

## **ROLE OF IL-1 $\alpha$ IN IgG1 MEDIATED ANAPHYLAXIS**

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TITLE: Role of IL-1 $\alpha$  in IgG1 Mediated Anaphylaxis

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## ABSTRACT

**BACKGROUND:** There is growing evidence indicating that skin can be an initiating site for allergic sensitization to peanut. Additionally, anaphylaxis can also be mediated by IgG1 and macrophages, known as the alternative pathway. In this setting, the allergen binds to serum IgG1 forming immune complexes which can bind to macrophages, basophils and neutrophils and cause the release of anaphylactic mediators.

**METHODS:** The model of epicutaneous sensitization used in this study relied on tape stripping the skin followed by direct application of peanut. Knockout mice and/or antibody neutralization studies were used to characterize anaphylaxis in this model. For the *in vitro* experiments, we used either peritoneal or bone marrow derived macrophages. We also collected samples from mice undergoing anaphylaxis at different time points to measure mediators involved.

**RESULTS:** We found that anaphylaxis in this model of epicutaneous sensitization was dependent on IgG1, macrophages and PAF but not IgE, mast cells, basophils, neutrophils, monocytes and histamine. Additionally, IL-1 $\alpha$  was critically required for anaphylaxis. Interestingly, this role was intracellular as both anti-IL-1 $\alpha$  treatment and a deficiency in IL-1R failed to prevent anaphylaxis. Using macrophage cultures, we found that the activity of cPLA<sub>2</sub>, the enzyme responsible for PAF production, was intact in the absence of IL-1 $\alpha$ . Likewise, the activity of PAF-AH, the enzyme that degrades PAF, was also unaffected in IL-1 $\alpha$ <sup>-/-</sup> mice. We also showed that PAF signalling was intact in IL-1 $\alpha$ <sup>-/-</sup> mice. Lastly, we showed that MDR1, the transporter for PAF was not critical for anaphylaxis in this model.

**CONCLUSION:** We developed a model of skin sensitization in which anaphylaxis was driven by IgG1, macrophages and PAF. We identified intracellular IL-1 $\alpha$  as a critical component of the alternative pathway of anaphylaxis. We also showed that this effect is not related to defects in PAF metabolism or signalling. This allows us to direct the focus on other pathways affected by IL-1 $\alpha$  in our future studies.

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## TABLE OF CONTENTS

Chapter 1: Introduction.....	1-5
The Problem	
Phases of Peanut Allergy	
Skin as a Potential Site of Sensitization	
Mechanisms of Peanut Induced Anaphylaxis	
PAF as an Anaphylactic Mediator	
PAF Metabolism	
Regulation of cPLA <sub>2</sub>	
PAF Signalling	
MDR-1 and PAF Secretion	
Intracellular Role of IL-1 $\alpha$	
Thesis Objectives	
Chapter 2: Materials and Methods.....	6-10
Chapter 3: Results.....	11-22
Epicutaneous Sensitization and its Anaphylactic Features	
<i>In Vitro</i> Testing of cPLA <sub>2</sub> Activity in Macrophages	
cPLA <sub>2</sub> Activity in IL-1 $\alpha$ <sup>-/-</sup> Macrophages	
Peritoneal Macrophages as a Model of Tissue Resident Macrophages	
Assessment of cPLA <sub>2</sub> Activity in Vivo	
PAF Degradation in IL-1 $\alpha$ <sup>-/-</sup> mice	
PAF Signaling in IL-1 $\alpha$ <sup>-/-</sup> mice	
MDR-1 and PAF Secretion	
Chapter 4: Discussion, Future Directions, and Conclusions.....	23-26
IgE and its Role in Peanut Induced Anaphylaxis	
Epicutaneous Sensitization to Foods	
Role of PAF in Peanut Induced Anaphylaxis	
Role of IL-1 $\alpha$ in Peanut Induced Anaphylaxis	
Future Directions	
Conclusion	
References.....	27-33

## **CHAPTER 1: INTRODUCTION**

### **1.1 The Problem**

Peanut allergy (PA) is a detrimental immunological reaction to inherently innocuous peanut (PN) antigens (Ag). The prevalence of PA in North America has doubled in the last 10 years, and is currently estimated at 1.5%.<sup>1,2</sup> PA often develops early in childhood and, unlike most food allergies, is lifelong in >80% of individuals.<sup>3</sup> Symptoms range from mild urticaria, wheezing, vomiting and diarrhea to anaphylaxis, a rapid systemic reaction that can cause death.<sup>4</sup> PA is the most common cause of food-induced anaphylactic reactions.<sup>5</sup> The management of PA is limited to strict avoidance and administration of rescue epinephrine once an anaphylactic reaction has started.<sup>6</sup> Accidental ingestion of PN has been reported in up to 50% of patients within a 3-4 year period.<sup>7</sup> As a result of its potential severity as well as its rising prevalence, PA has emerged as a major health concern in dire need of novel preventative and therapeutic strategies.

### **1.2 Phases of Peanut Allergy**

The development of PA can be conceptualized within a timeline encompassing two distinct phases: the sensitization phase, *i.e.* the generation of immunoglobulins (Ig) specific for PN, and the effector phase, *i.e.* the clinical and physiological manifestations arising from PN exposure in sensitized individuals.<sup>8</sup> Sensitization is thought to develop due to either a lack of induction or a disruption of oral tolerance, the immune process that establishes systemic hypo-responsiveness to ingested Ag.<sup>8</sup> Despite significant progress in recent years, the mechanisms that mediate sensitization or oral tolerance to food allergens remain to be fully elucidated.

Currently, anaphylaxis is defined as “a serious allergic reaction that is rapid in onset and may cause death”.<sup>9</sup> It is a syndrome with diverse clinical presentations, including diffuse erythema, pruritus, urticaria, angioedema, bronchospasm, laryngeal edema, hyperperistalsis, hypotension, and cardiac arrhythmias. Unfortunately, the identification of the molecules that actually precipitate the anaphylactic reaction remains incomplete. Advances in our understanding of PN induced anaphylaxis (PIA) should uncover new mediators and, thus, the development of novel diagnostics and therapeutics.

### **1.3 Skin as a Potential Site of Sensitization**

Traditionally, the gut has been considered the route of allergic sensitization because it is the primary site of food absorption. However, in a landmark paper, Lack *et al.* (2003) showed that the use of PN oil containing creams to treat rashes in infants within the first six months of life was positively correlated with increased incidence of PA in childhood.<sup>10</sup> Later, it was discovered that a loss-of-function mutation in the gene encoding filaggrin was correlated with PA.<sup>11</sup> Filaggrin is a protein that helps maintain the integrity of the skin barrier.<sup>11</sup> This suggests that an increase in permeability of the skin could be a factor in causing allergic sensitization.

### **1.4 Mechanisms of Peanut Induced Anaphylaxis**

PIA is a type 1 hypersensitivity reaction that is primarily mediated by IgE, FcεRI and mast cells in mice.<sup>12</sup> IgE is the antibody isotype typically associated with Th2 immunity *i.e.*

helminth infections and allergy.<sup>13</sup> It has a short half-life (typically 12 hours in mice and 2 days in humans) in circulation resulting in lower serum titres compared to IgG.<sup>14</sup> IgE is bound to the surface of mast cells through the high-affinity FcεRI.<sup>14</sup> Mast cells, first identified by Paul Ehrlich in 1878, are found at perivascular sites in tissues readily exposed to the environment, such as the skin and the respiratory and gastrointestinal mucosae.<sup>15, 16</sup> They have been shown to participate in both innate and adaptive immunity as a first line of defense.<sup>17</sup> In the case of PIA, PN allergens interact with PN-specific IgE bound to the mast cell surface. This leads to receptor cross-linking causing mast cell degranulation and release of bioactive molecules.<sup>13</sup>

