



Cardiac mMCP-4+ mast cell expansion and elevation of IL-6, and CCR1/3 and CXCR2 signaling chemokines in an adjuvant-free mouse model of tree nut allergy

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ARTICLE INFO

Article history:

Received 16 October 2014

Received in revised form

13 November 2014

Accepted 14 November 2014

Available online 2 December 2014

Keywords:

Hazelnut allergy
Cardiac mast cell
Intestinal mast cell
Mouse model
Cytokines
Chemokines

ABSTRACT

Background: Nut allergy is a growing and potentially fatal public health problem. We have previously reported a novel mouse model of near-fatal hazelnut (HN) allergy that involves transdermal sensitization followed by oral elicitation of allergic reactions. Here we studied the cardiac mast cell and cardiac tissue responses during oral nut induced allergic reaction in this mouse model.

Methods: Groups of mice were sensitized with HN and specific and total IgE were measured by ELISA. Oral allergic reaction was quantified by rectal thermometry and plasma mouse mast cell protease (mMCP)-1 by ELISA. Cardiovascular functions were determined by a non-invasive tail cuff method. Mucosal mast cells (MMC) and intestinal connective tissue MC (CTMC) were studied by immunohistochemistry (IHC) for mMCP-1 and mMCP-4 protein expression respectively. Cardiac MC were studied by toluidine blue (TB) as well as by the above IHC methods. Cytokines and chemokines in the tissues were quantified by a multiplex protein array method.

Results: Oral allergen challenge (OAC) of transdermal sensitized mice results in hypothermia, hypotension, tachycardia and rapid elevation of circulating mMCP-1. The IHC analysis of small intestine found significant expansion of mMCP-1+ MMCs and mMCP-4+ CTMCs. The TB analysis of cardiac tissues showed degranulation of majority of cardiac MCs. The IHC analysis of cardiac tissues showed very little mMCP-1 expression, but marked mMCP-4 expression. Furthermore, repeated OAC resulted in significant expansion of mMCP-4+ cardiac MCs in both the pericardium and the myocardium. Protein array analysis revealed significant elevation of cardiac IL-6 and CCR1/3 and CXCR2 signaling chemokines upon oral elicitation compared to sensitization alone.

Conclusion: These results demonstrate that: (i) besides the intestine, cardiac mast cells and the cardiac tissue respond during oral nut induced allergic reaction; and (ii) repeated oral elicitation of reaction is associated with cardiac mMCP-4+ mast cell expansion and elevation of cardiac IL-6, and CCR1/3 and CXCR2 signaling chemokines.

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Introduction

Food allergy is a growing, significant and serious public health problem in many countries (Sicherer and Sampson 2014; Gupta et al. 2011, 2013; Prescott et al. 2013). Tree nuts and peanuts in

particular, are the leading causes of near-fatal or fatal systemic allergic reactions to foods with incompletely understood mechanisms (Sicherer and Sampson 2014; Gupta et al. 2011). In view of the life-threatening nature of nut allergies, and the growing prevalence especially among the children, improved and highly effective therapeutic and preventive strategies are urgently needed (Wang and Sampson 2012; Berin and Sampson 2013). Animal models that simulate the human near-fatal nut induced allergies are particularly helpful to elucidate the mechanisms of such allergic reactions and on the long run may provide clues to develop novel

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preventive/therapeutic approaches (Wang and Sampson 2012; Berin and Sampson 2013; Gonipeta et al. 2015).

We previously developed, characterized and reported a novel adjuvant-free mouse model of life-threatening oral allergy using hazelnut as a model tree nut (Birmingham et al. 2005, 2007; Parvataneni et al. 2009; Gonipeta et al. 2010, 2013, 2015). This model uses a transdermal route for sensitization (TS) to hazelnut protein (without adjuvants such as alum or cholera toxin or tape-stripping), followed by oral elicitation (OE) of severe allergic reactions as measured by hypothermia shock response and pathological indication of intestinal tissue response (Birmingham et al. 2007). Major characteristics of this model include: (i) robust systemic Th2 cytokine (IL-4, IL-5 and IL-13) and GATA3 responses (Birmingham et al. 2005, 2007; Parvataneni et al. 2009; Gonipeta et al. 2010); (ii) expansion of specific subsets of T and B cells in the bone marrow and the mesenteric lymph nodes (Gonipeta et al. 2013); (iii) a major hazelnut allergen (*Cor a 9*) in this model is the same protein that was linked to life-threatening human oral allergic reaction to hazelnut (Birmingham et al. 2007; Beyer et al. 2002); (iv) dominant, non-MHC genetic control of oral allergic reaction with a key role for IL-4 but not IL-5/IL-13 (Parvataneni et al. 2009); and (v) long-term persistence of both sensitization as well as oral reactivity similar to most human nut allergies that are rarely outgrown (Gonipeta et al. 2010). Because this model simulates many aspects of the human life-threatening oral allergic reactions, it provides a valuable and unique tool to elucidate the mechanisms underlying such oral reactions (Gonipeta et al. 2015).

The extent of participation of heart tissue during tree nut induced oral allergic reactions is incompletely understood at present. Previous human studies show that cardiac mast cells respond to systemic exposure to allergens in situations such as wasp sting induced systemic reactions particularly in subjects with mastocytosis (Marone et al. 1995; Mueller 2007; Genovese et al. 2010). Furthermore, anaphylactoid reactions to radio contrast media are also thought to involve cardiac mast cell responses (Genovese et al. 1996). It is also well known that food induced systemic allergic reactions are associated with cardiovascular dysfunction such as hypotension and tachycardia (Sicherer and Sampson 2014; Berin and Sampson 2013; Wang and Sampson 2012). However, it is unclear at present whether such symptoms are associated with cardiac mast cell and cardiac tissue responses or simply due to compensatory physiological responses of the cardiovascular system to the fluid loss during systemic anaphylaxis. Although, it is assumed that all systemic anaphylaxis might involve cardiac mast cell responses, there is very little direct evidence for cardiac mast cell and cardiac tissue responses in tree nut induced systemic reactions in humans or in animal models. Elucidating cardiac tissue responses is critical because, it may provide novel clues to develop newer methods to manage/treat/prevent such reactions (Wang and Sampson 2012).

