through the entirety of the 30-minute period (Figure 7.17 A-D).

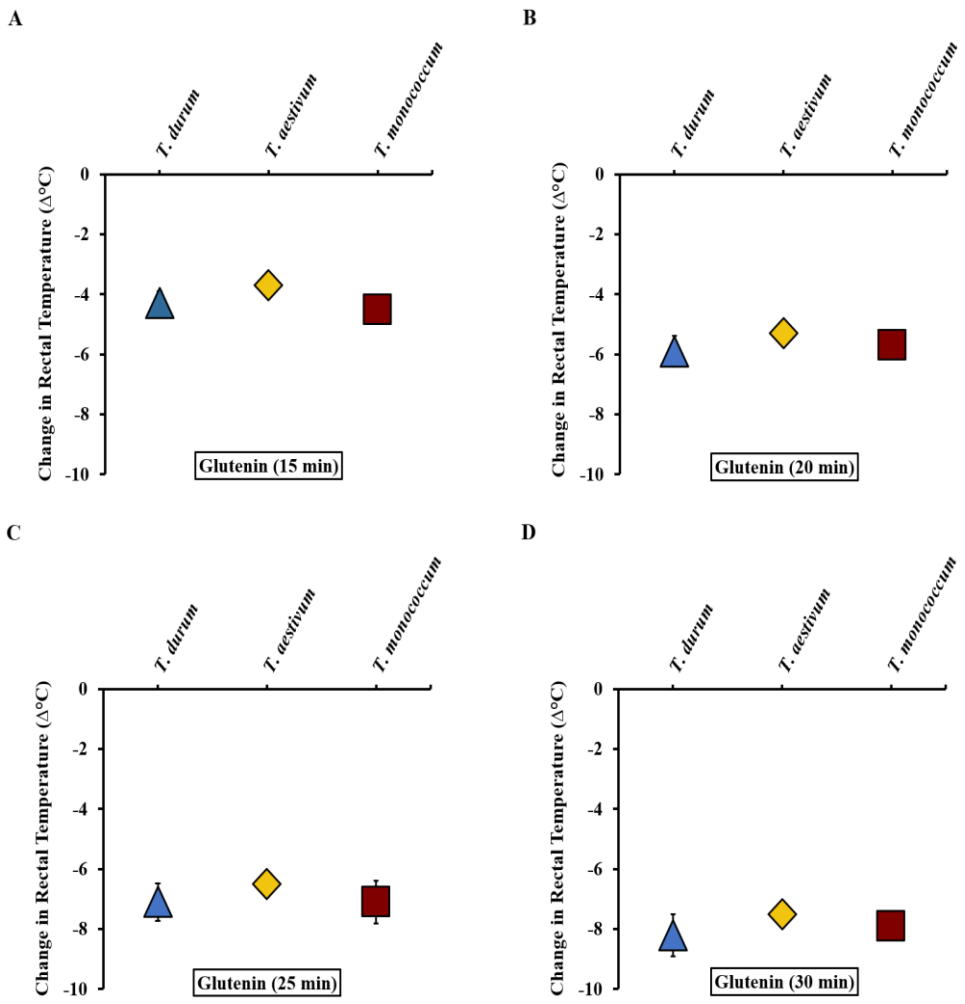

Figure 7.17 Comparative map of the intrinsic allergenicity disease elicitation potential of acid-soluble gluten extracts from diploid, tetraploid, and hexaploid wheat.

(**A,B**) HSRs at 15 and 20 min after systemic challenge with 0.5 mg of *Triticum durum*, *Triticum aestivum*, and *Triticum monococcum* respectively. (**C,D**) HSRs at 25 and 30 min after systemic challenge with 0.5 mg of *Triticum durum*, *Triticum aestivum*, and *Triticum monococcum* respectively.

7.3.6.2 Comparative Map of the Mucosal Mast Cell Response (MMCR) Elicitation Potential of Acid-Soluble Gluten Extracts from the Diploid, Tetraploid, and Hexaploid Wheats

We utilized the MMCP-1 data obtain from systemic allergen challenges in the validation studies of *T. durum*, *T. monococcum*, and *T. aestivum* wheats, along with previously reported durum wheat studies to construct a comparative Mast Cell Degranulation (MMCR) elicitation potential map (Jorgensen et al., 2023). Figure 7.18 presents the MMCR elicitation potential map at 0.5 mg systemic allergen challenge. It is apparent that *T. aestivum* induced the highest MMCR. *T. durum* and *T. monococcum* elicited similar levels of MMCR.

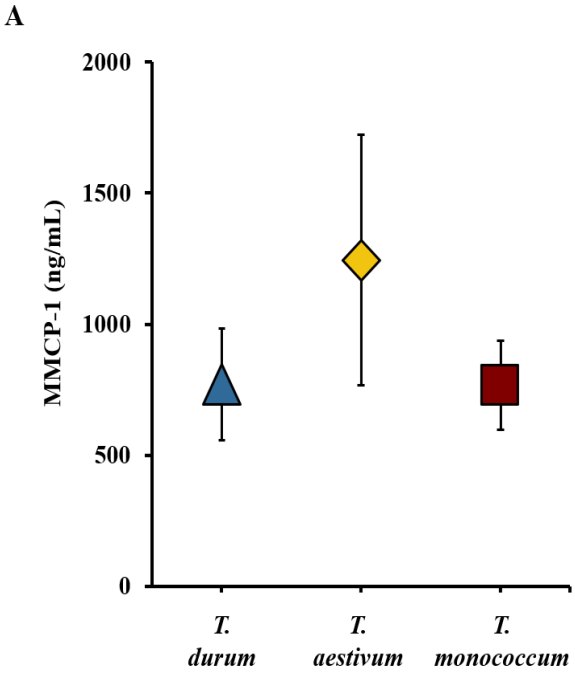

Figure 7.17 Comparative map of the mucosal mast cell response (MMCR) elicitation potential of acid-soluble gluten extracts from diploid, tetraploid, and hexaploid wheats.

Average MMCP-1 blood level after 0.5 mg systemic allergen challenge dose. *n* = 10/group. MMCP-1: mucosal mast cell protease-1. *n*: number of mice.

7.4 Discussion

In this study, we sought to develop a standardized comparative map of the intrinsic allergenicity of native glutens from the three commonly cultivated and consumed wheat species. We hypothesized that these three different wheat species will exhibit natural variations in their sensitization capacity as well as their ability to elicit systemic anaphylactic disease. Our results in general support this hypothesis.

This study yielded eight novel findings, encompassing: i) the first-time validation of the model for intrinsic allergenicity of alcohol-soluble gluten (gliadin) extracts from two commercially cultivated wheat species namely *Triticum aestivum (*common bread wheat hexaploid of AABBDD genome*)* and *Triticum monococcum* (Ancient diploid Einkorn wheat of AA genome); ii) creation of the first comparative map of intrinsic sensitization potential of alcohol-soluble gluten extracts from *T. aestivum*, *T. monococcum,* and *T. durum*; iii) establishment of a comparative map showcasing the intrinsic hypothermic shock response (HSR) elicitation potential of alcohol-soluble gluten extracts from the three commonly cultivated wheat species; iv) development of a comparative map highlighting the mucosal mast cell response elicitation potential of alcohol-soluble gluten extracts from the three commonly cultivated wheat species; v) the first-time validation of the model for intrinsic allergenicity of acid-soluble gluten (glutenin) extracts from two commercially cultivated wheat species namely *Triticum durum (*tetraploid of AABB genome*)* and *Triticum monococcum* (Ancient diploid Einkorn wheat of AA genome); vi) creation of the first comparative map of intrinsic sensitization potential of acid-soluble gluten extracts from *T. aestivum*, *T. monococcum,* and *T. durum*; vii) establishment of a comparative map showcasing the intrinsic hypothermic shock response (HSR) elicitation potential of acid-soluble gluten extracts from the three commonly cultivated wheat species; and

viii) development of a comparative map highlighting the mucosal mast cell response elicitation potential of acid-soluble gluten extracts from the three commonly cultivated wheat species.

Previously, we had developed and characterized the transdermal sensitization and systemic disease elicitation mouse model using the alcohol-soluble gluten extract (gliadin) from *Triticum durum*, and the acid-soluble gluten extract (glutenin) from *Triticum aestivum* (Jorgensen et al., 2023A,B). In this study, we further validated this model for alcohol-soluble gluten extracts from *Triticum aestivum* and *Triticum monococcum* and acid-soluble gluten extracts from *Triticum durum* and *Triticum monococcum*. We chose these three wheat species because of the following reasons: i) *T. aestivum* wheat (variety Ambassador, a hexaploid AABBDD genome) is the common wheat primarily employed in cracker and cookie production (Martin et al., 2012); ii) *Triticum durum* wheat is the tetraploid genome (AABB genome) is the common wheat primarily used making pastas such as spaghetti or penne (Banach et al., 2021); and iii) *Triticum monococcum* (known as Einkorn, AA genome) is a commercially available ancient wheat species with considerable research interest in its potential health-promoting properties (Hidalgo et al., 2014).

In this study, the order of sensitization capacity for three wheat species were as follows: i) for gliadins, *T. aestivum* > *T. durum* = *T. monococcum*; ii) for glutenins, *T. aestivum* > *T. durum* > *T. monococcum*. There are no previous studies comparing allergenic sensitization capacity of gluten proteins in mice. There is one previous study comparing the sensitization capacity of non-gluten proteins in mice (Gao et al., 2023). They found the order of sensitization capacity for the three wheat species as follows: *T. aestivum* ≥ *T. durum* > *T. monococcum*. Thus, these studies together suggest that *T. monococcum* has the least sensitization capacity

independent of the type of allergen. In contrast, *T. aestivum* has the highest sensitization capacity.

There are two studies conducted using IgE from wheat allergic human subjects to investigate the relative allergenicity of different wheat species/varieties. Nakamura et al., 2005 used urea-soluble wheat allergens that included both gluten (gliadin and glutenin) and non-gluten proteins extracted from 321 wheat varieties belonging to various species as follows: (*Triticum monococcum*), tetraploid (*T. durum, T. dicoccum, T. polonicum, T. turgidum*), and hexaploid (T. *aestivum, T. compactum, T. spelta*). They reported that Einkorn was among the least allergenic wheats and *T. aestivum* was the highest. Larre et al., 2011 compared non-gluten proteins allergenicity from two wheat species *T. monococcum* (Engrain) and *T. aestivum* (cv Recital). They used IgE ELISA for testing using serum from 20 wheat allergic subjects. They reported that the majority of patient's IgE reacted most with *T. aestivum* and least with *T. monococcum*. Thus, in humans Einkorn appears to be the least sensitizer and *T. aestivum* appears to be the most prolific sensitizer for allergic reactions. These results in principle are consistent with the mouse model studies on gluten as well as non-gluten allergenicity with *T. monococcum* being the least sensitizer and *T. aestivum* being the greatest sensitizer. Therefore, the adjuvant-free mouse model used in these two studies appears to mimic human sensitization capacities of different wheat species. These findings further support the utility of this model to predict the human allergenicity of novel wheat proteins in the future.