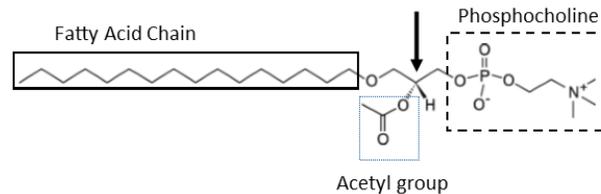
Macrophages, first discovered in 1883 by Ilya Mechnikov, are present in every tissue and are best known for their phagocytic properties.<sup>18, 19</sup> However, our lab and others have demonstrated a significant role for macrophages in PIA.<sup>20, 21, 22</sup> After the systemic administration of PN (done intraperitoneally in mice), PN-specific IgG1 in the serum binds to PN and forms immune complexes.<sup>23, 24</sup> This process is rapid and the resulting immune complexes are soluble due to the excess antibody. The immune complexes diffuse into the tissues where they encounter resident macrophages and bind to the FcγII/III receptors leading to the secretion of multiple mediators including cytokines (*i.e.* tumor necrosis factor  $\alpha$ ) and arachidonic acid metabolites (*i.e.* leukotrienes, prostaglandins, and platelet-activating factor (PAF)).<sup>24, 25</sup> These molecules are synthesized rapidly upon stimulation.<sup>26</sup> These mediators act on endothelial cells and cause vasodilation leading to systemic hypotension and consequently hypothermia, a sensitive measure often used in murine studies.<sup>27</sup> Other cell types such as neutrophils and basophils can also respond to IgG1 immune complexes leading to similar outcomes seen in the alternative pathway mentioned above.<sup>28, 29</sup>

### **1.5 PAF as an Anaphylactic Mediator**

A multitude of molecules are released in the course of an allergic/anaphylactic reaction. Hence, it is important to distinguish between biomarkers and mediators. Mediators are directly responsible for a biological process while biomarkers are molecules that are indicative of a process but do not play a significant role in its manifestation.<sup>30</sup> For example, elevated levels of cysteinyl-leukotrienes (Cys-LTs), serotonin, and tryptase are often detected during PIA but their limited clinical impact on PIA classifies them as biomarkers.<sup>31, 32</sup> Since mast cells and basophils are the key cells in human anaphylaxis, histamine, the major mediator stored in these cells, was thought to be a critical mediator for PIA.<sup>33</sup> However, Vadas *et al.* showed a direct correlation between serum levels of PAF, but not histamine, with the severity of anaphylaxis in allergic patients.<sup>34, 35</sup> In an experimental murine system, we demonstrated that PAF is a major mediator of PIA. Furthermore, we showed that concurrent blockade of PAF and histamine signaling achieved a greater abrogation of anaphylaxis than just histamine blockade alone which had a minor effect.<sup>32</sup> PAF exerts this effect primarily by decreasing peripheral resistance, systemic hypotension, pulmonary hypertension (leading to the drop in blood pressure and consequently temperature) and plasma extravasation (leading to an increase in the viscosity of the blood).<sup>36</sup>

## 1.6 PAF Metabolism

PAF is a glycerophospholipid with three distinct moieties, namely a sixteen carbon fatty acid chain, an acetyl group and a phosphocholine group, attached to a glycerol backbone (Figure 1).<sup>37</sup>



**Figure 1.** Schematic diagram of PAF. The arrow points at the sn-2 group of the glycerol backbone which is targeted by cPLA<sub>2</sub>.

PAF can be formed through two different pathways: the synthesis pathway and the remodeling pathway.<sup>38</sup> The synthesis pathway involves PAF production through sequential biosynthetic reactions and is important in lipid homeostasis. The remodeling pathway works through cleavage of existing phospholipids by group IV cytosolic phospholipase A2 (cPLA<sub>2</sub>) to produce PAF.<sup>38</sup> Due to its rapid output, this is the primary pathway associated with acute reactions such as anaphylaxis. cPLA<sub>2</sub> is expressed ubiquitously *in vivo* and is specific for phospholipids that contain an arachidonic acid fatty acid tail.<sup>39</sup> Upon recognition, cPLA<sub>2</sub> cleaves these phospholipids releasing arachidonic acid, which can be further processed to produce leukotrienes, prostaglandins, and lysophospholipids (phospholipids missing one fatty acid tail) such as lyso-PAF.<sup>40</sup> Lyso-PAF is further acylated by PAF-acetyltransferase to produce PAF. Unlike arachidonic acid derivatives, the acetyl group on the sn-2 position of PAF is essential for its biological activity.<sup>37</sup>

PAF-acetylhydrolase (PAF-AH) is a serine esterase that deacylates PAF and causes its degradation to lyso-PAF, which does not have any anaphylactic effects.<sup>41</sup> PAF-AH is detected both in the blood and intracellularly, particularly in macrophages and monocytes.<sup>42</sup> The fact that serum levels of PAF-AH are inversely correlated to the severity of anaphylaxis in humans supports the role of PAF in anaphylaxis.<sup>34</sup>

## 1.7 Regulation of cPLA<sub>2</sub>

Since cPLA<sub>2</sub> is the critical enzyme for PAF production via the remodeling pathway, its regulation has important physiological consequences. cPLA<sub>2</sub> has a N-terminal C2 domain, responsible for targeting the protein to cellular membranes, attached to a C-terminal catalytic domain by a short flexible linker.<sup>43</sup> Both these domains are used to control cPLA<sub>2</sub> activity in the cell. Normally, acidic residues such as aspartic acid and asparagine in the membrane binding portion of the C2 domain render it electronegative, which is not favorable for proper interaction with cellular membranes. As intracellular calcium concentration increases, positively charged calcium ions bind to the calcium binding loops in the C2 domain and neutralize the electronegativity, allowing cPLA<sub>2</sub> to translocate from the cytosol and bind to intracellular membranes such as the nuclear membrane, the Golgi and the endoplasmic reticulum.<sup>43</sup>

The catalytic domain of cPLA<sub>2</sub> has multiple functionally important phosphorylation sites including serines 505, 727 and 515 that are phosphorylated by mitogen-activated protein kinase (MAPKs), mitogen-activated protein kinase interacting kinase (MNK1) and calmodulin kinase II (CamKII), respectively.<sup>44</sup> Serine 505 seems to be the most important site and its phosphorylation increases the catalytic activity of the enzyme. Phosphorylation at serine 505 is also implicated in proper translocation of the enzyme to the membrane. Furthermore, it increases the binding efficiency, which is especially important in low calcium concentrations; phosphorylation seems to be less important at high sustained concentrations of calcium.<sup>43</sup>

### **1.8 PAF Signaling**

PAF is an extremely bioactive molecule with a half-life of 3-4' in mice and 7' in humans.<sup>45</sup> It binds exclusively to the PAF receptor (PAFR), a G-protein coupled receptor with seven transmembrane helices. The lack of a physiological response to injected PAF in PAFR<sup>-/-</sup> mice proves this conclusively.<sup>46</sup> PAFR is expressed on multiple hematopoietic and structural cells including granulocytes, platelets, macrophages, epithelial and endothelial cells. The widespread expression of the receptor allows PAF to rapidly exert its effects systemically.<sup>47</sup> PAF binding to PAFR causes coupling of the receptor to various G proteins leading to a multitude of effects. One of these is the activation of Erk which, in turn, activates cPLA<sub>2</sub> leading to the production of arachidonic acid metabolites and PAF in a positive feedback loop.<sup>47</sup> Erk activation is also involved in cell growth and induction of multiple inflammatory cytokines. The activation pathway can be different depending on the cell type. PAFR also induces phosphatidylinositol-3,4,5-triphosphate synthesis which regulates cell polarization, motility and survival.<sup>47</sup> In addition, PAFR signaling activates phospholipases D and C as well. Molecules similar in structure to PAF such as lyso-phosphatidylcholine 16:0, lyso-PAF, etc. cannot elicit the same response upon binding to the receptor.<sup>48</sup>

### **1.9 MDR-1 and PAF Secretion**

Originally, PAF was thought to be transported out of the cell via a vesicular transport system. However, blocking this system with brefeldin A, which induces a retrograde transport of all vesicles from the Golgi back to the ER, failed to alter PAF secretion.<sup>49</sup> Raggars *et al.* (2001) later demonstrated that PAF is transported across the plasma membrane by the multi-drug resistance protein 1 (MDR1), also known as p-glycoprotein (pgp).<sup>49, 50</sup> MDR1 is an ubiquitously expressed ATP-binding-cassette transporter responsible for maintaining the integrity of the blood-brain barrier as well as facilitating the transport of chemotherapeutic drugs out of cancer cells, hence causing the drug resistance.<sup>51</sup> Interestingly, MDR1 recognizes analogs of phosphatidylcholine (PCs) and transports them out of the cell. The mechanisms underlying this transport are not yet fully understood. However, it is known that MDR-1 has multiple binding partners such as HAX1 that influence its function.<sup>52</sup>

### **1.10 Intracellular Role of IL-1 $\alpha$**

IL-1 $\alpha$  has been traditionally viewed as a cytokine acting extracellularly involved in host defense in pathogenic infection, bone metabolism and activation of the acute phase

response.<sup>53</sup> However, the fact that most IL-1 $\alpha$  is rarely found extracellularly points to potentially important intracellular roles. IL-1 $\alpha$  is translated as a 31kDa precursor that is cleaved upon activation signals, by the calcium dependent calpain, to produce two fragments: mature IL-1 $\alpha$ , produced from the C-terminus of the precursor, which is eventually released and a so-called pro-piece from the N-terminus of the precursor.<sup>53</sup> There is a nuclear localization sequence within the pro-piece, and by extension the precursor, which implies that they can translocate to the nucleus and induce transcription.<sup>54, 55</sup> Since IL-1 $\alpha$  lacks a DNA binding domain *i.e.* a zinc finger, this effect is most likely indirect caused by protein-protein interactions. In fact, IL-1 $\alpha$  has been shown to interact with histone deacetyltransferases and HAX-1.<sup>56, 57</sup> HAX1 interaction with MDR-1 provides a potential link between IL-1 $\alpha$  and PAF secretion.