To address this critical gap in the knowledge here we studied the cardiac mast cells and the cardiac tissue responses during oral nut induced allergic reaction in an adjuvant-free mouse model of hazelnut allergy discussed above. We analyzed the cardiac responses in addition to the intestinal mast cell responses. We also performed protein array analysis for identifying specific changes in cytokine and chemokine protein levels in the cardiac tissues and determined whether such changes are due to sensitization alone or sensitization plus oral elicitation of allergic reactions. The results demonstrate that: (i) besides the intestine, cardiac mast cells and the cardiac tissue respond during oral nut induced allergic reaction; and (ii) repeated oral elicitation of reaction is associated with cardiac mMCP-4+ mast cell expansion and elevation of cardiac IL-6, and CCR1/3 and CXCR2 signaling chemokines.

Methods

Transdermal sensitization to hazelnut, followed by oral elicitation of systemic anaphylaxis

BALB/c female mice (6 weeks old) were purchased from The Jackson Lab (Bar Harbor, ME, USA). Animal protocols were approved by the Michigan State University Animal Care Committee. After pre-bleeding, mice were rendered allergic to hazelnuts via transdermal sensitization using the protocol described earlier (Birmingham et al. 2005, 2007). Briefly, groups of mice were exposed to hazelnut protein extract (Greer lab) (1 mg per mouse per dose) via transdermal route, once a week, for four weeks. Blood samples were collected every 15 days and used in measuring hazelnut specific IgE antibody response and total IgE levels by an optimized ELISA method as described before (Birmingham et al. 2003, 2005, 2007). After confirming sensitization, oral elicitation of immediate hypersensitivity reactions was examined by oral hazelnut protein challenge. In one experiment, oral challenge was done twice and in other experiments, repeated oral challenge was done for five times at weekly interval.

Quantitation of oral anaphylactic reaction: rectal thermometry and elevation of mouse mast cell protease (mMCP)-1 protein level in the plasma

After the oral challenge, anaphylaxis was quantified by hypothermia shock responses using rectal thermometry (Probe, Physitemp Instruments Inc., NJ, USA) as described earlier (Birmingham et al. 2007). Blood samples were collected immediately after rectal thermometry (1 h) and plasma separated and used in measuring protein levels of mMCP-1 using a commercial kit (eBioscience).

Acquisition of cardiovascular functions in mice

A non-invasive cardiovascular function acquisition system (Kent Scientific Inc.) that employs tail-cuff method was used to obtain all cardiovascular function data. Cardiovascular functions were obtained in naïve mice before sensitization, and after transdermal sensitization and after oral allergen challenge.

Tissue collection, histopathology and mast cell response analysis

After the last oral challenge mice were euthanized and small intestine and heart tissues were collected for histological analysis using an optimized method as described (Higuchi et al. 2008; Kitaura-Inenaga et al. 2003; Birmingham et al. 2007). Heart tissues were fixed in 10% buffered formalin (48 h) and then placed in cassettes and the cassettes were then placed in 70% alcohol containers. For small intestine, tissue was flushed with cold sterile saline and then placed into cassettes, fixed with 10% buffered formalin (48 h) and then stored in 70% alcohol containers for future analysis. The tissues were processed by the Michigan State University Histopathology and Cytology Service Laboratory using standard methods. Paraffin sections (Reichert Jung 2030 rotary microtome) were obtained and stained with mast cell specific staining with toluidine blue (1%) in aqueous sodium chloride solution pH 2.3 for 3 min followed by 3 distilled water rinses and quick dehydration through 95% and 100% ethanol, cleared in several changes of xylene.

Toluidine blue positive metachromatic mast cells were counted in the tissue sections from hazelnut allergic mice and non-allergic age and gender matched control mice. In general, 2–4 sections per tissue per animal were examined (objective: 43×; eyepiece: 10×; AmeriScope digital microscope). Numbers of mast cells (intact and

degranulated) in 20 high power fields (HPF) were counted and percent degranulation was calculated.

Immunohistochemistry analyses for mMCP-1 and mMCP-4 protein expression by tissue mast cells

The immunohistochemistry (IHC) staining was optimized for the best concentration of detection antibodies for mMCP-1 and mMCP-4 (US Biological, MA) by the Michigan State University Histopathology and Cytology Service Laboratory. Using optimized protocol, tissue sections were stained. Positively staining mast cells were counted and number of mMCP-1 and mMCP-4 positive cells in 20 high power fields was determined.

Protein array analyses of heart and intestinal tissues for cytokines and chemokines

The protein array study was conducted in collaboration with the Myriad RBM Inc. and the method optimized by the Myriad RBM Inc., was used to make the tissue extracts for protein array studies. Tissues collected after oral challenge (1 h) were snap frozen in liquid nitrogen and then stored at -70°C . Briefly, frozen vials containing samples were directly transferred to liquid nitrogen followed by addition of extraction buffer (T-PER, Thermo Scientific; 78510) containing protease inhibitors (Sigma, P8340). The ratio of tissues to buffer volume was kept constant for all the extractions. Tissues were homogenized immediately without the tissue being thawed for 30 s, twice. Then the samples were centrifuged at 4°C and supernatants collected and stored at -70°C . Sample aliquots were used in protein array analysis by the Myriad RBM Inc. Rodent custom MAP and Luminex technology were used in a multiplex analysis of samples for cytokines and chemokine proteins. This method requires the detection of a minimum of 50 beads per analyte per sample. The median fluorescence intensity value of the measured beads is then derived for each protein in the multiplexed assay. All samples were analyzed twice on two independent days in a blinded fashion.

Statistical analysis

Data from control and hazelnut allergic groups were compared using unpaired Student's *t* test (SlideWrite Program), with $p < 0.05$ indicating significant difference.

Results

Analysis of gut mast cell responses in a transdermal sensitization/oral elicitation adjuvant-free mouse model of tree nut allergy

Groups of adult female BALB/c mice were transdermal sensitized to hazelnut protein and systemic allergen specific IgE antibody responses and total plasma IgE levels were measured by ELISA. As evident, robust exposure dependent IgE responses were observed (Fig. 1A and B). Upon oral hazelnut protein challenge mice exhibited systemic anaphylaxis as quantified by hypothermia shock responses (Fig. 1C).

Since mouse mast cell protease (mMCP)-1 is a biomarker specifically for mucosal mast cell (MMC) responses and for IgE (but not IgG1) mediated anaphylaxis, we studied this protein (Khodoun et al. 2011). As evident, upon oral challenge sensitized mice showed elevated plasma levels of mMCP-1 protein providing direct evidence of IgE mediated MMC degranulation response (Fig. 1D) (Khodoun et al. 2011). Transdermal sensitization alone did not cause marked mMCP-1 elevation in the circulation.