The gluten allergy mechanism occurs in two distinct phases. In the initial stage, genetically predisposed individuals encounter gluten through various pathways, such as the skin. This exposure triggers the production of gluten specific IgE antibodies (Platts-Mills, 2015; Renz et al., 2018; Sicherer & Leung, 2015). These antibodies subsequently bind to mast cells and

basophils through high-affinity IgE receptors, establishing a sensitized immune state. The second phase unfolds upon re-exposure to gluten in sensitized individuals. Gluten allergens bind to IgE-loaded mast cells and basophils, initiating the release of chemical mediators that culminate in clinical manifestations, including systemic anaphylaxis (Jin et al., 2019). To assess the sensitization potential of glutens from the three wheat species used in this study, we employed a highly sensitive ELISA-based method, as previously described (Gao et al., 2023; Jorgensen et al., 2023a,b). Our findings reveal that both types of gluten extracts from all three species inherently possess the capability to induce sensitization through skin exposure.

The hypothermic shock response (HSR) serves as an indicator of the impact of anaphylaxis on the neurological and cardiovascular functions associated with thermoregulation. Employing rectal thermometry, HSR is a widely utilized method to assess the severity of anaphylaxis in mouse models of food allergy, as it is considered a surrogate marker for cardiac output, which is adversely affected during food-induced systemic anaphylactic reactions (Gao et al., 2023; Gonipeta et al., 2015; Gouel-Chéron et al., 2023; Jorgensen et al., 2023a,b). Notably, in mouse models of anaphylaxis, HSR can arise from both IgE and IgG-mediated reactions following intraperitoneal challenge (Miyajima et al., 1997; Tsujimura et al., 2008). Our findings demonstrate that HSR can effectively quantify systemic anaphylaxis induced by both gluten extracts from all three wheat species in this mouse model.

Previously identified as a specific biomarker for IgE-antibody-mediated systemic anaphylaxis in mouse models of food allergy, mucosal mast cell protease-1 (MMCP-1) has been established as a reliable indicator (Khodoun et al., 2011). Given that hypothermic shock response (HSR) reflects both IgE and IgG1-mediated systemic anaphylaxis, we opted to incorporate MMCP-1 as an additional biomarker. This allowed us to specifically assess whether glutens from

the three wheat species can instigate life-threatening anaphylaxis through the IgE pathway, and whether there are distinctions in the capacity of the two gluten types to initiate the IgE-mediated pathway of disease elicitation. It is crucial to note that MMCP-1 is exclusively sourced from mucosal mast cells present in the gut. These gastrointestinal mucosal mast cells contain MMCP-1 within granules, a unique protein absent in connective tissue mast cells (Andersson et al., 2008). These mast cells in the gut possess receptors for IgE antibodies. Following sensitization to gluten, IgE antibodies attach to mucosal mast cells through high-affinity IgE antibody receptors (FcεRI). In pre-challenge samples, blood exhibits minimal MMCP-1 levels, as the mucosal mast cells remain inactive. However, upon gluten challenge, the gluten binds to IgE antibodies on the gut mast cells, triggering mast cell activation and degranulation. This process releases MMCP-1 into the systemic circulation. Therefore, MMCP-1 levels one hour after challenge serve as an indicator of IgE-antibody-mediated anaphylaxis in the gut, simulating the natural IgE-mediated reactions observed in gluten allergy upon ingestion of gluten-containing products. Our data reveals that both gluten extracts can activate this pathway, with the acid-soluble gluten extract showing a slightly superior capacity to elicit MMCP-1 release.

Numerous studies indicate that the allergenicity of food proteins, including those found in wheat, can be influenced by food processing (Gao et al., 2021). For example, employing novel processing methods on wheat allergens may lead to the generation of new epitopes or the revelation of previously concealed epitopes, potentially heightening their allergenicity. The proposed methodology facilitates the development of intrinsic allergenicity maps using processed wheat. By comparing the allergenicity potential maps of native and processed wheat proteins, it becomes feasible to quantify the effects of processing on intrinsic allergenicity within each wheat genotype. In essence, creation of the first gluten allergenicity will assist the

development of novel potentially hypoallergenic glutens. It also helps to prevent inadvertent introduction of hyper-allergenic processed glutens into food and cosmetic products. Currently, GM/GE wheat is not available for consumption in the United States. However, USFDA recently approved a GE wheat produced by Argentina and Brazil (Ning et al., 2022; Yankelevich, 2021). GE wheat has recently been approved for eating and growing in Argentina and Brazil, and it has been approved for consumption in Australia, New Zealand, and Nigeria (Yankelevich, 2021). However, the allergenic potential of this GM wheat or other such novel wheats must be carefully evaluated to establish whether they may be hyper/hypo/non-allergenic compared to the common non-GM counterparts. The methodology described here can be used for this purpose. The comparative map will provide a reference standard to objectively quantify the relative allergenicity potential of GM/GE wheat versus non-GM/GE counterparts. By comparing the allergenicity potential maps of the novel wheat lines versus the traditional non-GM/GE wheat, it becomes feasible to quantify the impact of genetic engineering on intrinsic allergenicity of wheat – a major long-standing concern for all GM foods.

It is important to note that we selected one representative variety from each of the three wheat species for this study, because of the limited resources available. Further research is necessary to validate whether the chosen variety adequately represents the intrinsic allergenicity potential of all other varieties within the species (Shewry, 2009). This same methodology could be employed to investigate the differences in intrinsic allergenicity potential between various wheat lines/varieties. This approach has the potential to identify hypoallergenic and non-allergenic wheat lines by utilizing comparative mapping. In the same vein, by utilizing comparative potential maps of allergenicity, it is also possible to identify and exclude potentially hyperallergenic wheat lines, thus safeguarding individuals sensitive to wheat allergens.

7.5 Materials and Methods

7.5.1 Chemicals and reagents

Biotin-conjugated antibodies specific to mouse IgE were obtained from BD Bio-Sciences (San Jose, CA, USA). The p-nitrophenyl phosphate compound was sourced from Sigma (St. Louis, MO, USA), and Streptavidin alkaline phosphatase was acquired from Jackson ImmunoResearch (West Grove, PA, USA). Folin reagent was obtained from Bio-Rad (Hercules, CA). Specific reagents, including the IgE Mouse Uncoated ELISA Kit with Plates, Streptavidin-HRP, TMB substrate, MCPT-1 (mMCP-1) Mouse Uncoated ELISA Kit with Plates, Avidin-HRP, and TMB substrate, were procured from Invitrogen (MA, USA). The Tissue Protein Extraction Reagent (T-PER), a proprietary detergent with a composition of 25mM bicine and 150mM sodium chloride at pH 7.6, was obtained from ThermoFisher Scientific (MA, USA). For protease inhibition, a cocktail of serine, cysteine, and acid proteases, along with aminopeptidases, was acquired from Sigma-Aldrich (MO, USA).

7.5.2 Mice breeding and establishment of a plant-protein-free mouse colony

Breeder pairs of adult Balb/c mice were procured from The Jackson Laboratory (Bar Harbor, ME). Upon their arrival, the mice were introduced to a stringent plant-protein-free diet (AIN-93G, Envigo, Madison, MI). After a one-week acclimation period, conventional breeding methods were employed to initiate reproduction. Female mice aged 6-8 weeks from the litters were specifically chosen for this study. Throughout the entire research period, all mice were consistently maintained on the strict plant-protein-free diet (AIN-93G). All animal procedures strictly adhered to the guidelines established by Michigan State University.

7.5.3 Preparation of gluten extract from wheat flour

Protein extraction from wheat flour targeted the isolation of alcohol- and acid-soluble wheat gluten using the Osborne sequential extraction method (Osborne, 1924). Briefly, a mixture of flour and filter-sterilized 0.5 M NaCl (1:10, m/v) underwent continuous agitation for 2 hours, followed by centrifugation at 20000 x g for 30 minutes. The resulting pellets were preserved for alcohol extraction. The salt-insoluble pellets were then mixed in a 1:10 ratio with 70% ethanol for 2 hours, and centrifuged at 20000 x g for 15 minutes, yielding the alcohol-soluble gluten extract supernant and alcohol-insoluble pellets. The latter was saved for acid extraction. The alcohol-insoluble pellets were combined in a 1:4 ratio with 0.05 M acetic acid for two hours and centrifuged at 20000 x g for 15 minutes. The resulting supernatants from both extractions were frozen at -70°C overnight and subjected to freeze-drying the next day. The lyophilized alcohol-soluble gluten extract was re-constituted using 70% ethanol and the acid-soluble gluten extract was reconstituted using 0.05 M acetic acid to achieve a concentration of 1 mg protein per 100 µL for topical application. For challenges involving intraperitoneal (IP) injections, the gluten extracts were re-constituted with phosphate-buffered saline (PBS) to attain concentrations of 0.5 mg/mouse. Protein content was quantified using the LECO total combustion method from LECO (St. Joseph, MI). SDS-PAGE testing was conducted to assess protein quality.

7.5.4 Skin sensitization, bleeding, and plasma sample preparation

Female adult Balb/c mice were utilized for experimental purposes. To facilitate the procedures, the hair on the mice's rumps was bilaterally removed using a Philips hair clipper (Amsterdam, Netherlands). The acid-soluble wheat glutenin (WG) was administered onto the rump at a dosage of 1 mg per mouse in 100 µL, alternately using a vehicle solution of 0.05 M

acetic acid. Following application, the treated area was covered with a non-latex bandage sourced from Johnson & Johnson (New Brunswick, New Jersey) and left in place for one day. This process was repeated on a weekly basis, occurring nine times over a span of nine weeks. Blood samples were collected from the saphenous vein before the initial exposure and after the sixth exposure. The blood was drawn into tubes coated with the anticoagulant lithium heparin (Sarstedt Inc MicrovetteCB 300 LH, Germany). The collected blood was subsequently subjected to centrifugation to isolate plasma, which was then stored individually at -70°C until required for subsequent testing of (s)IgE and (t)IgE.

7.5.5 Elicitation of systemic anaphylaxis

Two weeks after the final cutaneous exposure to alcohol or acid-soluble gluten extract or the vehicle, the mice underwent an intraperitoneal (IP) injection. This injection involved either 0.5 mg of alcohol or acid-soluble gluten extract, or the vehicle (phosphate-buffered saline, PBS).

7.5.6 Determination of hypothermic shock responses

Rectal temperature (°C) measurements were recorded both before the challenge and at 5-minute intervals following the challenge, up to a 30-minute duration. A rectal thermometer (DIGI-SENSE, MA, USA) was used for these measurements. The recorded values included the specific temperatures and the corresponding differences (Δ°C) compared to the pre-challenge temperatures for each individual mouse. These recorded data points were subsequently utilized for further analyses.

7.5.7 Measurement of specific IgE antibody levels

Gluten-specific (s) IgE antibody levels were quantified using a highly sensitive ELISA method, as previously detailed with certain modifications (Gao et al., 2019; Jin et al., 2020; Jin et al., 2017; Jorgensen et al., 2023a,b). Initially, 96-well Corning 3369 plates were coated with WG and subsequently blocked using a 5% gelatin solution. After a thorough washing step, plasma samples were introduced onto the plate. Further washing ensued, followed by the addition of a biotin-conjugated anti-mouse IgE antibody. Subsequent washes were performed before introducing streptavidin alkaline phosphatase, and eventually, p-nitrophenyl phosphate was added to enable quantification, following established methodologies (Gao et al., 2019; Jin et al., 2017; Jorgensen et al., 2023a,b).