### **1.11 Thesis Objectives**

The objective of this thesis is to study the cellular and molecular features of the anaphylactic response in a model of epicutaneous sensitization to peanut. By looking at the mechanism of action, we hope to identify new therapeutic targets to prevent anaphylaxis.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Animals

Female and male C57BL/6 mice were purchased from Charles River Laboratory (Ottawa, Ontario). IL-1 $\alpha$ <sup>-/-</sup> mice (B6-Il1a<sup>tm1Yiw</sup>) were provided by Dr. Iwakura (University of Tokyo, Tokyo) and bred in-house. PAF-R<sup>-/-</sup> mice (B6-Ptafr<sup>tm1a(KOMP)Wtsi</sup>) were provided by Dr. Elaine Tuomanen (St. Jude Children's Research Hospital, Memphis, Tennessee) and bred in-house. IgE<sup>-/-</sup> mice were provided by Dr. Hans Oettgen (Boston Children's Hospital, Boston, Massachusetts) and bred in-house. CCR2<sup>-/-</sup> mice (B6.129S4-*Ccr2*<sup>tm1Ifc</sup>/J) and MC<sup>-/-</sup> mice (B6-*Kit*<sup>W-v</sup>/J) were purchased from Jackson Laboratory (Bar Harbor, Maine). MDR-1<sup>-/-</sup> mice (FVB.129P2-*Abcb1a*<sup>tm1Bor</sup>*Abcb1b*<sup>tm1Bor</sup>N12) and the corresponding control mice were purchased from Taconic (Hudson, New York). The mice were housed in a specific pathogen-free environment and maintained on a 12-hour light-dark cycle. All experiments described were approved by the Animal Research Ethics Board of McMaster University.

### 2.2 Reagents

Cyanogen-treated agarose beads and anti-ovalbumin (OVA) IgG1 were purchased from Sigma-Aldrich (Oakville, Ontario). Endotoxin-free OVA was purchased from Invivogen (Carlsbad, California). Anti-cPLA<sub>2</sub> antibody and anti-phospho-cPLA<sub>2</sub> antibody were purchased from New England Biolabs (Whitby, Ontario). Anti-actin antibody was purchased from Santa Cruz Biotechnologies (Dallas, Texas).

### 2.3 Pharmacologic interventions

Blockade of PAF and histamine receptors was conducted as previously described with slight variations.<sup>32</sup> Briefly, allergic mice were treated with a PAF receptor antagonist (50 mg/kg), ABT491 (EMD Millipore, Etobicoke, Ontario), in 0.5 mL PBS either orally or intraperitoneally 1 hour before challenge. A separate group of sensitized mice were injected histamine receptor antagonists (mepyramine [3 mg/kg], an H1 receptor antagonist; and cimetidine [10 mg/kg], an H2 receptor antagonist (Santa Cruz Biotechnology, Dallas, Texas) in 0.5 mL PBS intraperitoneally 1h before challenge.<sup>32</sup>

Blockade of IgG-mediated anaphylaxis: mice were injected intraperitoneally with 500 mg of anti-Fc $\gamma$ RII/III mAb in PBS 24 hours before challenge as previously described.<sup>20</sup>

### 2.4 *In vivo* depletion of cell lineages

Basophil depletion was conducted by using the basophil-depleting anti-mouse CD200R3 Antibody (BioLegend, San Diego, California). For phagocyte depletion, mice were given an intraperitoneal injection of 300 $\mu$ L of clodronate-containing liposomes or PBS liposomes (FormuMax Scientific, Sunnyvale, California) 1 day before challenge. Neutrophil depletion was accomplished by using anti-mouse Ly-6G (BioXcell, West Lebanon, New Hampshire).

## **2.5 Model of Peanut Allergy and Anaphylaxis**

*Skin sensitization:* Epicutaneous sensitization was performed by directly applying 20  $\mu$ L of 10 mg/mL CPE (Greer laboratories, Lenoir, North Carolina) onto shaved and tape-stripped skin daily for 10 consecutive days. The dorsal hair was removed with a hair clipper and a mechanical razor (Gillette) on day 1 followed by daily tape-stripping for the subsequent 9 days. Sensitized mice were challenged with 5 mg of CPE in 500  $\mu$ L PBS intraperitoneally (i.p.) two weeks after the last PN application.

## **2.6 Measurement of Systemic Anaphylaxis**

Rectal body temperature was measured immediately before and after challenge at 10' intervals for 40' using a rectal probe (VWR International, Mississauga, Ontario). Clinical scores were recorded as described previously (5-point grading scheme: 0 = no clinical signs, 1 = pruritus: repetitive ear scratching and ear canal digging with hind legs, 2 = periorbital/periauricular edema; piloerection, 3 = lethargy/decreased activity; lying prone on stomach, 4 = no response to whisker provocation, 5 = End point (seizures or death)).<sup>58</sup> Hematocrit readings were taken 40' post challenge by whole blood centrifugation at 6000-6200 rpm for 1' (HemataSTAT-II, Separation Technology Inc.).

## **2.7 Intravenous Challenge with PAF**

Wild type and IL-1 $\alpha$ <sup>-/-</sup> mice were administered PAF (Sigma) intravenously. PAF was dissolved in PBS and flash frozen immediately. At the time of administration, each aliquot was used only once and diluted in PBS to the appropriate concentration. Mice were injected with a range of doses from 100 ng to 500 ng in a final volume of 200  $\mu$ L. Core temperature, clinical scores and hematocrit were measured as indicated in section 2.4. In experiments involving anti-histamines, Mepyramine maleate (H<sub>1</sub> antihistamine) and Cimetidine (H<sub>2</sub> antihistamine) were injected intravenously 30' prior to the PAF challenge as described in 2.3.

## **2.8 Serum Collection**

Mice were anaesthetized with isoflurane and peripheral blood was collected via retro-orbital bleeding using lime glass Pasteur pipettes (VWR International). Approximately 100  $\mu$ L of whole blood was collected per mouse per time point into redtop collection tubes with clot activator (Fisher Scientific, Ottawa, Ontario). Collected samples were incubated at room temperature for 30' and were then centrifuged at 13200 rpm for 10' at 4°C for 10'. Supernatants (sera) were then collected and stored at -20°C for further analysis.

## **2.9 Plasma Collection during the Challenge**

Mice were anaesthetized with isoflurane at the indicated time after challenge. Blood was collected via retro-orbital bleeding using heparinized Pasteur pipettes (VWR International). Approximately 200  $\mu$ L of whole blood was collected per mouse into lavender top tubes with EDTA (Fisher Scientific, Ottawa, Ontario). Samples were spun at 13200 rpm for 5' at 4°C. Plasma was then collected and flash frozen in liquid nitrogen and stored at -80°C for further analysis.

## **2.10 Formation of Immune Complexes**

Cyanogen bromide treated sepharose beads (Sigma) were coupled to endotoxin-free OVA (Invivogen, San Diego, California) following the manufacturer instructions with a final suspension in 1 M NaCl. The OVA-coupled beads were incubated with anti-OVA IgG1 at 37°C for 30' in an end-over-end mixer. The beads were then spun down in a benchtop centrifuge for 30'' and the supernatant removed. Then, they were then re-suspended in 1M NaCl and the washing procedure was repeated thrice more. The beads were finally re-suspended in cRPMI to further stimulate the macrophages. Beads and IgG1 alone were used as controls.