We previously reported marked pathological changes particularly in the small intestine (duodenum) upon oral allergen challenge in sensitized mice and that transdermal sensitization alone does not cause disease/pathological changes in the gut (Birmingham et al. 2007). Then we studied MCs in the duodenum of mice that were orally challenged and compared them to vehicle control mice for mMCP-1 and mMCP-4 protein expression in the gut MCs using immunohistochemistry (IHC) methods. We noted significant increase in the total number of MMCs in the duodenum tissues suggesting tissue mastocytosis in HN allergic mice (Fig. 1E and F). Similarly significant expansion of mMCP-4+ CTMCs was also noted in HN allergic mice (Fig. 1G).

Analyses of cardiovascular functions in this mouse model of tree nut allergy

We studied the cardiovascular function before and after transdermal sensitization and after oral allergen challenge. As evident, transdermal sensitization alone did not markedly alter the systolic blood pressure (BP) (Fig. 2A), diastolic BP (Fig. 2C), the mean arterial pressure (MAP) (Fig. 2E) or the heart rate (Fig. 2G). Doses for oral challenge were optimized. Mice that were orally challenged with near-fatal dose (15 mg/mouse) elicited responses that were below the detection limit of the equipment. The mice that were orally challenged at this dose were clinically very moribund, but alive. Since most of these mice were alive until 1–2 h after oral challenge, it is unlikely that their heart had completely failed. Oral allergen challenge (10 mg/mouse dose) yielded consistent signals with significant drop in systolic BP (Fig. 2B), diastolic BP (Fig. 2D), MAP (Fig. 2F) and tachycardia (Fig. 2H).

Analyses of cardiac mast cell responses in this mouse model of tree nut allergy

We conducted initial histopathology studies using TB staining that stain cardiac MCs, to compare the cardiac MC degranulation responses upon two vs. repeated five oral allergen challenges. Significant degranulation of TB positive MCs was noted after two oral challenges (heart: allergic mice: $62 \pm 2.9\%$ vs. control mice $22 \pm 3.2\%$, $p < 0.01$); however, stronger cardiac MC responses were noted after repeated five oral challenges (Fig. 3A–C). Consequently, for rest of the studies, five oral challenge tissues were used in analysis.

Testing cardiac MCs for mMCP-1 expression using IHC method revealed that they were largely mMCP-1-ve in both non-allergic as well as hazelnut allergic mice (number of mMCP-1+ cells in the heart: allergic mice: 0 ± 0 ; $n = 12$ vs. control mice: 0.34 ± 0.08 ; $n = 20$; p : n.s.). However, majority of cardiac MCs expressed mMCP-4 in both the pericardium as well as the myocardium (Fig. 4A and B). We found significant expansion of mMCP-4+ MCs in both the pericardium as well as in the myocardium of allergic mice relative to non-allergic age matched control mice (Fig. 4C–E).

Analyses of cardiac tissue response for local pro-inflammatory cytokine and chemokine mediators in this mouse model of tree nut allergy

We tested whether oral allergic reactions are associated with specific changes in the cardiac chemokine and cytokine protein levels. The fold changes in protein levels in the heart tissue relative to the vehicle controls were determined using a protein array method (see Section “Methods”). Elevation of 2 fold or higher was considered significant. The result for cytokine protein fold elevation in the heart tissue is shown (Fig. 5A). There was significant elevation of only IL-6 by 6.3 fold (control mice: 0.86 ± 0.1 pg/mL vs. allergic mice: 5.45 ± 0.25 pg/mL, $p < 0.01$). Among the other cytokines