7.5.8 Quantification of mucosal mast cell protease-1 (MMCP-1) level

One-hour post-challenge, blood samples were obtained and employed to measure the levels of mucosal mast cell protease-1 (MMCP-1) in the plasma. The quantification was performed using an ELISA-based method developed by Invitrogen, following established procedures (Jorgensen et al., 2023a,b). In detail, 96-well Corning Costar 9018 plates were initially coated with a capture antibody (anti-mouse MMCP-1). Subsequently, both samples and standards (recombinant mouse MMCP-1) were added to the plate. A biotin-conjugated anti-mouse MMCP-1 antibody was then introduced as the secondary antibody. Detection was achieved using an avidin-HRP/TMB substrate system. It is noteworthy that the assay has a limit of detection set at 120 pg/mL, and the range of standards covered from 120 to 15000 pg/mL. Testing was conducted in quadruplicate for each individual mouse's plasma.

7.6 Conclusions

For the first time, we illustrate similarities and distinctions in the inherent allergenic properties among the three chosen genetically distinct wheat species. This preclinical comparative mapping approach could serve in identifying potentially hyperallergenic, hypoallergenic, and non-allergenic wheat varieties through crossbreeding and genetic engineering. Additionally, it offers a means to evaluate alterations in wheat protein allergenicity resulting from various processing methods.

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CHAPTER 8 EVALUATION OF INTRINSIC ALLERGENICITY OF GLUTEN EXTRACTS FROM *AEGILOPS TAUSCHII*-THE ANCIENT DD GENOME PROGENITOR OF MODERN HEXAPLOID WHEAT, IN AN ADJUVANT-FREE MOUSE MODEL

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8.1 Abstract

Wheat allergy is a major type of food allergy with the potential for life-threatening anaphylactic reactions. Currently, consumed wheat belongs to three distinct species that are either diploid (AA genome), tetraploid (AABB genome), or hexaploid (AABBDD genome). The hexaploid wheat was developed using tetraploid wheat and an ancient diploid wheat progenitor (DD genome)-*Aegilops tauschii*. The potential allergenicity of gluten from the ancient *Ae. tauschii* wheat is unknown. In this study, using a recently reported adjuvant-free gluten allergy mouse model, we evaluated the intrinsic allergenicity potential of gluten extracts from *tauschii* wheat. Ancient wheat was grown and harvested at our university. Alcohol-soluble and acid-soluble gluten extracts were prepared and used in testing. A plant protein-free colony of Balb/c mice was established and used in this study. The allergic sensitization (IgE) potential of glutens were determined using a transdermal exposure method. Life-threatening anaphylaxis eliciting potential of glutens were determined by intraperitoneal injection followed by hypothermic shock response and mucosal mast cell responses. Both gluten proteins elicited robust allergic

sensitization as measured specific IgE responses. Life-threatening HSR reactions were induced by both glutens. Similarly, both glutens elicited significant mucosal mast cell responses as evident by elevated MMCP-1 in the blood. These results demonstrate that both glutens from the ancient *tauschii* wheat have the inherent potential to elicit clinical sensitization for life-threatening anaphylaxis.

8.2 Introduction

The identification of wheat as a prevalent instigator of immune-mediated food allergies has emerged as a significant global health concern for public health and food safety. As wheat has the potential for life-threatening anaphylaxis, this poses a substantial health and financial burden (Cianferoni, 2016). In a recent global meta-analysis of wheat allergy, it was found that 0.22% to 1.93% of individuals are affected by this condition (Liu et al., 2023). The prevalence varies across different regions of the world, based on self-reported physician diagnosed wheat allergy (Liu et al., 2023). With no cure for wheat allergy at the present, individuals are required to adhere to strict elimination diets, impacting their quality of life through an elevated risk for severe allergic reactions at any time (Gupta et al., 2018).

Wheat proteins encompass both gluten and non-gluten components. The gluten fraction constitutes 80–85% of the overall wheat proteins, while the remaining 15–20% is comprised of the salt-soluble, non-gluten fraction (Jin et al., 2019)**.** Gluten serves as storage proteins found in wheat and other cereals like rye or barley, all cereals belonging to the *Poaceae* family (Cabanillas, 2020). The alcohol-soluble glutens, known as gliadins, are found as individual proteins that interact via hydrogen bonds and predominantly feature intramolecular disulfide bonds. In contrast, the acid-soluble glutens, known as glutenins are polymeric proteins that

establish connections through both intermolecular and intramolecular disulfide bonds. Moreover, gliadins can also be linked to the glutenin network through intermolecular disulfide bonds (Gao et al., 2021). Among alcohol-soluble gluten proteins, ω−1, 2, 5 gliadin, and α/β/γ-gliadins have been thoroughly characterized and are recognized for inducing allergic reactions in susceptible individuals (Cianferoni, 2016). Within the acid-soluble fraction, both high-molecular weight (HMW) and low-molecular weight (LMW) glutenin subunits can bind IgE antibodies causing severe allergic reactions (Cianferoni, 2016; Juhász et al., 2018; Pastorello et al., 2007).

Wheat exhibits significant genetic diversity within both domesticated and wild species, spanning various ploidy levels. Durum wheat, *Triticum durum* (AABB genome), is a tetraploid wheat containing the A and B genomes. *Triticum aestivum* (AABBDD genome) is a hexaploid wheat containing the A, B, and D genomes, originating from two interspecific hybridization events between the tetraploid emmer wheat, *Triticum turgidum* (AABB genome) and the diploid wheat, *Aegilops tauschii* (DD genome) progenitors (Obenland & Riechers, 2020). Most studies of gluten allergenicity have used tetraploid and hexaploid wheats, and in some cases, diploid Einkorn (AA genome) wheat (Fu et al., 2022; Jorgensen, Gao, Selvan, et al., 2023; Jorgensen, Gao, Chandra, et al., 2023; Kohno et al., 2016; Lombardo et al., 2015; Yu Wang et al., 2021). However, the intrinsic allergenicity of glutens from the ancient wheat *Aegilops tauschii* (DD genome), which is not commercially available, is unknown. Elucidating the allergenicity potential of ancient wheats such as *Ae. tauschii* is critical because: i) *Ae. tauschii* whole genome has previously been sequenced (Luo et al., 2017); it is proposed that this genetic information will be valuable to improve the genetics of modern wheat crops to enhance their quality and safety; and ii) there are scientific discussions in the literature that ancient wheat species may vary in

immunogenicity and allergenicity compared the modern widely consumed wheats (Shewry, 2018).

Various animal models, including dogs, rats, and mice have been employed to investigate gluten allergenicity (Adachi et al., 2012; Ballegaard et al., 2019; Buchanan et al., 1997; Frick et al., 2005; Kohno et al., 2016; Kroghsbo et al., 2014; Yamada et al., 2019). These models maybe classified into two general types based on the sensitization protocols: i) intraperitoneal administration of gluten plus adjuvants such as alum or Fruend's adjuvant, to enhance immune response to gluten so that marked IgE responses are initiated; and ii) administering gluten transdermally without adjuvant to induce robust IgE responses. The former approach has been widely used. However, despite their utility and unique strengths, these adjuvant-based models cannot be used to evaluate inherent allergenicity potential of glutens, because of enhancement of non-specific immune stimulation due to adjuvant's action on innate immune cells as well as adaptive immune responses (Dearman & Kimber, 2009; Dearman & Kimber, 2007). In contrast, the latter approach that does not utilize adjuvants to elicit IgE responses has the attractive capacity to decipher the inherent allergenicity potential of glutens.

Therefore, in this study, we utilized an adjuvant-free mouse model to investigate the hypothesis that skin application of alcohol- and acid-soluble glutens from *Aegilops tauschii* will induce clinical sensitization for life-threatening anaphylaxis. The study encompassed four objectives: i) To assess the sensitization (IgE antibody) eliciting capacity of alcohol- and acid-soluble gluten extracts from the ancient wheat *Ae. tauschii*; ii) to quantify systemic anaphylaxis eliciting potentials of both glutens in this model as quantified by the hypothermic shock responses (HSR) upon intraperitoneal injections of the two gluten extracts; iii) to quantify the mucosal mast cell degranulation responses (MMCR) induced by intraperitoneal injection of the

two gluten extracts; and iv) to create comparative intrinsic sensitization and disease elicitation maps of both types of glutens.

This study collectively demonstrates for the first time that both gluten extracts from the ancient *Ae. tauschii* wheat are intrinsically allergenic with largely similar sensitization as well as disease elicitation capacities.

8.3 Results

8.3.1 Testing Alcohol-Soluble Gluten Extract from *Ae. tauschii* for Sensitization Potential

Validation of the Transdermal Sensitization and Systemic Elicitation of Disease Mouse Model for *Aegilops tauschii* Using Alcohol-Soluble Gluten Extract

8.3.1.1 Transdermal Exposure to Alcohol-Soluble Gluten Extract from *Ae. tauschii* Elicits Robust Specific-IgE Antibody Response in Balb/c Mice

Balb/c female mice were divided into groups and subjected to transdermal exposure to alcohol-soluble protein extract from *Aegilops tauschii* (genome DD), or vehicle, through repeated weekly exposure following the outlined methods. Blood samples obtained before the first (pre) and after the sixth skin exposure (6R) were analyzed for specific (s) IgE levels. Notably, a significant elevation of sIgE antibody levels were observed after transdermal exposure to alcohol-soluble gluten extract from *Ae. tauschii* in allergic mice compared to the vehicle control mice, as depicted in Figure 8.1A and B.

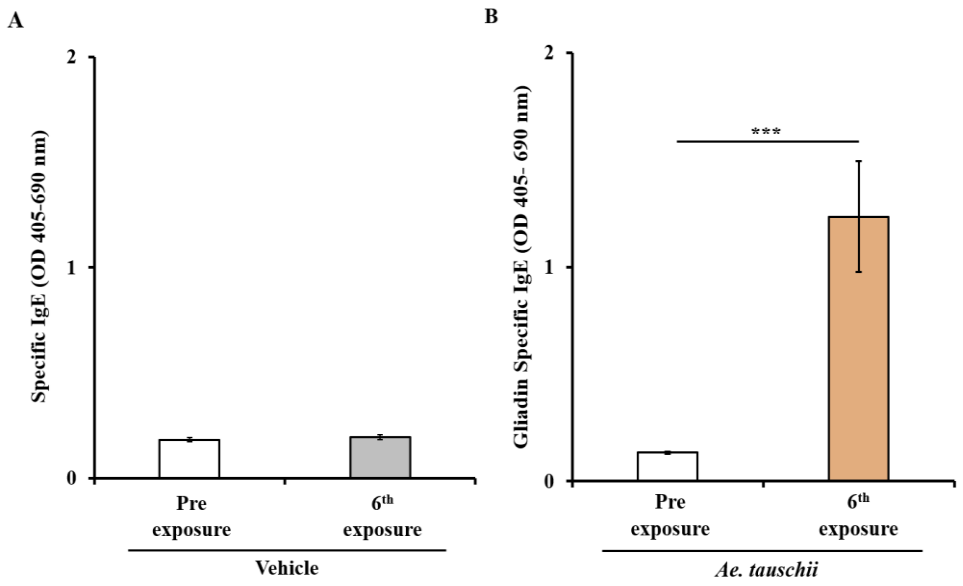

Figure 8.1 Transdermal exposure of Balb/c mice to alcohol-soluble gluten extract (gliadin) from *Aegilops tauschii* (genome DD) elicited robust specific (s) IgE antibody responses.