## **2.11 Peritoneal Lavage and Macrophage Cultures**

Peritoneal macrophages were isolated and cultured as described previously.<sup>59</sup> Briefly, mice were anesthetized with isoflurane and euthanized. Under sterile conditions, 4 mL of PBS containing 10% FBS and 10 mM EDTA was injected intraperitoneally and the abdomen was gently massaged for 15''. The injected fluid was then retrieved using a 1 mL pipette and immediately put on ice. The lavage samples were spun down at 1160 rcf for 10' at 4°C and the cells were re-suspended in cRPMI. Peritoneal macrophages were counted using Turks (cells with a prominent cytoplasmic halo around their nucleus). Viability was also tested using Trypan Blue exclusion and shown to be greater than 95%. Cells were then seeded on tissue culture treated plates and incubated at 37°C and 5% CO<sub>2</sub> for two hours to allow the macrophages to adhere. After two hours, wells were washed twice with warm PBS and freshly prepared media was added. Stimulations started 30' after the washing.

## **2.12 Bone Marrow Derived Macrophage Culture**

Bone marrow derived macrophages (BMMCs) were cultured as described previously.<sup>59</sup> Spines were isolated from mice in sterile conditions and kept in ice cold PBS. The spines were cleaned to remove the excess tissue and expose the vertebral column. The column was first cut in three pieces and then each piece was cut in a way to expose the interior of the column. The cord tissue was removed and the spinal pieces crushed in a mortar and pestle containing cold PBS. PBS containing spinal cells was removed occasionally and fresh PBS was added to the pestle. The resulting cell suspension was filtered through a 40 µm filter to remove the debris. The cells were spun down and re-suspended in cRPMI. The progenitor cells (brightest cells with a round morphology) were counted using Trypan Blue exclusion to determine the viability. After diluting the suspension to  $5 \times 10^6$  cells/mL with medium containing Macrophage Colony Stimulating Factor (M-CSF) at 20 ng/mL, 20 mL was plated on 120 mm non-tissue culture treated polystyrene dishes (day 0) and incubated at 37°C and 5% CO<sub>2</sub>. On day 3 of the culture, 20 mL of warm media with M-CSF was added to the plates. On day 6, all the media was removed and 25 mL of M-CSF containing medium was added to each plate. All the media was removed on day 7 and 10 mL of Accutase (Sigma) was added to each plate and incubated for 15' at 37°C. The macrophages were removed from each plate using special cell lifters and counted using Trypan blue exclusion. The macrophages were then re-suspended to the appropriate concentration, plated in tissue culture treated plates and allowed to adhere overnight at 37°C and 5% CO<sub>2</sub>. The cells were then stimulated on day 8.

### **2.13 Protein Isolation**

Cells were put on ice immediately after stimulation and the supernatants were flash frozen in liquid nitrogen. The cells were washed twice with cold PBS and then lysed with cold lysis buffer (1% IgePal C-680, 150 mM NaCl, 50 mM Tris and 5 mM EDTA) for 5' while shaking. Each well was scraped using a cell scraper to collect the cells into the lysis buffer. The cells were left on ice for one hour and then sheared by passing through a 21 gauge needle (30 passages). The resulting suspension was flash frozen, thawed on ice and spun down to collect the protein fraction.

### **2.14 Immunoblotting**

The protein concentration of cell lysates from *in vitro* experiments was quantified using a Bradford Assay, with samples run in triplicate. Using a BSA protein standard as a comparison, samples were diluted 1/200 in distilled water and 20% Bio-Rad Protein Assay Dye Reagent Concentrate (Bio Rad, Hercules, California). The optical density at 595 nm of the standard and each sample was measured using a plate reader. Microsoft Excel was used to quantify the protein concentration in each sample based on the optical density readings.

10-20  $\mu$ g of protein was subsequently run on a 10 or 12% SDS-PAGE gel alongside a molecular weight ladder at 120 V for 60'. The SDS-PAGE gel was transferred onto a nitrocellulose membrane (Pall Corp., Port Washington, New York) by electrical transfer using a BioRad Mini-Protean II equipment (Bio Rad) at 400 mA for 60'. Membranes with transferred proteins were blocked using Odyssey blocking buffer (Licor, Lincoln, Nebraska) diluted 1:1 in 1x tris buffered saline (TBS) for 1 hour at room temperature. The membranes were probed while rocking overnight at 4°C using antibodies targeting cPLA<sub>2</sub> and pcPLA<sub>2</sub> (New England Biolabs) diluted at 1:1000 and actin (Santa Cruz Biotechnology) diluted at 1:2000 in 1:1 mixture of Odyssey buffer and 1x TBS containing 0.15% Tween 20. Blots were washed the following day three times using 1xTBS containing 0.15% Tween 20 for 10' at room temperature. Blots were incubated with an IRdye secondary antibody (Licor). The antibodies were diluted in 1:1 mixture of odyssey buffer and 1X TBS and used at the following concentrations: Donkey anti-goat IgG (1:10000), donkey anti-rabbit IgG (1:2000). Membranes were then washed two times for 10' each at room temperature with 1x TBS containing 0.15% Tween 20, and two times for 10' each at room temperature with 1x TBS. The membranes were imaged using Odyssey scanner (Licor). The densitometry was performed using the ImageStudio Lite software from Licor.

### **2.15 Detection of Prostaglandin E2 (PGE2) and Cys-LTs**

Both PGE2 and Cys-LTs were measured using ELISA immunoassays from Cayman chemicals (Ann Arbor, Michigan). The protocol was followed as indicated by the supplier. Plasma was used for *in vivo* studies while cell culture supernatants were used for *in vitro* studies.

### **2.16 Serum Peanut-Specific IgE and IgG1**

PN-specific IgE and IgG1 were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously.<sup>58</sup> For PN-specific IgG1, Maxi-Sorp 96-well plate (Nunc; VWR Canlab) was coated with CPE (2  $\mu$ g/mL) in 50 nM carbonate-bicarbonate

buffer (pH 9.6; Sigma-Aldrich, Oakville, Ontario) at 4°C overnight. Coated plates were blocked with BSA (1%) in PBS for 2 hours at room temperature. Plates were washed and incubated with serum samples overnight at 4°C. Biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates) were added and incubated with the samples the next day for 2 hours before washing and a 1 hour incubation with alkaline-phosphatase streptavidin (Sigma-Aldrich, Oakville, Ontario) for 1 hour at room temperature. P-nitrophenyl phosphate tablets were used to develop the assay and H<sub>2</sub>SO<sub>4</sub> (2 M) was added to stop the reaction before Absorbance readings taken at 450 nm. PN-specific IgE. Maxi-Sorp 96-well plate was coated with rat anti-mouse IgE Abs (2 µg/mL; BD Pharmingen) in PBS overnight at 4°C. Coated plates were washed and blocked with Tween buffer (10% bovine serum; 1% bovine serum albumin; 0.5% Tween in PBS) for 1 hour at 37°C and washed. Serum samples were then incubated for 2 hours at room temperature before CPE-digoxigenin (DIG) conjugate solution was added for coupling with CPE. Peroxidase-conjugated anti-DIG was added at 37°C for 1 hour before tetramethylbenzidine (0.1 mg/mL) solution was added to develop the colour reaction. 2N H<sub>2</sub>SO<sub>4</sub> was added last to stop the reaction for absorbance reading at 450 nm.

### **2.17 Data Analysis**

Data were analyzed using GraphPad Prism version 5.0 and expressed as mean ± SEM. Results were interpreted using either a student's t-test or a one-way analysis of variance (ANOVA) with a Tukey's or Bonferroni's *post hoc* test. Differences were considered statistically significant when p-value were less than 0.05 (\*).