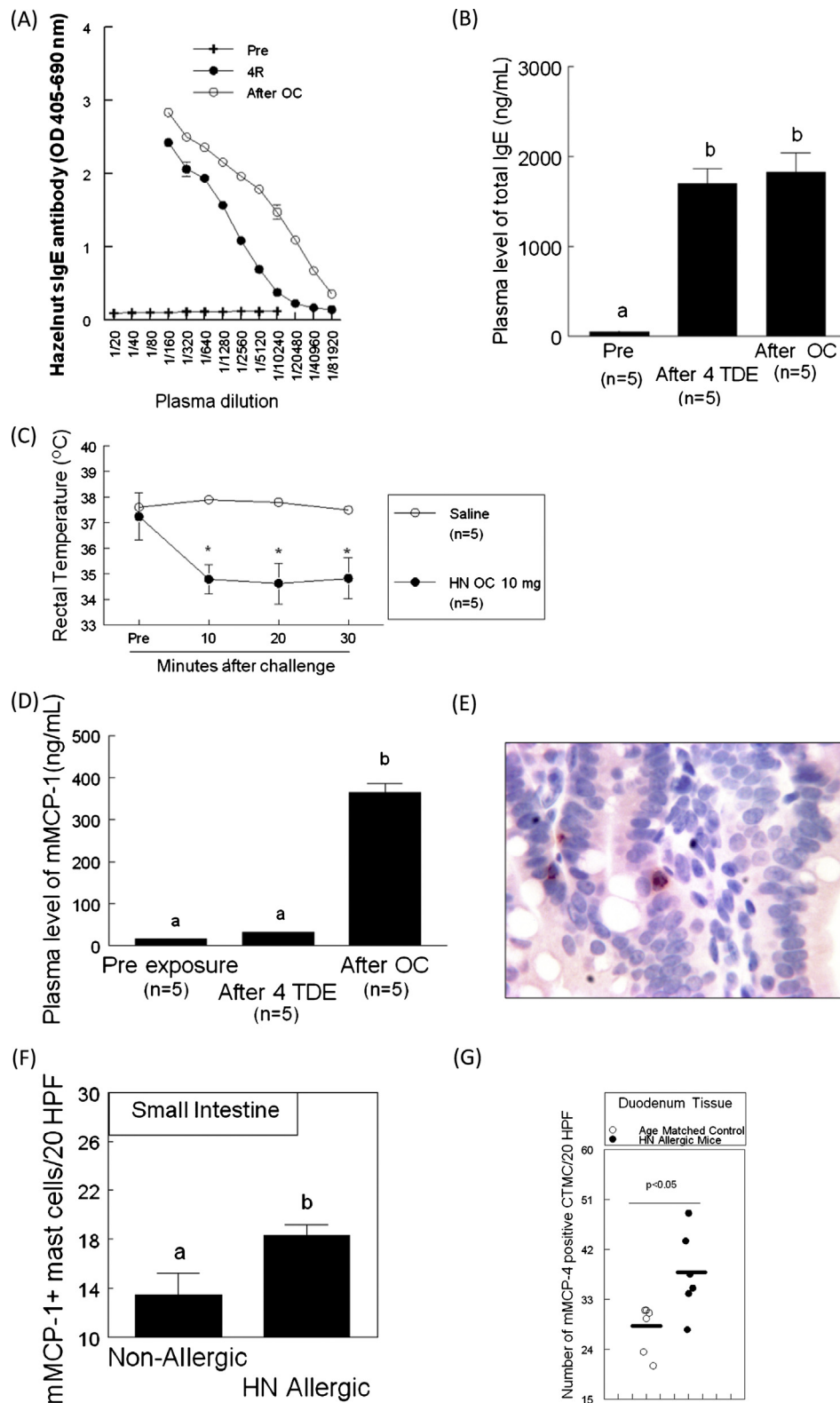


Fig. 1. (A–G) Characteristics of gut mast cell responses associated with hypothermia shock response in an adjuvant-free mouse model of hazelnut allergy. Groups of BALB/c mice were sensitized to hazelnut protein via transdermal exposure. (A) shows blood levels of hazelnut specific IgE antibody levels before (pre) and after 4 transdermal exposures (4R) and in the last blood sample that was collected after oral challenge (OC). (B) shows total plasma IgE levels in these samples; (C) shows kinetics of hypothermia shock response (indicator of systemic anaphylaxis) upon oral allergen challenge; (D) shows elevation of plasma mouse mast cell protease-1 (mMCP-1) in the last blood sample collected post OC (~1 h), but not after transdermal exposure; background levels of mMCP-1 in pre-exposure sample and after 4th transdermal allergen exposure (4TDE) are also shown; * Student's *t* test, $p < 0.05$; bars with different letters are significantly different, $p < 0.05$. (E) shows representative duodenum tissue section stained with anti-mMCP-1 antibody illustrating mucosal mast cells; 40× Objective; 10× Eye Piece; AmScope; images were captured using a digital camera (10 MP) and AmScope MT software; (F) shows total number of mMCP-1 positive mast cells in the duodenum from control age matched non-allergic vs. hazelnut allergic mice; bars with different letters are significantly different, $p < 0.05$; (G) shows total number of mMCP-4 positive connective tissue mast cells in the duodenum tissue from control age matched non-allergic vs. hazelnut allergic mice; * $p < 0.05$, Student's *t* test.

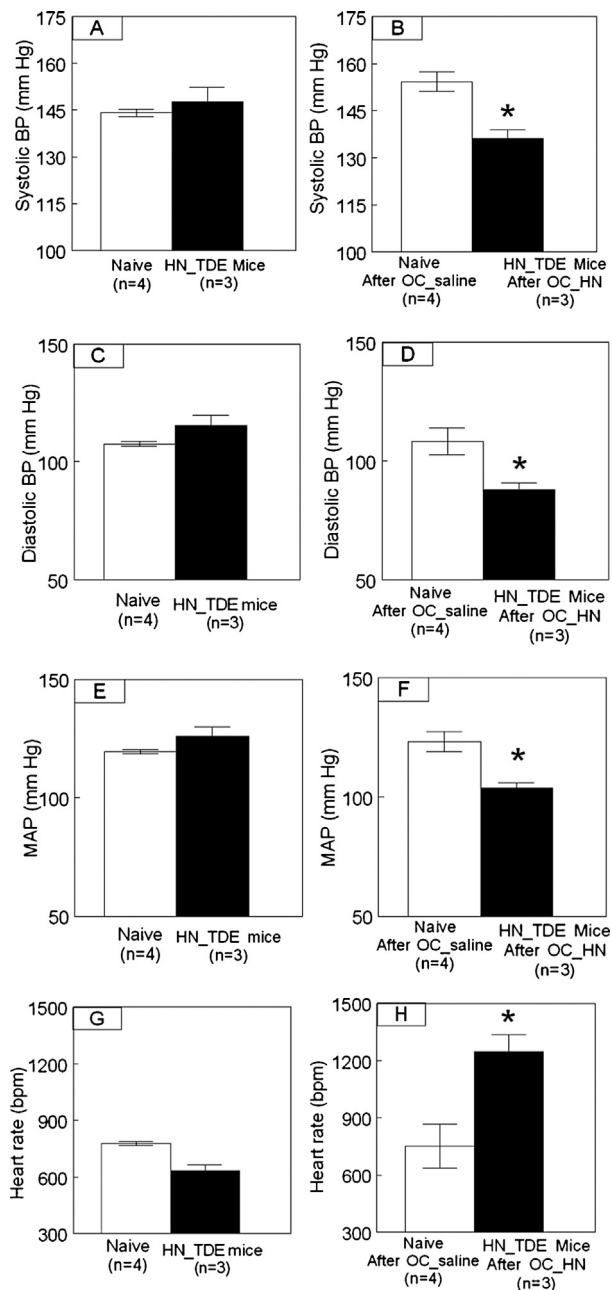


Fig. 2. (A–H) Transdermal sensitization does not alter the cardiovascular function, but oral elicitation of allergic reaction causes acute cardiac dysfunction. Cardiovascular function was assessed using a non-invasive method (Kent Scientific Inc.) as described in the text. (A, C, E and G) show profile of systolic BP (A), diastolic BP (C), mean arterial pressure (E) and heart rate (G) in naïve mice vs. mice after transdermal sensitization to hazelnut. (B, D, F and H) show changes in systolic BP (B), diastolic BP (D), mean arterial pressure (F) and heart rate (H) cardiovascular function after oral gavage with saline vs. hazelnut (10 mg/mouse). Data shows mean $\pm$ SE of read-outs collected for over 2 h time period; * $p < 0.05$, Student's *t* test; HN: hazelnut; TDE: transdermal exposure.

tested, only IL-17A was found to be elevated relative to the control mice; however it did not reach the 2 fold cut-off level used in this study. In contrast to heart tissue, in the duodenum, IL-18 was markedly elevated by ~ 17 fold. Other cytokine elevation did not reach the 2 fold cut levels.