Mice were exposed to alcohol-soluble gluten extract from *Ae. tauschii* or vehicle as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 6th exposure (6R) was used in the measurement of sIgE levels (OD 405–690 nm). n = 10/group. (A) sIgE levels in control mice. (B) sIgE levels in sensitized mice. *** $p < 0.005$, student's t-test. Ab: antibody; n: number of mice; OD: optical density

8.3.1.2 Systemic Challenge with Alcohol-Soluble Gluten Extract from *Aegilops tauschii* Elicits Hypothermic Shock Responses in Skin-Sensitized Mice

Parallel groups of skin-sensitized mice were used to induce anaphylaxis by performing systemic challenge with alcohol-soluble gluten extract from *Ae. tauschii* (0.5 mg/mouse) or vehicle. Anaphylactic reactions were quantified by hypothermic shock reactions (HSR) using rectal thermometry, as described in methods. There was no HSR upon vehicle (i.e., zero allergen) or alcohol-soluble gluten extract challenge in control mice (Figure 8.2 A-B). In contrast, acute HSRs were observed upon systemic allergen challenge in sensitized mice (Figure

8.2 C-D). Significant HSRs were noted from 10 to 30 min post-systemic allergen challenge ($p < 0.05$).

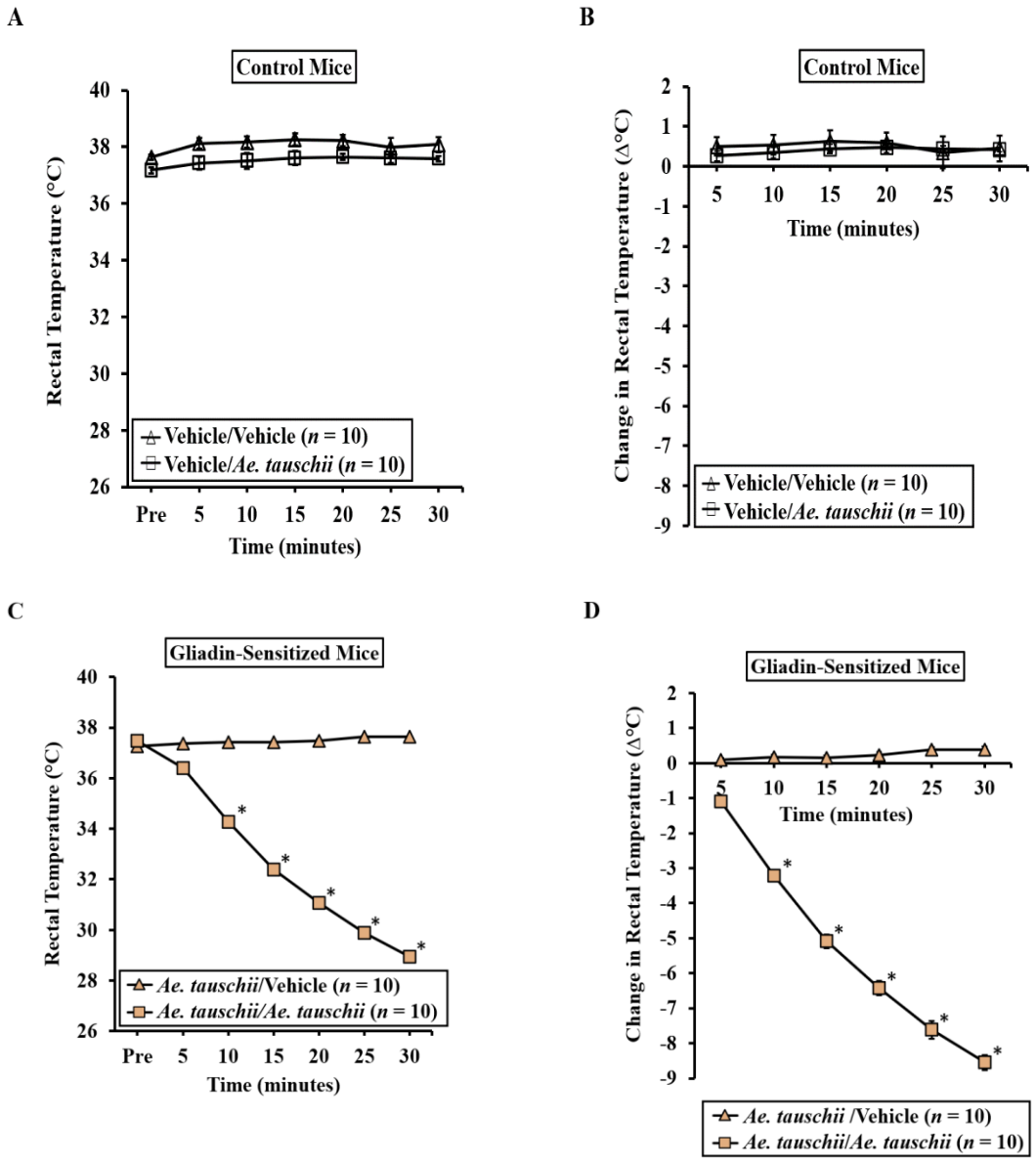

Figure 8.2 Transdermal exposure to alcohol-soluble gluten extract (gliadin) from *Aegilops tauschii* is sufficient for clinical sensitization to elicit systemic anaphylaxis in Balb/c mice.

Mice were sensitized and systemically challenged with alcohol-soluble gluten extract from *Ae. tauschii* or vehicle, as described in Materials and Methods. n = 10/group. (**A**) Actual rectal temperature at indicated time points in control mice challenged with alcohol-soluble gluten

extract from *Ae. tauschii* or vehicle. (**B**) Change in rectal temperature at indicated time points in control mice challenged with alcohol-soluble gluten extract from *Ae. tauschii* or vehicle. (**C**) Actual rectal temperature at indicated time points in gliadin-sensitized mice challenged with alcohol-soluble gluten extract from *Ae. tauschii* or vehicle. (**D**) Change in rectal temperature at indicated time points in gliadin-sensitized mice challenged with alcohol-soluble gluten extract from *Ae. tauschii* or vehicle. *n*: number of mice.

8.3.1.3 Balb/c Mice Sensitized by Alcohol-soluble Gluten Extract Exhibit Strong Mucosal Mast Cell Response (MMCR) Upon Intraperitoneal Injection

Figure 8.3A and B illustrate the MMCP-1 responses in both control mice and allergic mice. It is evident that the systemic challenge with *Ae. tauschii* alcohol-soluble gluten extract, as opposed to vehicle, results in a significant increase in MMCP-1 levels in the blood, as depicted in Figure 8.3A and B.

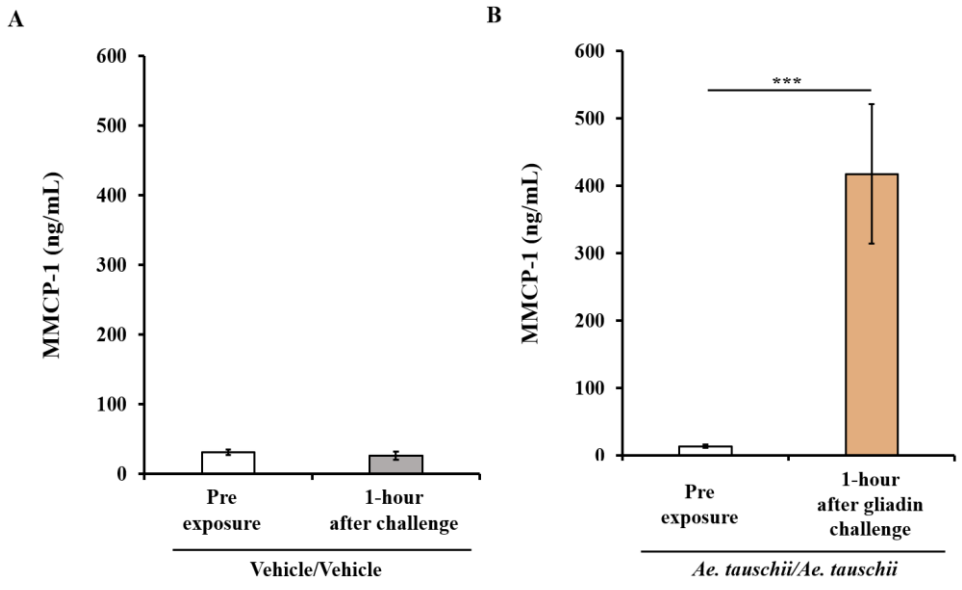

Figure 8.3 Systemic challenge with alcohol-soluble gluten (gliadin) extract from *Aegilops tauschii* elicits a robust mucosal mast cell response (MMCR) in Balb/c mice.

Mice underwent sensitization and systemic challenge with alcohol-soluble gluten extract from *Ae. tauschii* or the control vehicle, as outlined in the Materials and Methods section. Plasma levels of mucosal mast cell protease (MMCP)-1 (ng/mL) in pre and one-hour after challenge were measured via ELISA, with a sample size of $n = 10$/group. (**A**). MMCP-1 levels in control mice challenged with vehicle. (**B**) MMCP-1 levels in allergic mice challenged with alcohol-soluble gluten extract from *Ae. tauschii*. *** $p < 0.005$, student's t-test; n = number of mice.

8.3.2 Evaluation of the Transdermal Sensitization and Systemic Elicitation of Disease Mouse Model for *Aegilops tauschii* Using Acid-Soluble Protein Extract

8.3.2.1 Transdermal Exposure to Acid-Soluble Gluten Extract from *Ae. tauschii* Elicits Robust Specific-IgE Antibody Response in Balb/c Mice

Balb/c female mice were grouped and exposed to transdermal application of acid-soluble gluten extract from *Aegilops tauschii* (genome DD) or vehicle, following a repeated weekly exposure regimen as outlined in the specified methods. Blood samples collected before the initial exposure (pre) and after the sixth skin exposure (6R) were analyzed for specific (s) IgE levels. Significantly, there was a substantial elevation in sIgE antibody levels after transdermal exposure to acid-soluble gluten extract from *Ae. tauschii* in allergic mice compared to the vehicle control mice, as depicted in Figure 8.4A and B.