## CHAPTER 3: RESULTS

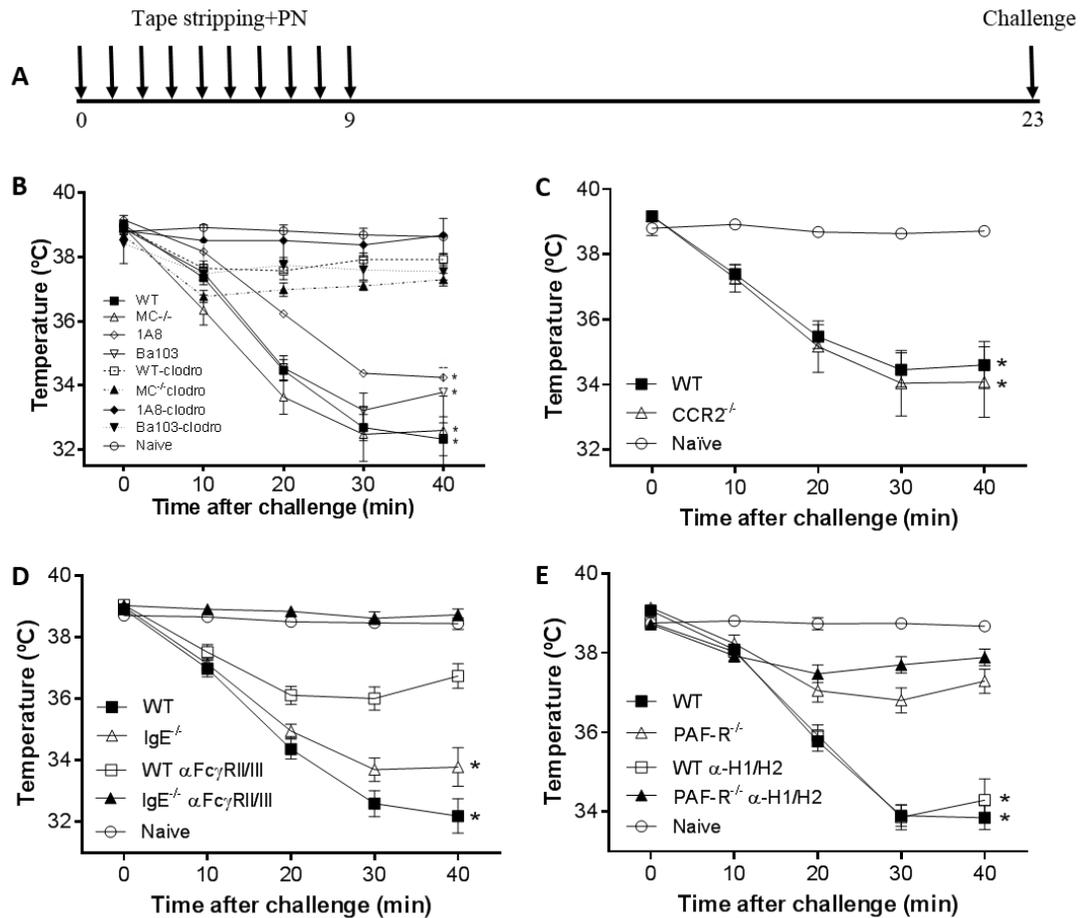
### *Epicutaneous Sensitization and its Anaphylactic Features*

Since there is growing evidence that the skin might be an initiating site for food sensitization (section 1.3), our laboratory established a model of epicutaneous sensitization and PIA that relies on the removal of the *stratum corneum* (the outer layer of the skin) by tape stripping prior to direct application of PN on the damaged skin (Figure 2A).

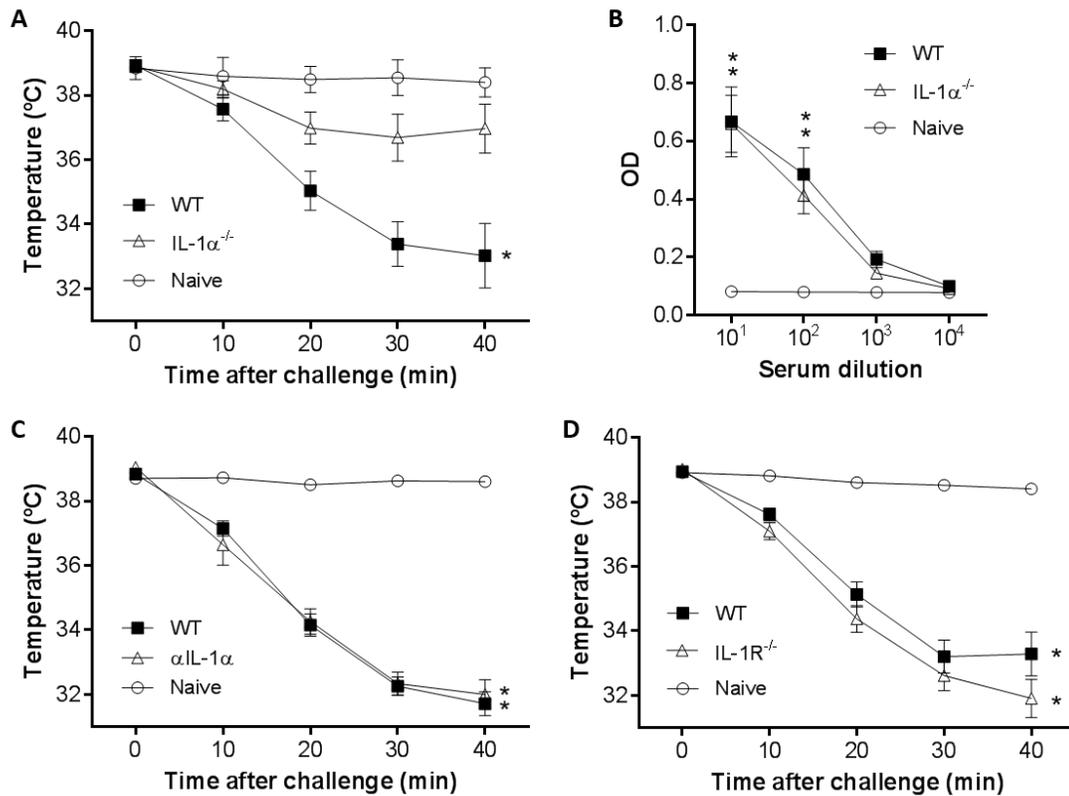
As shown in figure 2B, depletion of macrophages using clodronate containing liposomes caused a significant abrogation of anaphylaxis while depletion of neutrophils (1A8 antibody) or basophils (Ba103 antibody), and mast cell deficiency ( $MC^{-/-}$ ) did not result in a significant effect. Since clodronate containing liposomes deplete monocytes along with macrophages, we used  $CCR2^{-/-}$  mice, which only lack circulating monocytes.<sup>60</sup> Figure 1C shows the redundancy of monocytes to anaphylaxis, thus indicating that macrophages are the major cell mediating this response.

In a similar fashion, blockade of IgG1 signaling with anti-FcγRII/III dramatically reduced the anaphylactic response while IgE deficiency ( $IgE^{-/-}$  mice) had no significant effect (Figure 2D). Finally, histamine receptor blockade, using  $H_1$  and  $H_2$  antihistamines, failed to cause a significant effect while PAF inhibition (both with the  $PAF-R^{-/-}$  mice and PAFR antagonist ABT-491) abrogated the response in a magnitude similar to macrophage depletion or IgG1 signaling blockade (Figure 2E) Overall, these data indicate that anaphylaxis in this model is primarily driven by the alternative pathway.

Since IL-1 family cytokines are released following macrophage activation, we next sought to investigate their contribution to anaphylaxis. We explored the role of IL-1 $\alpha$ , IL-1 $\beta$ , IL-18 and IL-33 in genetically deficient mice. The absence of IL-1 $\beta$ , IL-18 or IL-33 had no impact on the anaphylactic response (data not shown). Conversely, anaphylaxis was dramatically abrogated in  $IL-1\alpha^{-/-}$  mice (Figure 3A) even though the mice were sensitized similarly to the wild type controls (Figure 3B). Interestingly, blockade of extracellular IL-1 $\alpha$  signaling using an anti-IL-1 $\alpha$  antibody or  $IL-1R^{-/-}$  mice did not replicate this phenotype, suggesting that the role of IL-1 $\alpha$  is likely intracellular within the macrophages, the predominant cell type (Figure 3C, 3D).



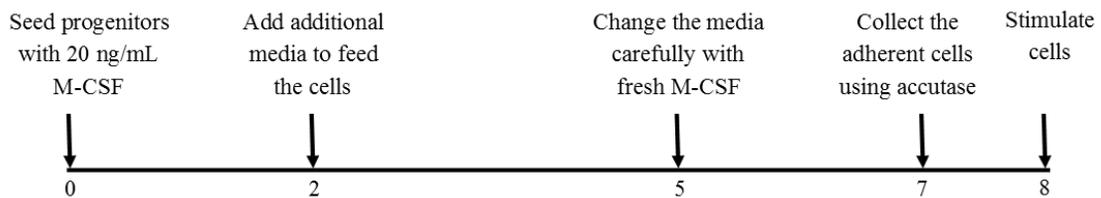
**Figure 2.** Changes in core body temperature in a model of epicutaneous sensitization. **A**, Model of epicutaneous sensitization. **B**, Depletion of various cell types to assess their contribution during the anaphylactic response. Monoclonal antibodies Ba103 and 1A8 were used to deplete basophils and neutrophils respectively. Clodronate containing liposomes were used for macrophage/monocyte depletion while *Kit<sup>W-sh<sup>c</sup></sup>* sash<sup>+</sup> mice were used for mast cell depletion. **C**, *CCR2<sup>-/-</sup>* mice were used for specific depletion of monocytes and account for the dual effect of clodronate on macrophages and monocytes. **D**, IgE deficient mice and blockade of FcγRII/III (critical for IgG1 signalling) were used to explore the role of IgE and IgG1 in this model. **E**, PAF-R<sup>-/-</sup> mice and anti-histamines were used to determine the involvement of these factors in anaphylaxis. Data is represented as mean ± SEM from two independent experiments. A minimum of 5 mice were used for each treatment. \*p<0.005 compared to the naïve controls.