The result for chemokine protein fold elevation in the heart tissue is shown (Fig. 5B). There was marked elevation of CCL11/eotaxin by ~ 2 fold (control mice: 46 ± 1 pg/mL vs. allergic

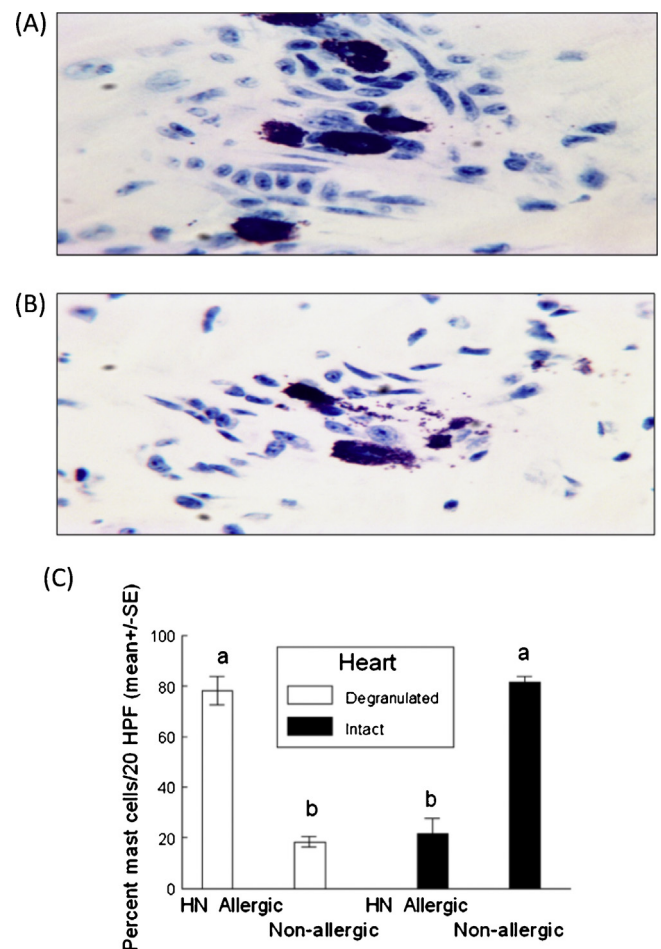


Fig. 3. (A–D) Cardiac mast cell degranulation response upon oral allergen challenge in hazelnut allergic mice. (A, B) show representative heart tissue section stained with toluidine blue illustrating cardiac mast cell degranulation in allergic mice compared to the intact cardiac MCs in age matched non-allergic control mice; 40 $\times$ Objective; 10 $\times$ Eye Piece; AmScope; images were captured using a digital camera (10 MP) and AmScope MT software. (C) shows results from toluidine blue analysis of relative % of degranulated vs. intact mast cells in the heart tissue collected from control age matched non-allergic mice and hazelnut allergic mice; bars with different letters are significantly different, $p < 0.05$.

mice: 91 ± 2.5 pg/mL, $p < 0.01$), CXCL1/KC/GRO by 5 fold (control mice: 8 ± 4 pg/mL vs. allergic mice: 40 ± 8 pg/mL, $p < 0.05$), CCL9/MIP-1 g by 2.2 fold (control mice: 425 ± 55 pg/mL vs. allergic mice: 930 pg/mL, $p < 0.01$), CXCL2/MIP-2 by 7.9 fold (control mice: 3.75 ± 0.95 pg/mL vs. allergic mice: 29.5 ± 1.5 pg/mL, $p < 0.01$), and CCL12/MCP-5 by 2.3 fold (control mice: 9.2 ± 0.8 pg/mL vs. allergic mice: 21.5 ± 0.5 pg/mL, $p < 0.05$). In the duodenum, there was marked elevation for CCL11/eotaxin (2.8 fold), CCL9/MIP-1 g (2.5 fold) and CCL5/RANTES (307 fold; control mice: 0.023 ± 0.001 pg/mL vs. allergic mice: 7.05 ± 1.45 pg/mL, $p < 0.05$) proteins.

We tested whether elevation of cytokines/chemokines noted in the heart was due to sensitization alone or due to the oral elicitation of allergic reaction in sensitized mice. Results for cytokines and chemokines are shown in Tables 1 and 2 respectively. As evident, among cytokines tested only IL-6 was significantly elevated in the heart tissue upon oral elicitation of allergic reaction relative to sensitization alone (Table 1). Among chemokines tested, CCL9, 11, 12 and CXCL2 were significantly elevated upon oral allergen challenge relative to sensitization alone; and CXCL1 elevation reached the border of significance ($p < 0.06$) (Table 2).

Table 1
Cardiac tissue cytokine protein levels in the heart of mice that were only sensitized vs. oral challenged sensitized mice.

Cytokine	Protein concentration ^a in the heart of mice after		<i>p</i> ^b
	Sensitization only (<i>n</i> = 5 mice) (mean ± SE)	Sensitization + oral elicitation (<i>n</i> = 5 mice) (mean ± SE)	
IFN-γ	30 ± 2	30 ± 1	<0.05
IL-1α	182.5 ± 10.5	214 ± 32	
IL-1β	0.72 ± 0.3	<0.69	
IL-2	41 ± 5	46.5 ± 2.5	
IL-3	1.42 ± 0.5	1.45 ± 0.1	
IL-4	15.5 ± 0.5	16.5 ± 0.5	
IL-5	<0.15	<0.15	
IL-6	1.57 ± 0.8	5.45 ± 0.2	
IL-7	<0.024	<0.024	
IL-10	383.5 ± 24.5	380 ± 59	
IL-11	<14	<14	
IL-12p70	<0.027	<0.027	
IL-17A	3.3 ± 0.1	3.5 ± 0.1	
IL-18	6.1 ± 0.6	6.3 ± 0.8	
TNF-α	33.5 ± 3.5	32.5 ± 0.5	

^a All values are in pg/mL except IL-1β, IL-5, IL-7, IL-12p70, IL-18 that are in ng/mL.
^b Student's *t* test; cytokine that is differentially elevated in the heart is identified in bold face.