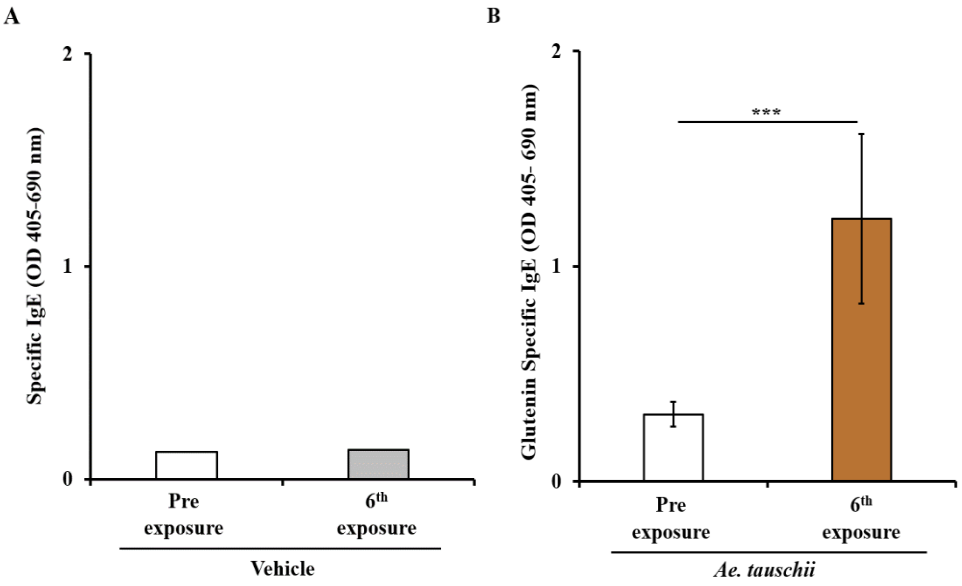

Figure 8.4 Transdermal exposure of Balb/c mice to acid-soluble gluten extract (glutenin) from *Ae. tauschii* (genome DD) elicited robust specific (s) IgE antibody responses.

Mice were exposed to acid-soluble gluten extract from *Ae. tauschii* or vehicle as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 6th exposure (6R) was used in the measurement of sIgE levels (OD 405–690 nm), with a sample size of $n = 10$/group. (**A**) sIgE levels in control mice. (**B**) sIgE levels in sensitized mice. *** $p < 0.001$, student's t-test. Ab: antibody; *n*: number of mice; OD: optical density.

8.3.2.2 Systemic Challenge with *Aegilops tauschii* Acid-Soluble Gluten Extract Elicits Hypothermic Shock Responses in Skin-Sensitized Mice

Groups of skin-sensitized mice were employed to induce anaphylaxis through systemic challenge with *Ae. tauschii* acid-soluble gluten extract (0.5 mg/mouse) or vehicle. Anaphylactic reactions were quantified using hypothermic shock reactions (HSR) and monitored with rectal thermometry, as detailed in the methods. No HSR was observed in control mice upon vehicle (i.e., zero allergen) or acid-soluble gluten extract challenge (Figure 8.5 A-B). In contrast, acute HSRs were evident upon systemic allergen challenge in sensitized mice (Figure 8.5 C-D).

Significant HSRs were noted from 10 to 30 minutes post-systemic allergen challenge, as indicated by ANOVA analysis ($p < 0.05$).

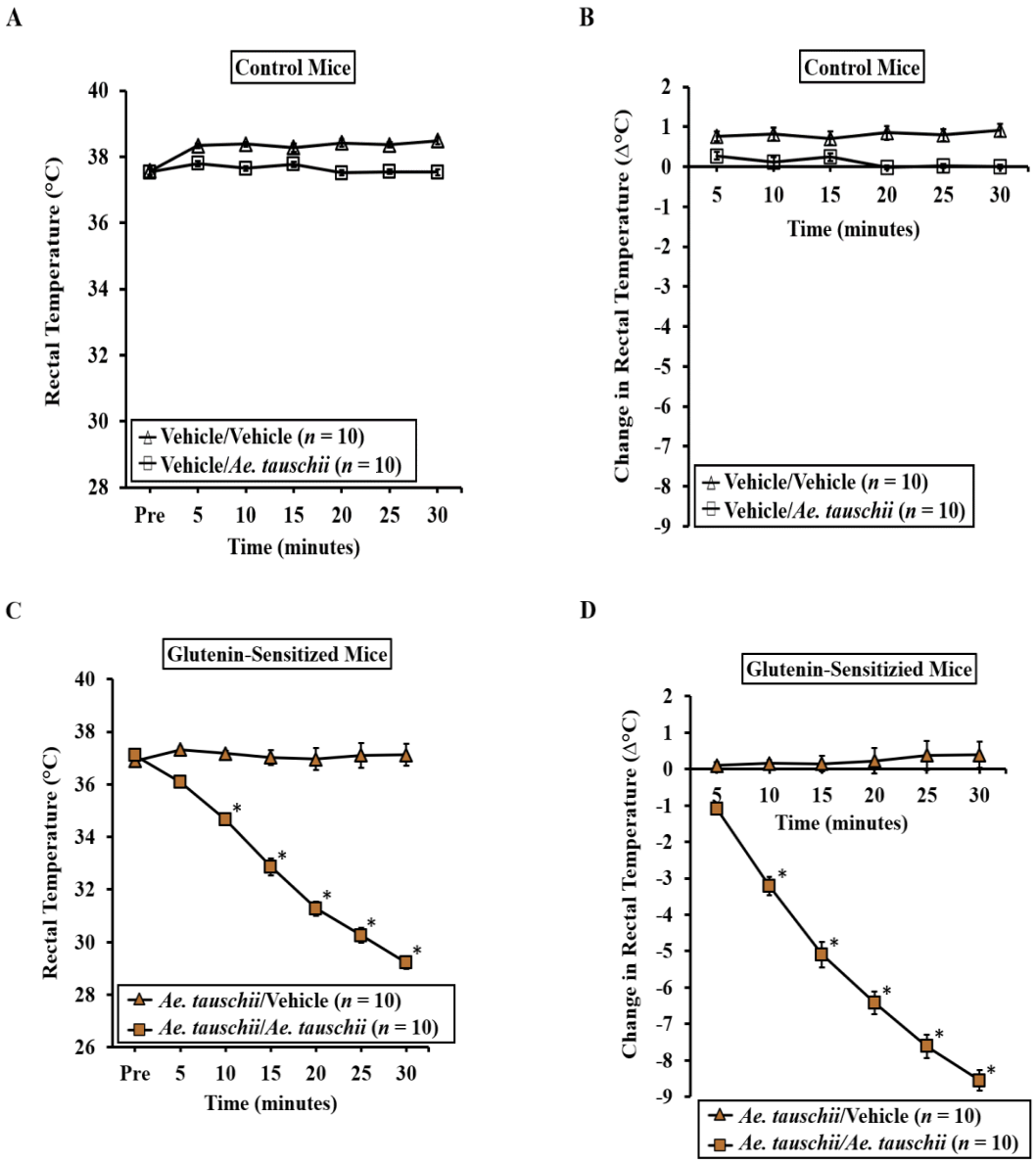

Figure 8.5 Transdermal exposure to acid-soluble gluten extract (glutenin) from *Aegilops tauschii* is sufficient for clinical sensitization to elicit systemic anaphylaxis in Balb/c mice.

Mice were sensitized and systemically challenged with acid-soluble gluten extract from *Ae. tauschii* or vehicle, as described in Materials and Methods. n = 10/group. (**A**) Actual rectal temperature at indicated time points in control mice challenged with acid-soluble gluten extract

from *Ae. tauschii* or vehicle. (**B**) Change in rectal temperature at indicated time points in control mice challenged with acid-soluble gluten extract from *Ae. tauschii* or vehicle. (**C**) Actual rectal temperature at indicated time points in acid-soluble gluten extract from *Ae. tauschii* sensitized mice challenged with acid-soluble gluten extract from *Ae. tauschii* or vehicle. (**D**) Change in rectal temperature at indicated time points in acid-soluble gluten extract from *Ae. tauschii* sensitized mice challenged with acid-soluble gluten extract from *Ae. tauschii* or vehicle. *n*: number of mice.

8.3.2.3 Balb/c Mice Sensitized by Acid-soluble Gluten Extract Exhibit Strong Mucosal Mast Cell Response (MMCR) Upon Intraperitoneal Injection

Figure 8.6A and B illustrate the MMCP-1 responses in both control mice and allergic mice. It is evident that the systemic challenge with *Ae. tauschii* acid-soluble gluten extract, as opposed to vehicle, results in a significant increase in MMCP-1 levels in the blood, as depicted in Figure 8.6A and B.

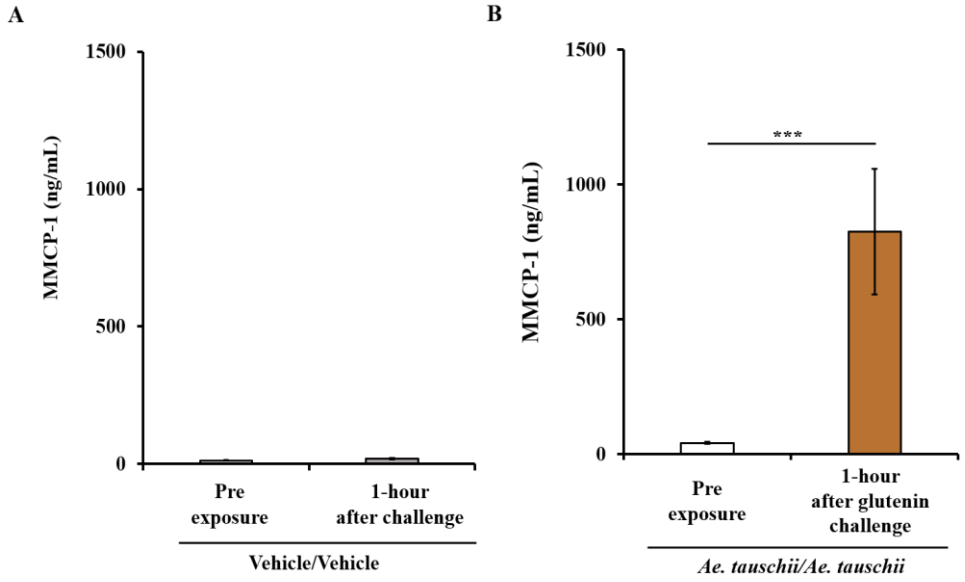

Figure 8.6 Systemic challenge with acid-soluble gluten (glutenin) extract from *Aegilops tauschii* elicits a robust mucosal mast cell response (MMCR) in Balb/c mice.

Mice were sensitized and systemically challenged with acid-soluble gluten extract from *Ae. tauschii* or vehicle, as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 (ng/mL) in pre and one-hour after challenge were measured via ELISA. n = 10/group. (A). MMCP-1 levels in control mice challenged with vehicle. (B) MMCP-1 levels in allergic mice challenged with acid-soluble gluten extract from *Ae. tauschii*. ***$p < 0.05$, student's t-test; *n*: number of mice.

8.3.3 Comparative Mapping of the Inherent Allergenicity Potential of Glutens from *Aegilops tauschii*

8.3.3.1 Comparative Map of the Inherent Sensitization Potential of Glutens from *Aegilops tauschii*

Utilizing the sIgE data from the validation studies mentioned earlier, and our previously reported *Triticum aestivum* wheat study (Jorgensen et al., 2023), we constructed a comparative sensitization map. The levels of sIgE antibodies induced by each gluten solubility were calculated by subtracting the baseline (pre) sIgE levels from the sIgE levels observed after the 6^{th} response (6R). The resulting map illustrates the intrinsic allergenicity and sensitization potential of the alcohol- and acid-soluble gluten extracts as visualized in Figure 8.7. Notably, the alcohol-soluble gluten extract yields slightly higher specific IgE response.