**Figure 3.** Role of IL-1 $\alpha$  during anaphylaxis in a model of epicutaneous sensitization. A, IL-1 $\alpha$ <sup>-/-</sup> mice did not undergo anaphylaxis but were sensitized as indicated by equal amount of IgG1 produced, B. C, D, Extracellular blockade of IL-1 $\alpha$  using either anti-IL-1 $\alpha$  antibody or IL-1R<sup>-/-</sup> mice respectively. Data is represented as mean  $\pm$  SEM from two independent experiments. n=5-10 mice. \*p<0.005 compared to the naïve group

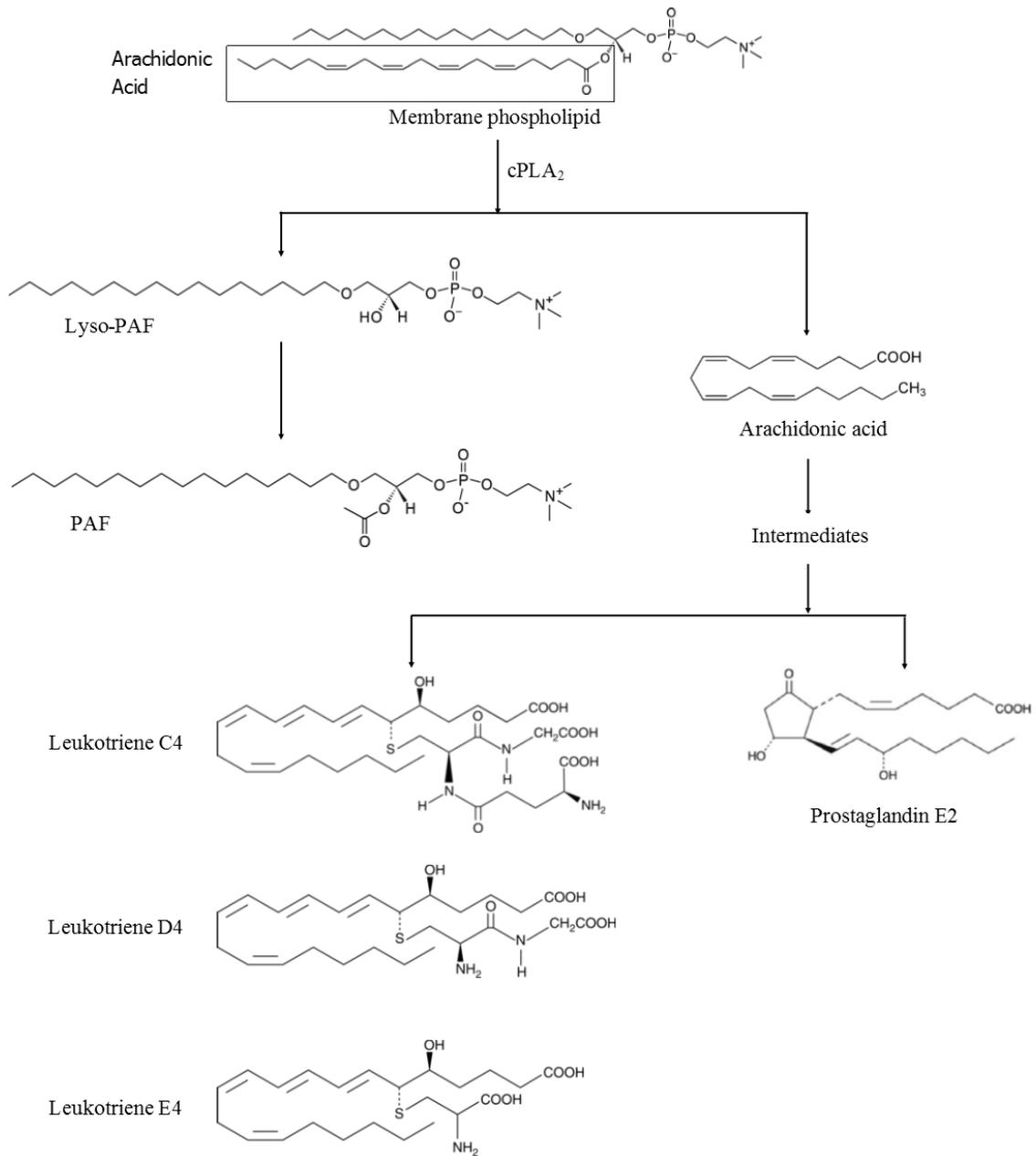
### ***In Vitro Testing of cPLA<sub>2</sub> Activity in Macrophages***

First, we established a system to test the role of IL-1 $\alpha$  in macrophages. We chose bone marrow derived macrophages (BMMCs) due to their greater availability per mouse and because they are thought to represent tissue resident macrophages.<sup>61</sup> Macrophages were grown in M-CSF without any additional M1/M2 polarizing cytokines such as IL-4 and TNF- $\alpha$  to produce a macrophage profile known as M0, which represents normal tissue resident macrophages in a homeostatic environment. A schematic of the culture protocol is shown in figure 4.



**Figure 4.** Schematic of the protocol to grow bone marrow derived macrophages. The seven day culture was done on bacteriological grade plastic while the final seeding and stimulation was in tissue culture treated multi-well plates.

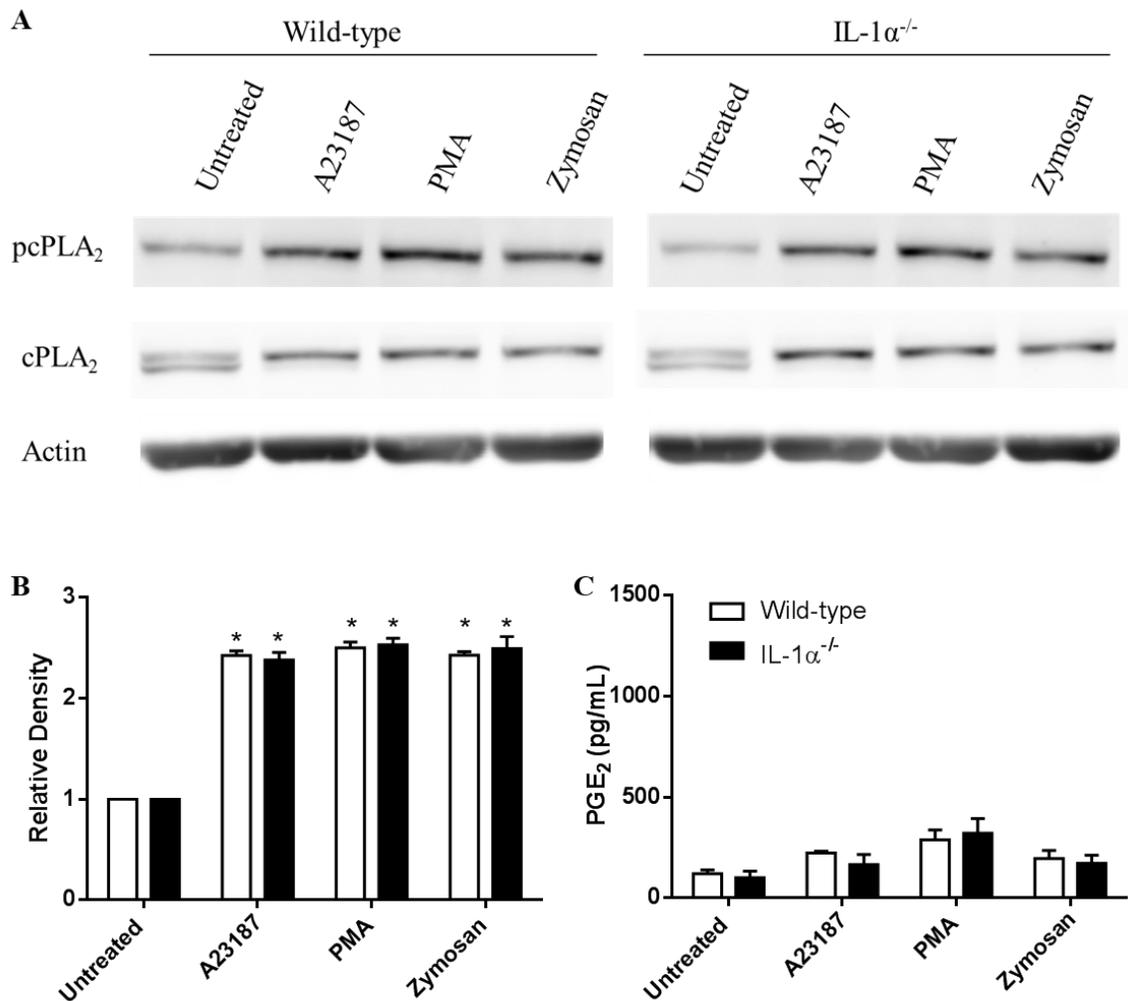
We used two measures to test cPLA<sub>2</sub> activity. First, we evaluated phosphorylation of serine residue 505 of cPLA<sub>2</sub> via immunoblotting. Phosphorylation of serine 505 is known to be required for the activation of the catalytic unit of the enzyme, thus making it a reliable marker for cPLA<sub>2</sub> activation. Second, we measured the levels of prostaglandin E2 (PGE2) in the cell supernatants to assess the functional capacity of cPLA<sub>2</sub> to process phospholipids and produce downstream mediators.<sup>40</sup> Initially, we focused on measuring PAF since it is the direct mediator in our model. However, technical issues hindered our ability to do so. Briefly, the commercially available ELISA was not accurate for PAF and failed to generate an accurate response curve with serial dilutions. In addition, the mass spectrometry method was unable to detect PAF even in the wild-type mice indicating a lack of sensitivity. Since PAF is produced in the same cascade as eicosanoids, they are an appropriate biomarker for PAF production (Figure 5).<sup>40</sup> For the purpose of these experiments, we measured PGE2 because it is highly stable in cell culture supernatants.