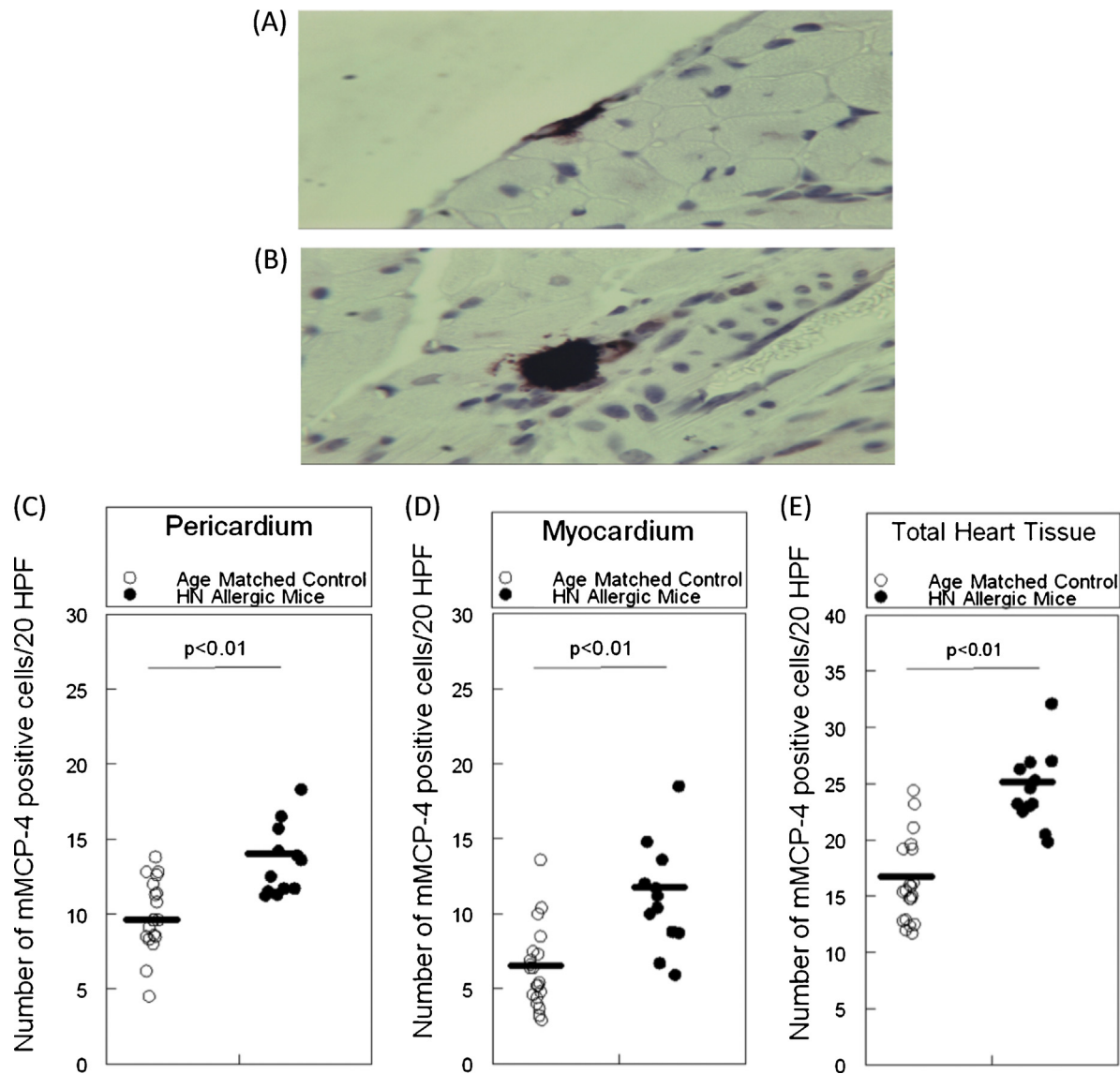


Fig. 4. (A–E) Significant expansion of mMCP-4 positive cardiac mast cells in hazelnut allergic mice. (A, B) show representative heart tissue section stained with antibodies against mMCP-4 illustrating mMCP-4 positive cardiac mast cells in the pericardium (A) and myocardium (B); 40× Objective; 10× Eye Piece; AmScope; images were captured using a digital camera (10 MP) and AmScope MT software; (C–E) show relative distribution of mMCP-4 positive mast cells in the pericardium (C), the myocardium (D) and the total heart tissue (E) of age matched non-allergic vs. hazelnut allergic mice; **p* < 0.01, Student's *t* test.

Table 2

Cardiac tissue chemokine protein levels in the heart of mice that were only sensitized vs. oral challenged sensitized mice.

Chemokine	Protein concentration ^a in the heart of mice after		<i>p</i> ^b
	Sensitization only (<i>n</i> = 5 mice) (mean ± SE)	Sensitization + oral elicitation (<i>n</i> = 5 mice) (mean ± SE)	
CCL11/Eotaxin	48 ± 0.0	90.5 ± 2.5	<0.003
CXCL6/GCP-2	305 ± 25	310 ± 30	
CXCL1/KC/GRO	13.15 ± 3.8	40 ± 6	<0.06
CXCL10/IP-10	11.9 ± 2.1	10.45 ± 0.5	
XCL1/Lymphotoxin	34.5 ± 5.5	30.5 ± 3.5	
CCL22/MDC	53 ± 5	78 ± 5	
CCL3/MIP-1a	<0.65	<0.65	
CCL4/MIP-1b	48.5 ± 11.5	100 ± 13	
CCL9/MIP-1g	540 ± 30	930 ± 0	<0.005
CXCL2/MIP-2	4.25 ± 1.2	29.5 ± 1.5	
CCL19/MIP-3b	375 ± 105	445 ± 55	<0.005
CCL2/MCP-1	20 ± 2	30.5 ± 2.5	
CCL7/MCP-3	15.5 ± 2.5	26.5 ± 2.5	
CCL12/MCP-5	11 ± 1	21.5 ± 0.5	
CCL5/RANTES	0.05 ± 0.005	0.03 ± 0.001	<0.01

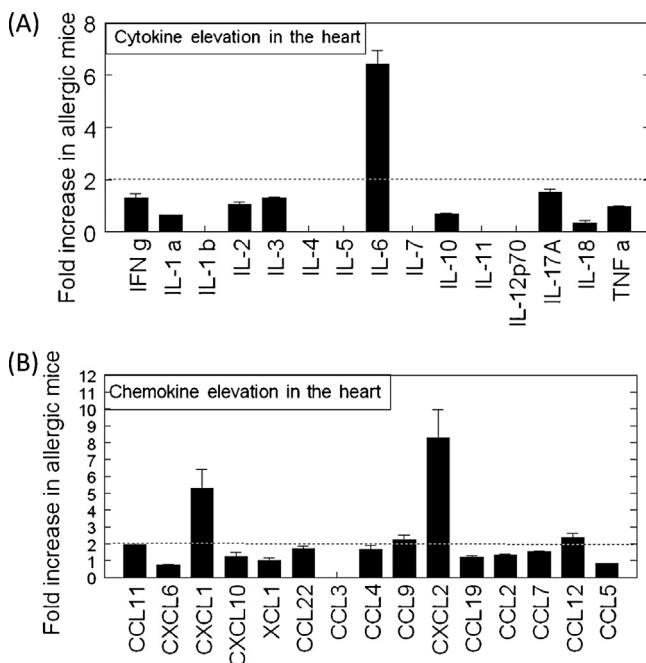
^a All values are in pg/mL except MIP-1a, that is in ng/mL.^b Student's *t* test; chemokines that are differentially elevated in the heart are identified in bold face.