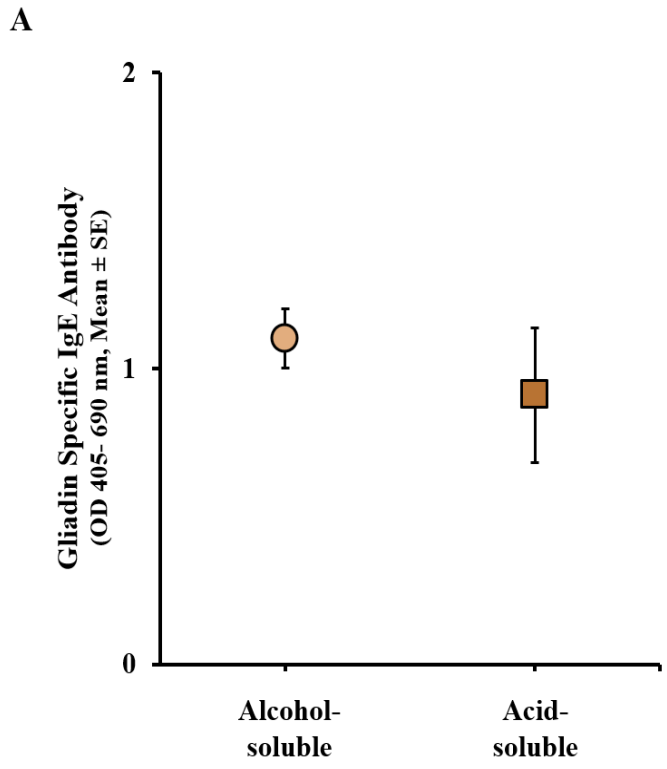

Figure 8.7 A comparative map of the intrinsic allergenicity sensitization potential of alcohol-soluble and acid-soluble gluten extracts from *Aegilops tauschii*.

The changes in alcohol- and acid-soluble gluten protein extract-specific IgE antibody (Ab) levels after the 6th skin exposure to alcohol or acid-soluble gluten protein extract from *Ae. tauschii*. *n* = 10/group. *n*: number of mice.

8.3.3.2 Comparative Map of the Inherent Disease Elicitation Potential of Glutens *from Aegilops tauschii* as Measured by HSR

We utilized the absolute changes in rectal temperature data resulting from systemic challenges in the validation studies of the alcohol- and acid-soluble gluten extracts from *Aegilops tauschii* to create a comparative disease elicitation map. Figures 8.8A through D display the disease elicitation potential map of the two gluten extracts after 15, 20, 25, and 30 minutes

respectively. As evident, the two gluten extracts display similar hypothermic shock responses throughout the 30 minutes after the challenge.

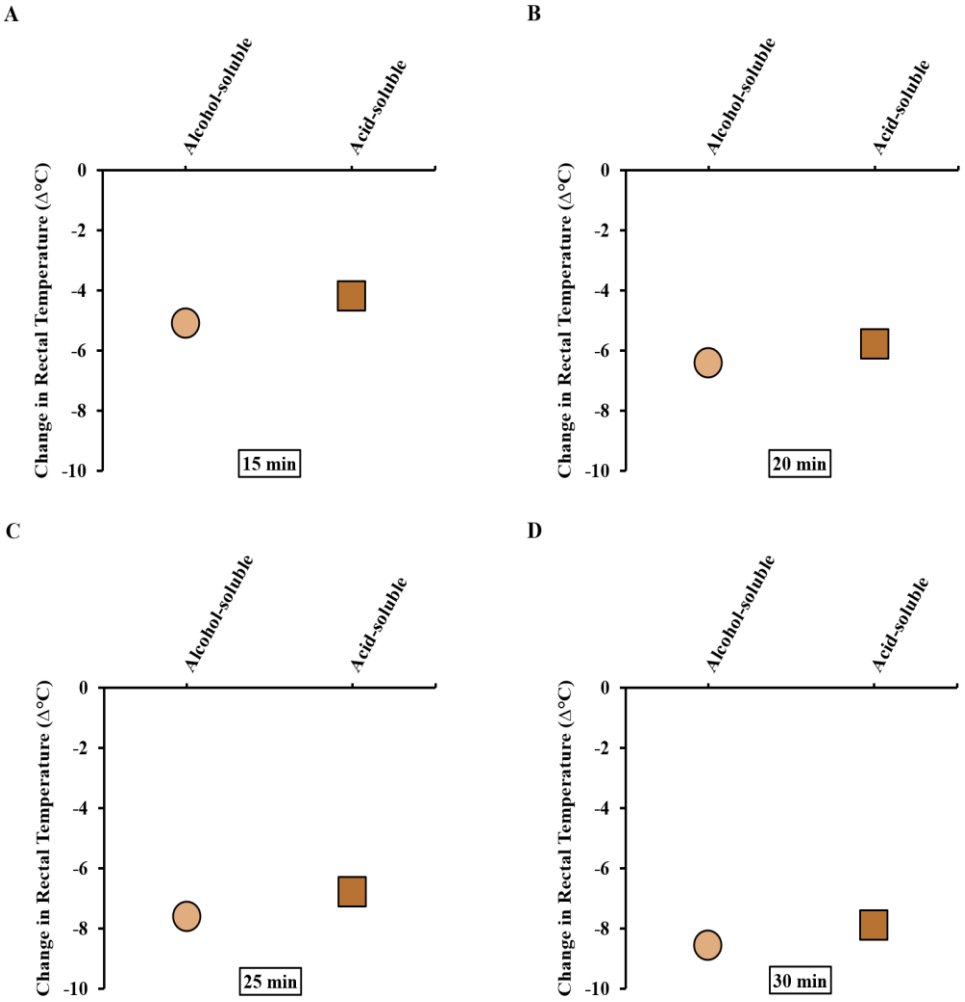

Figure 8.8 Comparative map of the intrinsic allergenicity disease elicitation potential of alcohol- and acid-soluble gluten protein extract from *Aegilops tauschii*.

(A, B) HSRs at 15 and 20 min after systemic challenge with 0.5 mg of alcohol- or acid-soluble gluten protein extract from *Aegilops tauschii*, respectively. (C, D) HSRs at 25 and 30 min after systemic challenge with 0.5 mg of alcohol- or acid-soluble gluten protein extract from *Aegilops tauschii*, respectively.

8.3.3.3 Comparative Map of the Inherent Disease Elicitation Potential of Glutens from *Aegilops tauschii* as Measured by MMCR

We utilized the MMCP-1 data obtained from systemic allergen challenges in the validation studies of alcohol- and acid-soluble gluten extracts from *Ae. tauschii* to construct a comparative Mast Cell Degranulation (MMCR) elicitation potential map. Figure 8.9 presents the MMCR elicitation potential map at 0.5 mg systemic allergen challenge. It is apparent that the acid-soluble gluten extract induced the highest MMCR.

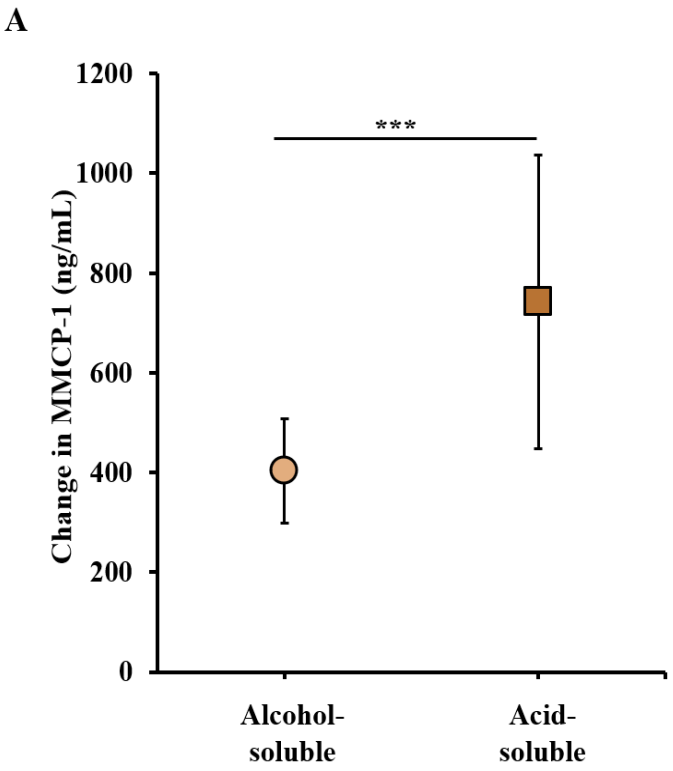

Figure 8.9 Comparative map of the mucosal mast cell response (MMCR) elicitation potential of alcohol- and acid-soluble gluten protein extracts from *Aegilops tauschii*.

Average MMCP-1 blood level after 0.5 mg systemic allergen challenge dose. n = 10/group. *** $p < 0.05$, student's t-test MMCP-1: mucosal mast cell protease-1. n: number of mice.

8.4 Discussion

Aegilops tauschii is the ancient progenitor with the DD genome that enabled the creation of the modern hexaploid bread wheat (AABBDD genome) (Awan et al., 2022). However, the potential allergenicity of gluten from the ancient *Ae. tauschii* wheat is unknown. Therefore, this study was undertaken to test the hypothesis that glutens from *Ae. tauschii* would induce clinical sensitization for life-threatening anaphylaxis in an adjuvant-free mouse model. Collectively, our data upholds this hypothesis.

There are 9 novel findings in this study: i) alcohol-soluble gluten (gliadin) extract from *Aegilops tauschii* is intrinsically allergenic because chronic skin exposure elicits specific IgE antibody response in Balb/c mice; ii) skin exposure to alcohol-soluble gluten extract from *Aegilops tauschii* is capable of clinically sensitizing mice for life-threatening anaphylaxis because dramatic hypothermic shock responses (HSR) were observed upon systemic challenge with alcohol-soluble gluten extract in skin-sensitized mice; iii) systemic anaphylaxis to alcohol-soluble gluten extract was associated with elevation of mucosal mast cell protease-1 (MMCP-1) in the blood suggesting that mucosal mast cell response (MMCR) is IgE-antibody mediated; iv) acid-soluble gluten (glutenin) extract from *Aegilops tauschii* is intrinsically allergenic because chronic dermal exposure to acid-soluble gluten extract from *Aegilops tauschii* induces a significant specific IgE antibody response in Balb/c mice; v) skin exposure to acid-soluble gluten extract from *Aegilops tauschii* is capable of clinically sensitizing mice for life-threatening anaphylaxis because dramatic hypothermic shock responses (HSR) were observed upon systemic challenge with acid-soluble gluten extract in skin-sensitized mice; vi) systemic anaphylaxis to acid-soluble gluten extract was associated with elevation of mucosal mast cell protease-1 (MMCP-1) in the blood suggesting that mucosal mast cell response (MMCR) is IgE-antibody

mediated; vii) the relative sensitization capacities of both the alcohol- and acid-soluble gluten extracts from *Ae. tauschii* were found to be similar in this model; viii) the relative anaphylactic disease eliciting capacity as measured by HSR, were strikingly similar between the two types of glutens from *Ae. tauschii*; and ix) Interestingly, although both glutens elicited significant MMCR, the acid-soluble gluten was more potent compared to the alcohol-soluble gluten.