**Figure 5.** cPLA<sub>2</sub> and the downstream cascade. cPLA<sub>2</sub> acts on membrane phospholipids to produce lyso-PAF and arachidonic acid. Lyso-PAF is further converted to PAF while arachidonic acid is processed to produce prostaglandins and leukotrienes.

### ***cPLA<sub>2</sub> Activity in IL-1 $\alpha$ <sup>-/-</sup> Macrophages***

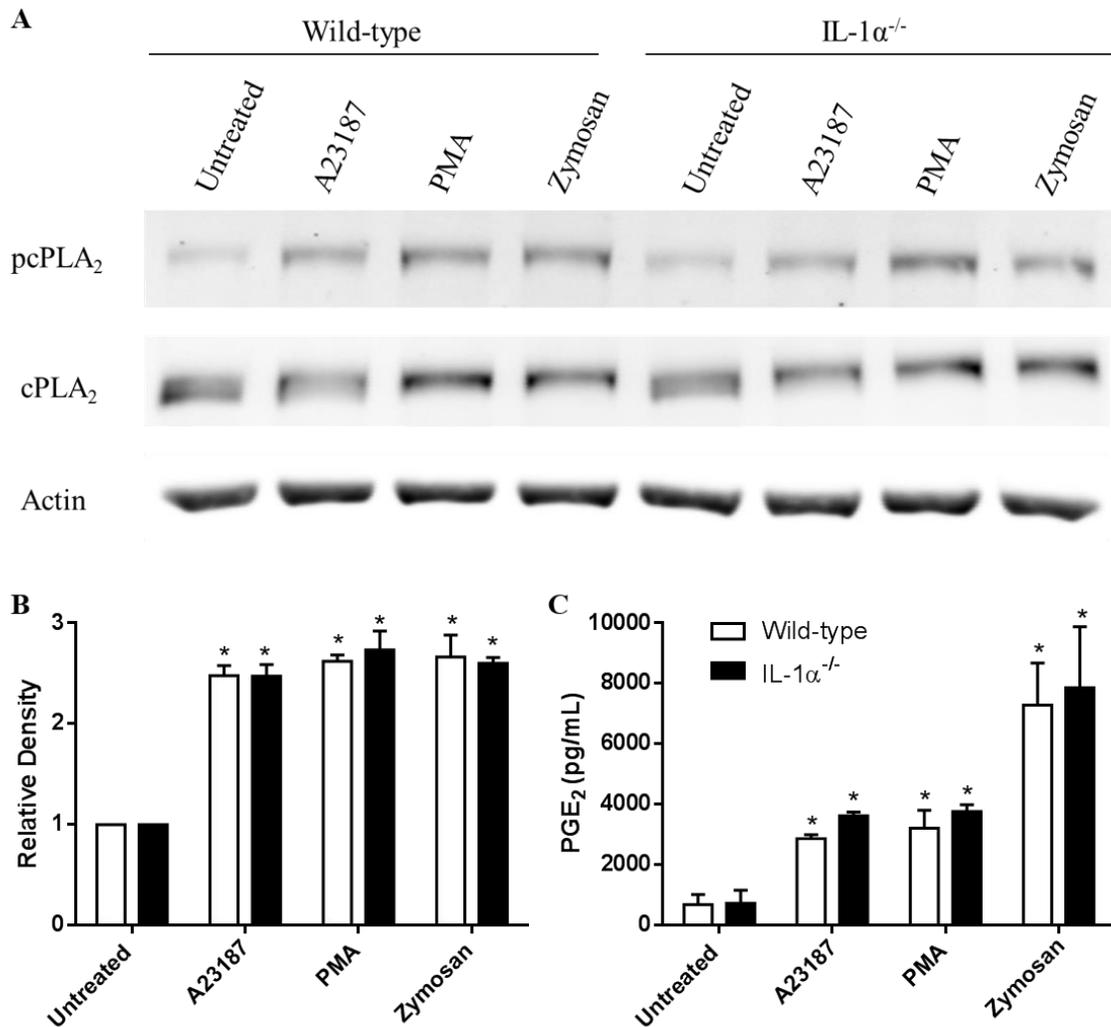
To validate our *in vitro* system, we initially used conventional agonists, namely calcium ionophore A23187, PMA and zymosan, that are known to cause cPLA<sub>2</sub> activation and the production of PAF.<sup>62</sup> As seen in Figure 6, both wild type and IL-1 $\alpha$ <sup>-/-</sup> macrophages responded comparably in terms of cPLA<sub>2</sub> phosphorylation. However, there was no significant production of PGE<sub>2</sub> in any condition. This raised the possibility that BMDCs may not precisely mimic tissue resident macrophages and might, in fact, lack key features of macrophage function.



**Figure 6.** A, B, Treatment of bone marrow derived macrophages with conventional agonists leads to cPLA<sub>2</sub> phosphorylation. Cells were treated for 20' with 2.5  $\mu$ M A23187, 100 nM PMA and 20 particles/cell of Zymosan. C, The level of PGE<sub>2</sub> produced in response to these agonists is not correlated with cPLA<sub>2</sub> phosphorylation. Data is represented as mean  $\pm$  SEM from three independent experiments. Representative western blot shown. \* $p$  < 0.005 compared to the untreated group.