Fig. 5. (A–B) Analyses of cardiac cytokine and chemokine responses in this mouse model of tree nut allergy. Groups of BALB/c mice (5/group) were sensitized to hazelnut protein via transdermal exposure as described in the text. After confirmation of IgE responses, mice received repeated five oral allergen challenges and then euthanized, organs collected and used in protein extract preparation. Indicated cytokine and chemokine protein levels were measured by a multiplex assay and fold enhancement in allergic mice relative to the non-allergic age matched control mice was determined as described in the text; (A) shows the fold change in the protein level of various cytokines in the heart tissue of allergic mice relative to the levels in the age matched control non-allergic mice. (B) shows the fold change in the protein levels of various chemokines in the heart tissue of allergic mice relative to the levels in the age matched control non-allergic mice. Broken line: Two fold or higher elevation in protein levels was considered significant.

Discussion

The goal of this study was to test the hypothesis that besides the intestine, cardiac mast cells and the cardiac tissue respond during oral nut induced allergic reaction in an adjuvant-free mouse model of tree nut allergy. Our data supports this hypothesis. Here we report several significant findings from this study: (i) oral

allergen challenge (OAC) results in intestinal mucosal mast cell (MMC) degranulation response as measured by elevated mMCP-1 in the circulation; allergic mice also show significantly increased number of mMCP-1+ MMCs and mMCP-4+ connective tissue MC (CTMC) in the duodenum; (ii) transdermal sensitization alone does not markedly alter the cardiovascular functions; however, OAC results in acute cardiac dysfunction with hypotension and tachycardia; (iii) oral allergic reactions are associated with degranulation of majority of cardiac MCs; and repeated OAC leads to cardiac mastocytosis characterized by expansion of mMCP-4+ but not mMCP-1+ MCs; and (iv) oral allergic reactions are associated with significant elevation of cytokine, IL-6 and a small group of chemokines that signal CCR1 (i.e., CCL9), CCR3 (i.e., CCL11/12) and CXCR2 (i.e., CXCL1/2) in the heart of allergic mice; transdermal sensitization alone does not markedly alter the cardiac levels of these mediators. Together these data suggest that besides the gut MC responses, cardiac MC and cardiac tissue responses also occur during nut induced oral allergic reactions in this mouse model.

There are no published reports on evaluating the gut MC responses in adjuvant-free mouse model of tree nut allergies. There are several studies examining the gut MC responses in adjuvant-based mouse models of peanut, ovalbumin and milk allergies and in a skin sensitization (by tape stripping) model of ovalbumin/peanut allergy (Li et al. 1999, 2000, 2001; Ganeshan et al. 2009; Ahrens et al. 2012; Khodoun et al. 2011; Bartnikas et al. 2013). Our data on MC degranulation responses and elevation of circulating mMCP-1 after oral allergen challenge suggest IgE mediated mucosal MC reactions; these results are consistent with previous studies in other adjuvant-based mouse models (cited above; Kulis et al. 2012). Our finding that both MMC and CTMCs expand in the gut is interesting because in an above cited study using tape-stripping skin sensitization model, expansion of sub-mucosal MCs (i.e., CTMCs) was noted (Bartnikas et al. 2013). This may be due to differences in the sensitization methods (skin tape-stripping method vs. no skin stripping) or the target tissue (jejunum vs. duodenum) studied or the method used to identify the MC (enzyme based method vs. IHC using specific antibodies to mMCP-1/4).

Involvement of cardiovascular system during systemic anaphylaxis to foods with symptoms of hypotension and tachycardia are reported in humans (Sicherer and Sampson 2014; Berin and Sampson 2013; Wang and Sampson 2012). Consistent with this situation, we noted acute hypotension and tachycardia in this mouse model as well suggesting similarity of the food induced severe reactions in both species. There are two previous studies

reporting changes in cardiovascular functions in mouse models of food allergy: (i) [Ganeshan et al. 2009](#) reported acute hypotension responses and tachycardia in a staphylococcus exotoxin adjuvant based mouse model of ovalbumin allergy; and (ii) [Liu et al. \(2008\)](#) reported hypotension responses in a soybean allergy mouse model. Here, we expand these findings further by providing evidence for acute cardiovascular dysfunction upon oral elicitation of reactions in an adjuvant-free tree nut allergy mouse model.

As noted above, a major goal of this study was to evaluate whether besides the gut, cardiac MCs and cardiac tissue participate during oral nut induced allergic reaction. It is noteworthy that cardiac MC responses and cardiac tissue mediator responses reported in this study are not available in the literature for human food allergy or in animal models of food allergy in general or for tree nut allergies in particular. It is generally assumed that cardiac mast cells might respond in food induced systemic reactions similar to what happens when allergens enter systemically in insect sting induced anaphylaxis or radio contrast media induced anaphylactoid reactions in some subjects particularly those with systemic mastocytosis ([Mueller 2007](#); [Marone et al. 1995](#); [Genovese et al. 1996, 2010](#)). It is noteworthy that BALB/c mice that were used in this study do not exhibit systemic mastocytosis phenotype. Furthermore, this mouse model of tree nut allergy does not use the systemic routes such as intraperitoneal or intramuscular routes of injection. Consequently, our data suggest that the observed cardiac MC/tissue responses most likely result from absorption of orally administered allergen. These findings are novel because this is the first study providing a direct evidence for the cardiac tissue responses in a mouse model of tree nut allergy.