We chose to utilize a recently described adjuvant-free mouse model to test the allergenic potential of glutens from *Ae. tauschii* because: i) previously, this model was validated using alcohol-soluble gluten extract from *Triticum durum* (AABB) and acid-soluble gluten extract from *Triticum aestivum* (AABBDD) (Jorgensen et al., 2023a,b); therefore the genetic contribution of the DD genotype to gluten allergenicity of modern wheat could be elucidated; ii) as opposed to adjuvant-based models, this model that does not use adjuvant to elicit sensitization to wheat proteins provides an opportunity to evaluate the inherent potential of glutens from ancient wheat; iii) this model uses a humane approach to elicit sensitization by application of wheat proteins on intact, undamaged skin, without the need of deliberate skin damage commonly used in other rodent models to develop skin-sensitization; iv) the quantitative readouts of systemic anaphylaxis (HSR) and mucosal mast cell responses (MMCP-1) had been validated providing opportunity to objectively determine disease eliciting potential of *Ae. tauschii*.

In this study we chose to evaluate the allergenicity potential of the ancestral *Aegilops tauschii* wheat because: i) *Ae. tauschii* is the ancestral uncultivated wild progenitor that provided modern bread wheat (*Triticum aestivum*, AABBDD genome) with the DD genome; however, its allergenicity was unknown (Awan et al., 2022; de Sousa et al., 2021); ii) at our university we had the unique access to *Ae. tauschii* seeds and experimentally cultivated the *Ae. tauschii* crop; iii) previously the *Ae. tauschii* whole genome has been deciphered with the hope of creating

improved varieties of modern wheat that are safer and more nutritious (Luo et al., 2017); and iv) there are scientific discussions in the literature debating the allergenicity of ancient wheats compared to that of the modern widely consumed wheats, however the results are still inconclusive (Shewry, 2018).

The mechanism of gluten allergy occurs in two phases. The first phase occurs when genetically predisposed individuals are exposed to gluten through the skin or other routes. This exposure leads to the production of gluten-specific IgE antibodies (Platts-Mills, 2015; Renz et al., 2018; Sicherer & Leung, 2015). These antibodies then attach to mast cells and basophils via high affinity IgE-receptors, resulting in a sensitized immune state. The second phase occurs when a sensitized individual is re-exposed to gluten. The gluten allergens bind to the IgE-loaded mast cells and basophils, triggering the release of chemical mediators that cause clinical disease including systemic anaphylaxis (Jin et al., 2019). Therefore, in order to evaluate sensitization potential of *tauschii* glutens, we measured specific IgE antibodies, using a ultra-sensitive ELISA-based method that we have reported previously (Gao et al., 2023; Jorgensen et al., 2023a,b). Our data demonstrates that both types of gluten extracts from *Aegilops tauschii* have the inherent capacity to elicit sensitization via skin-exposure.

The hypothermic shock response (HSR) represents the consequence of anaphylaxis on neurological and cardiovascular functions involved in thermoregulation. HSR, measured via rectal thermometry, is a widely used method to quantify the severity of anaphylaxis in mouse models of food allergy, as it is considered a proxy marker of cardiac output which is adversely affected in food-induced systemic anaphylactic reactions (Gao et al., 2023; Gonipeta et al., 2015; Gouel-Chéron et al., 2023; Jorgensen et al., 2023a,b). However, in mouse models of anaphylaxis, HSR can result from both IgE and IgG mediated reactions upon intraperitoneal challenge

(Miyajima et al., 1997; Tsujimura et al., 2008). Our data demonstrates that HSR can be used to quantify systemic anaphylaxis induced by both gluten extracts from *Ae. tauschii* wheat in this mouse model.

Mucosal mast cell protease-1 (MMCP-1) was previously reported as a specific biomarker of IgE-antibody, but not IgG-antibody mediated systemic anaphylaxis in food allergy mouse models (Khodoun et al., 2011). Since HSR reflects both IgE and IgG1-mediated systemic anaphylaxis, we chose to use MMCP-1 as an additional biomarker to specifically determine whether glutens from *Aegilops tauschii* are capable of eliciting life-threatening anaphylaxis via IgE pathway and whether the two types of glutens differ in their capacity to initiate the IgE-mediated pathway of disease elicitation. Furthermore, the only source of MMCP-1 in mice is the mucosal mast cell present in the gut. The gastrointestinal tract harbors mucosal mast cells containing MMCP-1 in the granules, which are a distinctive protein found exclusively in mucosal mast cells and absent in connective tissue mast cells (Andersson et al., 2008). These gut mucosal mast cells possess receptors for IgE antibodies. Following sensitization to gluten, IgE antibodies attach to mucosal mast cells through high-affinity IgE antibody receptors (FcϵRI). In the pre-challenge samples, blood exhibits minimal background levels of MMCP-1, since the mucosal mast cells remain inactive. However, upon challenge with gluten, the gluten binds to the IgE antibodies on the gut mast cells, triggering the activation and degranulation of the mast cell. This process releases MMCP-1 into the systemic circulation. Therefore, the MMCP-1 levels one hour after challenge indicates IgE-antibody mediated anaphylaxis in the gut which simulates the natural IgE-mediated reactions that occur in gluten allergy upon ingestion of gluten containing products. Our data demonstrates that both gluten extracts can activate this pathway; however, the acid-soluble gluten extract appears to be somewhat superior in eliciting MMCR.

In this study, we compared the sIgE eliciting capacity of alcohol-soluble gluten (gliadins) and acid-soluble gluten (glutenins) from *Aegilops tauschii*. Our data suggests that the gliadins are slightly more allergenic than glutenins in this model, although the difference was not statistically significant. There are no previous studies comparing allergenicity of two types of glutens from *Ae. tauschii* in animal or human systems. There are two studies comparing the two types of glutens from *Triticum aestivum* wheat cultivars for binding to IgE from human wheat allergic subjects (Lee et al., 2022; Yanbo Wang et al., 2021). Both studies report that gliadins are more allergenic than glutenins. It is interesting that two types of glutens from the ancient grain that contributed to the development of *T. aestivum* also show similar trend of allergenicity.

In this study, we found that two types of glutens from *Ae. tauschii* elicited strikingly similar HSR kinetics and intensity of HSR. However, they significantly differ in eliciting MMCR with glutenin being more potent than gliadin. The discrepancy between the two findings may be explained as follows: whereas in HSR, both IgG1 and IgE antibodies participate, in MMCR only IgE antibodies participate in eliciting reactions. Therefore, the two types of glutens may differ in the ability to activate the gut allergic reactions, because MMCR is entirely confined to the gastrointestinal tract. There are no previous studies comparing two types of glutens for eliciting HSR or MMCR.

There are suggestions that the ancient wheat grains may be more tolerable for individuals with wheat allergy compared to the modern wheat varieties (Shewry, 2018). This hypothesis was developed to explain why wheat allergies and other immune-mediated reactions to wheat have been increasing in recent decades. This is an area of great interest for scientists with very limited investigation (Shewry, 2018). Therefore, the present research was undertaken to evaluate this hypothesis using the ancient *Ae. tauschii* wheat progenitor. Our results clearly demonstrate that

both types of glutens are indeed highly allergenic in this model. We would like to caution that the results from this study may or may not be applicable to human situation because this is a mouse model study. Future investigation is needed to confirm the findings from this study in human subjects with wheat allergy. For example, *Ae. tauschii* gluten samples can be tested by ELISA, RAST, and Skin prick testing in wheat allergic individuals. Furthermore, oral challenge tests can also be conducted to confirm the preclinical mouse model findings.

In summary, this study collectively reveals for the first time that both the alcohol- and acid-soluble gluten from *Aegilops tauschii* are intrinsically allergenic in this mouse model. We also demonstrate that although both glutens show similar sensitization capacities and systemic anaphylaxis eliciting capacities, glutenins may be more potent in activating gut allergic reactions.

8.5 Materials and Methods

8.5.1 Chemicals and reagents

Biotin-conjugated antibodies specific to mouse IgE were obtained from BD Bio-Sciences (San Jose, CA, USA). The p-nitrophenyl phosphate compound was sourced from Sigma (St. Louis, MO, USA), and Streptavidin alkaline phosphatase was acquired from Jackson ImmunoResearch (West Grove, PA, USA). Folin reagent was obtained from Bio-Rad (Hercules, CA). Specific reagents, including the IgE Mouse Uncoated ELISA Kit with Plates, Streptavidin-HRP, TMB substrate, MCPT-1 (mMCP-1) Mouse Uncoated ELISA Kit with Plates, Avidin-HRP, and TMB substrate, were procured from Invitrogen (MA, USA). The Tissue Protein Extraction Reagent (T-PERTM), a proprietary detergent with a composition of 25mM bicine and 150mM sodium chloride at pH 7.6, was obtained from ThermoFisher Scientific (MA, USA). For

protease inhibition, a cocktail of serine, cysteine, and acid proteases, along with aminopeptidases, was acquired from Sigma-Aldrich (MO, USA).

8.5.2 Mice breeding and establishment of a plant-protein-free mouse colony

Breeder pairs of adult Balb/c mice were procured from The Jackson Laboratory (Bar Harbor, ME). Upon their arrival, the mice were introduced to a stringent plant-protein-free diet (AIN-93G, Envigo, Madison, MI). After a one-week acclimation period, conventional breeding methods were employed to initiate reproduction. Female mice aged 6-8 weeks from the litters were specifically chosen for this study. Throughout the entire research period, all mice were consistently maintained on the strict plant-protein-free diet (AIN-93G). All animal procedures strictly adhered to the guidelines established by Michigan State University.

8.5.3 Preparation of both alcohol-soluble and acid-soluble gluten extracts from ancient *Ae. tauschii* wheat

The ancient *Ae. tauschii* was grown at Michigan State University as described previously (Gao et al., 2023). Protein extraction from the hexaploid wheat flour targeted the isolation of alcohol- and acid-soluble wheat gluten using the Osborne sequential extraction method (Chen & Bushuk, 1969). Briefly, a mixture of flour and filter-sterilized 0.5 M NaCl (1:10, m/v) underwent continuous agitation for 2 hours, followed by centrifugation at 20000 x g for 30 minutes. The resulting pellets were preserved for alcohol extraction. The salt-insoluble pellets were then mixed in a 1:10 ratio with 70% ethanol for 2 hours, and centrifuged at 20000 x g for 15 minutes, yielding the alcohol-soluble gluten extract supernant and alcohol-insoluble pellets. The latter was saved for acid extraction. The alcohol-insoluble pellets were combined in a 1:4 ratio with 0.05 M

acetic acid for two hours and centrifuged at 20000 x g for 15 minutes. The resulting supernatants from both extractions were frozen at -70°C overnight and subjected to freeze-drying the next day. The lyophilized alcohol-soluble gluten extract was reconstituted using 70% ethanol and the acid-soluble gluten extract was reconstituted using 0.05 M acetic acid to achieve a concentration of 1 mg protein per 100 µL for topical application. For challenges involving intraperitoneal (IP) injections, the gluten extracts were reconstituted with phosphate-buffered saline (PBS) to attain concentrations of 0.5 mg/mouse. Protein content was quantified using the LECO total combustion method from LECO (St. Joseph, MI). SDS-PAGE testing was conducted to assess protein quality.