We conducted initial studies and found that whereas two oral allergen challenges induced little cardiac mastocytosis, five oral challenges induced significant cardiac mastocytosis suggesting dose-response pattern between the frequency of oral elicitation and cardiac mastocytosis. Thus, here we report that repeated oral allergen challenge causes marked cardiac mastocytosis with mMCP-4+/mMCP-1-mast cell expansion in both the pericardium and the myocardium. These data are consistent with the hypothesis that continued oral exposure to the nut allergens (at sub-lethal quantities) among nut allergic subjects will have adverse effect on the heart tissue mast cell composition. Further studies are required to test this hypothesis.

There are no published reports of cardiac mastocytosis in human hazelnut or other tree nut allergy or any model of food allergy. However, cardiac mastocytosis has been reported in few animal models of other diseases including a mouse models of *Trypanosoma cruzi* infection induced cardiomyopathy ([Meuser-Batista et al. 2008](#)), a rat model of myocardial remodeling following volume overload ([Janicki et al. 2006](#); [Forman et al. 2006](#)); and in dog models of mitral regurgitation ([Stewart et al. 2003](#)) and myocardial ischemia ([Frangogiannis et al. 1998](#)). Thus, the finding of cardiac mastocytosis with mMCP-4+ MC expansion in hazelnut allergy is a novel finding in the food allergy field.

In order to test whether cardiac tissue responds during oral elicitation of allergic reactions, we determined the local tissue levels of a large panel of pro-inflammatory cytokine and chemokine proteins. Our analysis suggests marked elevation of a single cytokine in the heart – IL-6 and elevation of a small group of chemokines (CCL9/11/12 and CXCL1/2). It is noteworthy that all these mediators are known to be important mediators in mast cell biology and allergic reactions ([Gurish and Austen 2012](#); [Jia et al. 1996](#); [Gangur et al. 2003](#); [Zhang et al. 2007](#); [Tsuji et al. 1993](#)). Furthermore, comparing mice that were sensitized vs. those that were sensitized plus oral challenged we noted that elevation of these mediators in the heart tissue was largely in response to oral allergen challenge. Local elevation of these mediators at the minimum, imply local inflammatory response in the heart tissue.

Previous studies report that the cytokine IL-6 is not only produced by mast cells upon activation, but is also an important growth factor for mast cell differentiation from bone marrow stem cell ([Gurish and Austen 2012](#)). A recent study using IL-6 knockout mice and IL-6 transgenic mice with selective over expression within the heart tissue reports a key role for IL-6 in myocardial inflammation ([Zhang et al. 2007](#)). Another previous report in a mouse model of virus induced myocardial inflammation identified CXCL2 as a key player in cardiac inflammation ([Kishimoto et al. 2001](#)). The chemokine CCL9, an agonist of CCR1 contributes to hypersensitivity reactions ([Lean et al. 2002](#)). Chemokines CCL11/12 are known to signal the chemokine receptor CCR3 – a critical chemokine receptor in allergic responses and in coordinating cellular traffic in the allergic inflammatory tissues ([Kita et al. 1996](#); [Gangur et al. 2003](#)). Thus, the elevation in these mediators in the heart tissue provide molecular evidence for allergic inflammation in the heart.

We do not yet know whether cardiac mast cell expansion we noted is due to local proliferation or new recruitment cells. There are controversial reports in the literature with some suggesting that cardiac mast cell can proliferate and others reporting limited capacity for them to divide ([Reid et al. 2007](#); [Frangogiannis et al. 1998](#); [Padawer 1961](#); [Gurish and Austen 2012](#)). In a canine model of myocardial ischemia and reperfusion, cardiac mastocytosis was found to be not due to proliferation but was associated with new mast cell precursor recruitment ([Frangogiannis et al. 1998](#)).

Previous studies show that the chemokine receptor CXCR2 is required for constitutive homing of mast cell precursor to the gut tissue ([Abonia et al. 2005](#)). We noted that CXCR2 agonists (CXCL1/2) are markedly elevated in the heart tissue in this model and this is associated with cardiac mast cell expansion. Consequently, these data supports the hypothesis that elevation of CXCR2 agonists in the heart may contribute to the mast cell expansion via recruitment of CXCR2 expressing mast cell precursors to the heart tissue.

We did not determine the precise cellular source of the mediators in the heart tissue. However, previous studies show that MCs can synthesize and release these mediators during allergic responses ([Gurish and Austen 2012](#); [Gangur et al. 2003](#)). Our data show cardiac MC degranulation upon oral allergen challenge. Therefore, we hypothesize that these mediators in the heart tissue are mostly derived from activated cardiac MCs with additional contribution from macrophages. Further studies are needed to pin point the cellular sources for these mediators and to test whether local elevation of these mediators directly contributes to heart dysfunction (i.e., hypotension, tachycardia) in this model.

The data from this study suggests that heart is a direct target of oral allergen induced reaction in this model and that repeated sub-lethal allergen exposures can elevate the cardiac mast cell numbers. Consequently, if this situation also happens in humans with nut allergies, it would have significant clinical implications in both developing novel treatments (e.g., novel cardiac mast cell stabilizers, antagonist to IL-6/Chemokines/their receptors identified here; novel methods to deliver these antidotes locally within the heart) and improving the diagnosis of near fatal reactions with novel cardiac function monitoring methods (e.g., via developing novel methods to monitor cardiac tissue levels of specific mediators and analyze elevated levels of mediators identified in this study in the blood and/or using non-invasive methods) ([Santone et al. 2008](#); [Hsu et al. 2014](#); [Guttmann et al. 2014](#)). Furthermore, a nut induced systemic allergic reaction may have to be dealt similar to acute myocardial infarction ('heart attack'). Furthermore, effect of chronic oral exposure to low doses of allergens on changes in long-term cardiac cellular composition, structure and function will need to be evaluated.

In summary, these results demonstrate that: (i) besides the intestine, cardiac mast cells and the cardiac tissue respond during oral nut allergic reaction; and (ii) repeated oral elicitation of reaction is associated with cardiac mMCP-4+ mast cell expansion and elevation of cardiac IL-6, and CCR1/3 and CXCR2 signaling chemokines.

Conflict of interest

Authors have no conflict of interests to disclose.

Acknowledgements

This work was supported by funding from: the United States Environmental Protection Agency STAR Grant RD83482201; Ag Bio-Research/NIFA/MSU, MICL02023; the McNair Scholars Program (He, Y); Food Science & Human Nutrition, Michigan State University; EJ Kim was supported by a scholarship from the Brain Korea 21 Program, The Ministry of Education, Science, and Technology, South Korea.

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