8.5.4 Skin sensitization, bleeding, and plasma sample preparation

Female adult Balb/c mice were utilized for experimental purposes. To facilitate the procedures, the hair on the mice's rumps was bilaterally removed using a Philips hair clipper (Amsterdam, Netherlands). The wheat gluten extracts were administered onto the rump at a dosage of 1 mg per mouse in 100 µL, alternately using a vehicle solution of 70% ethanol or 0.05 M acetic acid. Following application, the treated area was covered with a non-latex bandage sourced from Johnson & Johnson (New Brunswick, New Jersey) and left in place for one day. This process was repeated on a weekly basis, occurring nine times over a span of nine weeks. Blood samples were collected from the saphenous vein before the initial exposure and after the sixth exposure. The blood was drawn into tubes coated with the anticoagulant lithium heparin (Sarstedt Inc MicrovetteCB 300 LH, Germany). The collected blood was subsequently subjected to centrifugation to isolate plasma, which was then stored individually at -70°C until required for subsequent testing of (s)IgE.

8.5.5 Elicitation of systemic anaphylaxis

Two weeks after the final cutaneous exposure to alcohol or acid-soluble protein extract or the vehicle, the mice underwent an intraperitoneal (IP) injection. This injection involved either 0.5 mg of alcohol or acid-soluble gluten extract, or the vehicle (phosphate-buffered saline, PBS).

8.5.6 Determination of hypothermic shock responses

Rectal temperature (°C) measurements were recorded both before the challenge and at 5-minute intervals following the challenge, up to a 30-minute duration. A rectal thermometer (DIGI-SENSE, MA, USA) was used for these measurements. The recorded values included the specific temperatures and the corresponding differences (Δ°C) compared to the pre-challenge temperatures for each individual mouse. These recorded data points were subsequently utilized for further analyses.

8.5.7 Measurement of specific IgE antibody levels

Gluten-specific (s) IgE antibody levels were quantified using a highly sensitive ELISA method, as previously detailed with certain modifications (Gao et al., 2019; Jin et al., 2020; Jin et al., 2017; Jorgensen et al., 2023a,b). Initially, 96-well Corning 3369 plates were coated with WG and subsequently blocked using a 5% gelatin solution. After a thorough washing step, plasma samples were introduced onto the plate. Further washing ensued, followed by the addition of a biotin-conjugated anti-mouse IgE antibody. Subsequent washes were performed before introducing streptavidin alkaline phosphatase, and eventually, p-nitrophenyl phosphate was added to enable quantification, following established methodologies (Gao et al., 2019; Jin et al., 2017; Jorgensen et al., 2023a,b).

8.5.8 Quantification of mucosal mast cell protease-1 (MMCP-1) level

One hour post-challenge, blood samples were obtained and employed to measure the levels of mucosal mast cell protease-1 (MMCP-1) in the plasma. The quantification was performed using an ELISA-based method developed by Invitrogen, following established procedures. In detail, 96-well Corning Costar 9018 plates were initially coated with a capture antibody (anti-mouse MMCP-1). Subsequently, both samples and standards (recombinant mouse MMCP-1) were added to the plate. A biotin-conjugated anti-mouse MMCP-1 antibody was then introduced as the secondary antibody. Detection was achieved using an avidin-HRP/TMB substrate system. It is noteworthy that the assay has a limit of detection set at 120 pg/mL, and the range of standards covered from 120 to 15000 pg/mL. Testing was conducted in quadruplicate for each individual mouse's plasma.

8.6 Conclusion

These results demonstrate that both glutens from the ancient *Ae. tauschii* wheat have the inherent potential to elicit clinical sensitization for life-threatening anaphylaxis.

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CHAPTER 9 CONCLUSIONS AND FUTURE DIRECTIONS

In the present work an adjuvant-free mouse model was developed, characterized, and validated for testing allergenicity of two types of gluten extracts obtained from four different wheat species. A comparative map of intrinsic allergenicity of glutens from four wheat species was created for the first time. This model may be used to advance basic and preclinical research on wheat gluten allergy. Future research directions where the validated mouse model may be used are illustrated (Figure 9.1) and summarized below.

9.1 Safety assessment of glutens from genetically distinct wheats and novel wheats such as genetically engineered wheats for allergenicity

Thousands of genetically distinct wheat varieties, cultivars, and wheat lines are currently consumed worldwide (Shewry et al., 2009). The validated TS/SE mouse model as outlined in this dissertation offers a valuable preclinical testing approach to assess the varying levels of gluten allergenicity among these wheat types. Recent reports show reduced allergenicity of wheats upon gene editing using advanced technology such as (Kohno et al., 2016; Yu et al., 2023). Such genetically altered wheats can be tested for allergenicity using the TS/SE mouse model. This enables the identification of potential hypoallergenic, hyper-allergenic, and non-allergenic wheats.

Currently, genetically engineered (GE) wheats using recombinant DNA technology for commercial purpose are unavailable in the United States. However, recently the US FDA approved a GE wheat solely for cultivation in Argentina (US FDA 2022). This wheat is genetically engineered to have a modified HaHB4 transcription factor from *Helianthus annuus* (sunflower) allowing for greater tolerance to environmental stress such as drought resistance as

well as phosphinothricin acetyltransferase (PAT) from *Streptomyces hygroscopicus* for greater herbicide resistance.

Therefore, the TS/SE model could provide insight to the allergenic properties of this novel GE wheat line to determine substantial equivalency. Thus, this model holds a promise for evaluating the safety of gluten from GM/GE wheats during their developmental stages as new GM/GE wheats are on the near horizon (Yankelevich, 2021).

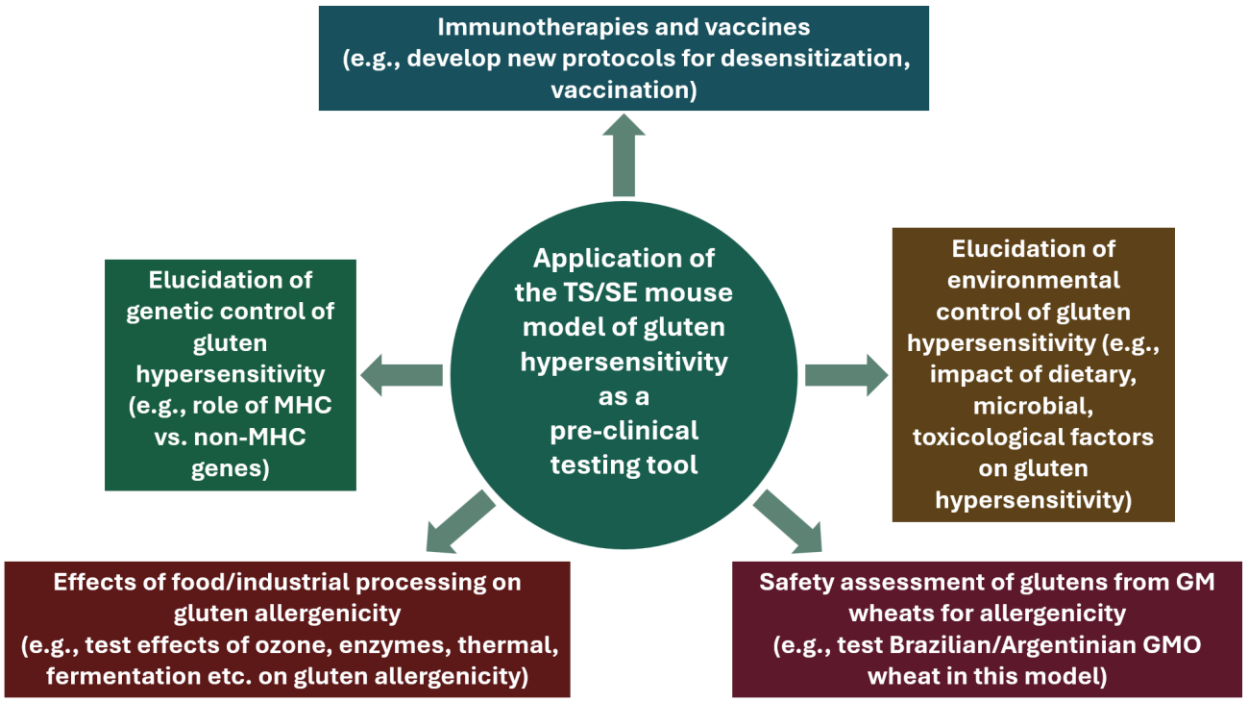

Figure 9.1 Potential application of the TS/SE model of gluten allergenicity. The validated pre-clinical mouse model reported in this dissertation may be used in at least five different areas of research on gluten hypersensitivity.

9.2 Effects of food/industrial processing on gluten allergenicity

As detailed previously, both food and industrial processing can exert substantial influence on wheat gluten allergenicity (Chapters 2 and 3; Gao et al., 2021; Lupi et al 2019; De Angelis et al., 2007). Utilizing the validated TS/SE mouse model allows for the examination of these processes on the intrinsic gluten allergenicity *in vivo* for the first time. Therefore, this model will

be useful in developing novel processing methods that are aimed at reducing wheat gluten allergenicity, with the hopeful prospect of creating hypoallergenic food products. In addition, potentially deadly hyper-allergenic glutens can be proactively identified and removed from human exposure.

9.3 Elucidation of genetic control of gluten hypersensitivity

The validated TS/SE mouse model offers a valuable method for unraveling the genetic underpinnings of sensitization as well as elicitation of systemic anaphylaxis in gluten. Gluten hypersensitivity is a complex immune disorder where both genetic susceptibility as well as environmental factors play key roles in pathogenesis (Jin et al., 2019; Fukunaga et al., 2021; Noguchi et al., 2019). Role of MHC vs. non-MHC genes in celiac disease has been established in humans (Sollid et al., 2012). However, the role of MHC or non-MHC genes in gluten hypersensitivity is understudied. This mouse model provides a unique opportunity to advance this area of research. For example, previous studies have shown that genetic mutations in a non-MHC gene (filaggrin) is associated with wheat-dependent exercise-induced anaphylaxis (WDEIA) in humans (Iga et al., 2013). Potential role of filaggrin gene mutations in mice can be tested using TS/SE model (Bodinier et al., 2009).

9.4 Elucidation of environmental control of gluten hypersensitivity

While genetical susceptibility is a perquisite for gluten allergy development, the recent surge in gluten allergy over a period of five decades suggests the involvement of unknown environmental triggers (Leoanrd & Vasagar, 2014; Calabriso et al., 2022). Various environmental candidates that might promote gluten hypersensitivity include detergents,

cosmetics, antibiotics, antimicrobial agents such as triclosan, as well as the influence of vitamin D and the microbiome (Wu et al., 2018; Kourosh et al., 2018; Marshall et al., 2017; Walters et al., 2017). However, the specific roles of these factors in gluten allergenicity remains unknown. Therefore, the utilization of the TS/SE model outlined in this dissertation allows for the elucidation of the effects of these environmental factors on the development of gluten allergy.

9.5 Developing improved immunotherapies and vaccines for gluten allergy

There are well established immunotherapy protocols for treating airways allergies and some food allergies such as egg allergy by inducing desensitization. (Sagara et al., 2021; Palosuo et al., 2021). For example, sub lingual immunotherapy SLIT. Further improvements to such immunotherapy protocols can be made using the preclinical TS/SE mouse model.

Currently, there is no available vaccine for preventing gluten allergy. However, there are recent efforts to develop vaccines for other types of food allergies such as peanut, egg, sesame etc. (US FDA, 2024). No vaccine efforts are underway for gluten allergy. The TS/SE mouse model outlined in this dissertation offers a promising avenue for the advancement of innovative strategies aimed at managing and preventing severe wheat food allergies.

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