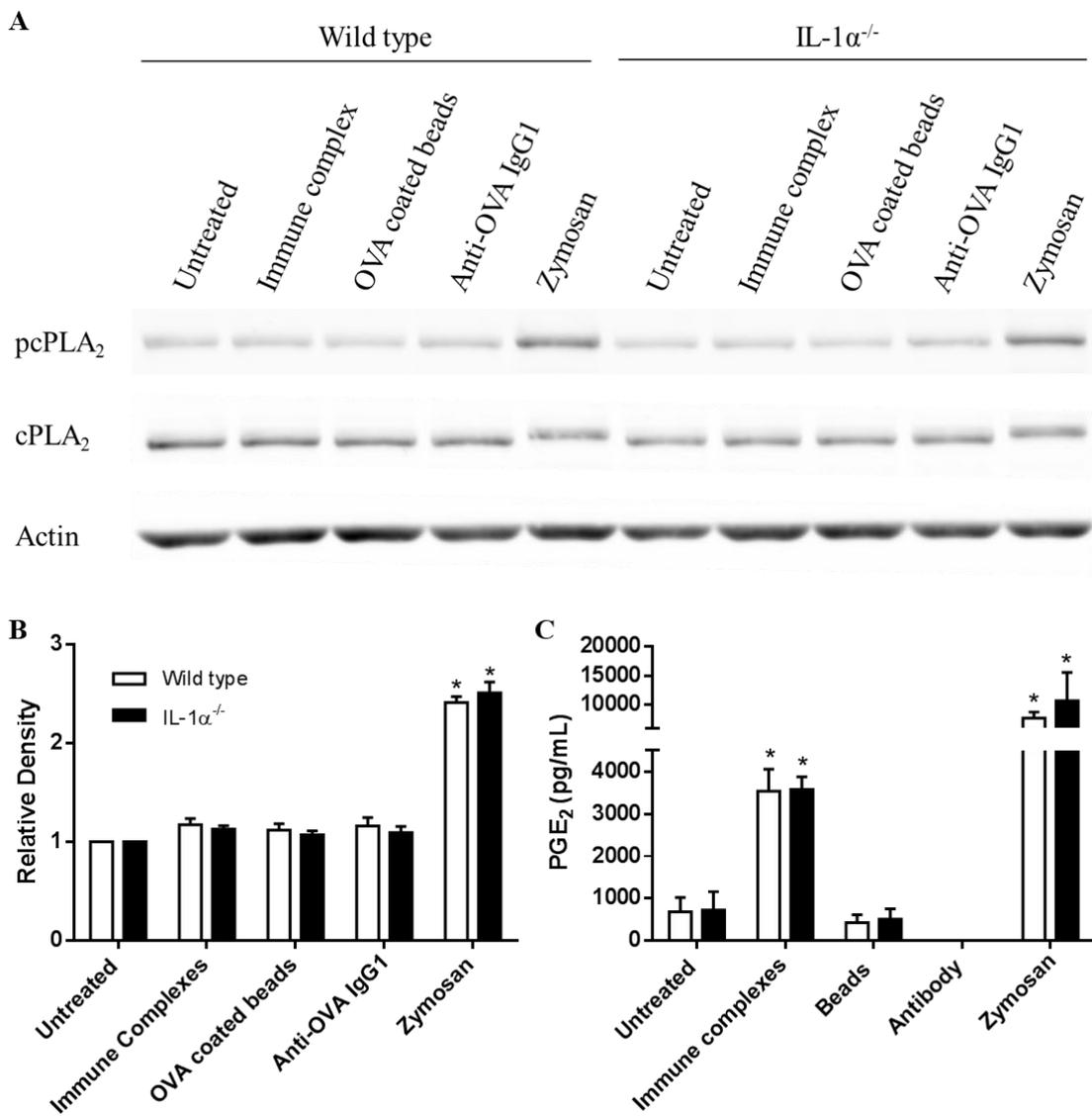
### Peritoneal Macrophages as a Model of Tissue Resident Macrophages

We reasoned that due to the lack of PGE<sub>2</sub> production, BMMCs were perhaps not the appropriate tool to assess cPLA<sub>2</sub> activity. Hence, we used peritoneal macrophages from naïve mice for the rest of the *in vitro* studies. Stimulation with conventional agonists showed that the response in wild type and IL-1 $\alpha$ <sup>-/-</sup> macrophages was very similar in terms of both cPLA<sub>2</sub> phosphorylation and PGE<sub>2</sub> production (Figure 7).



**Figure 7.** A, B, Treatment of peritoneal macrophages with conventional agonists leads to cPLA<sub>2</sub> phosphorylation. Cells were treated for 20' with 2.5  $\mu$ M A23187, 100 nM PMA and 20 particles/cell of Zymosan C. The extent of phosphorylation correlated with PGE<sub>2</sub> levels in the supernatants. Data is represented as mean  $\pm$  SEM from three independent experiments. Representative western blot shown. \*p<0.005 compared to the untreated group.

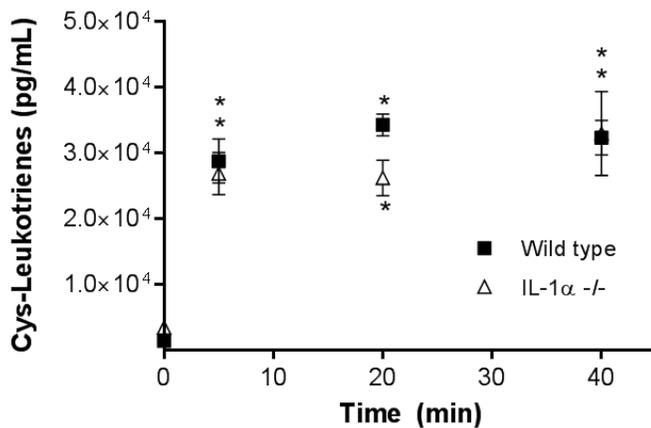
Next, we considered the possibility that the response of peritoneal macrophages to IgG1-Ag ICs could be different than that to conventional agonists. Making these ICs with PN was technically unfeasible because there is no commercially available source of pure PN-specific IgG1. Hence, we used OVA, a model Ag and OVA-specific IgG1 to form these ICs. Initially, we tried forming ICs by incubating IgG1 and OVA but these formulations were unable to stimulate the macrophages. Then, we used agarose beads as a stable surface to form the ICs. First, we coupled endotoxin-free OVA to agarose beads via cyanogen bromide treatment, which leads to an irreversible binding of the two constituents that is stable at cold temperatures indefinitely. Second, the beads were incubated with anti-OVA IgG1 at 37°C to form ICs.<sup>63</sup> The resultant ICs were used to test the cPLA<sub>2</sub> response in the peritoneal macrophage system. As shown in Figure 8, these ICs did not cause cPLA<sub>2</sub> phosphorylation but they did stimulate significant PGE<sub>2</sub> production. There is evidence that the phosphorylation of cPLA<sub>2</sub> is not essential for its function when there is a sustained high concentration of calcium.<sup>39</sup> It is plausible that ICs provide such conditions and can cause PGE<sub>2</sub> release without inducing phosphorylation.



**Figure 8.** A, Treatment of peritoneal macrophages with immune complexes does not lead to cPLA<sub>2</sub> phosphorylation. Cells were treated for 20' with immune complexes (100  $\mu$ g of antibody per 75  $\mu$ L of OVA coated beads) and 20 particles/cell of Zymosan. B, Densitometry analysis for the phosphorylation experiment. C, The treatment with immune complexes does causes significant release of PGE<sub>2</sub> in the supernatants. Data is represented as mean  $\pm$  SEM from three independent experiments. Representative western blot shown. \*p<0.005 compared to the untreated group.

### Assessment of cPLA<sub>2</sub> Activity *in Vivo*

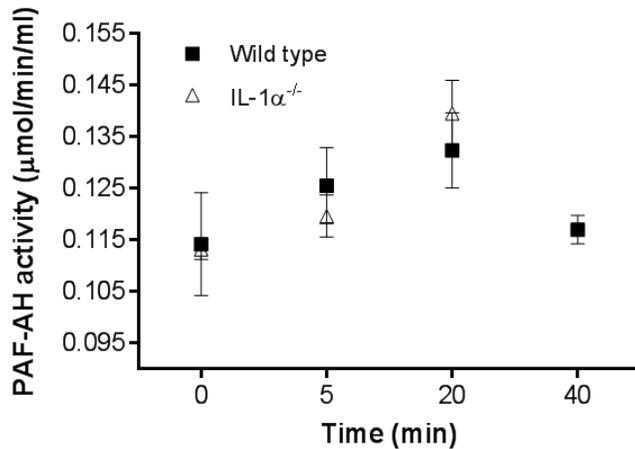
Since we observed similar responses in macrophages isolated from wild type and IL-1 $\alpha$ <sup>-/-</sup> mice in both our cell systems, we decided to test this *in vivo* by measuring downstream products in the blood. Given our inability to directly detect PAF, we measured arachidonic acid metabolites instead. Cys-LTs were used in this instance because PGE<sub>2</sub> has a very short half-life *in vivo*. For this experiment, we challenged multiple groups of wild type and IL-1 $\alpha$ <sup>-/-</sup> mice that were sacrificed at different time points. Plasma was collected and immediately frozen to prevent any significant degradation of leukotrienes. As shown in Figure 9, the levels of Cys-LTs increased rapidly after challenge comparably in the wild type and IL-1 $\alpha$ <sup>-/-</sup> mice. This indicates similar cPLA<sub>2</sub> activity in both strains at the systemic level, which confirms our *in vitro* data.



**Figure 9.** Levels of Cysteinyl-leukotrienes in plasma at different time points after the challenge. No differences are seen in the levels of cysteinyl-leukotrienes released in the plasma of peanut allergic wild type and IL-1 $\alpha$ <sup>-/-</sup> mice upon a systemic challenge. All data are represented as mean  $\pm$  SEM from two independent experiments. n=8-10 mice at each time point. \*p<0.005 compared to the naïve controls (at time 0)

### PAF Degradation in the IL-1 $\alpha$ <sup>-/-</sup> Mice

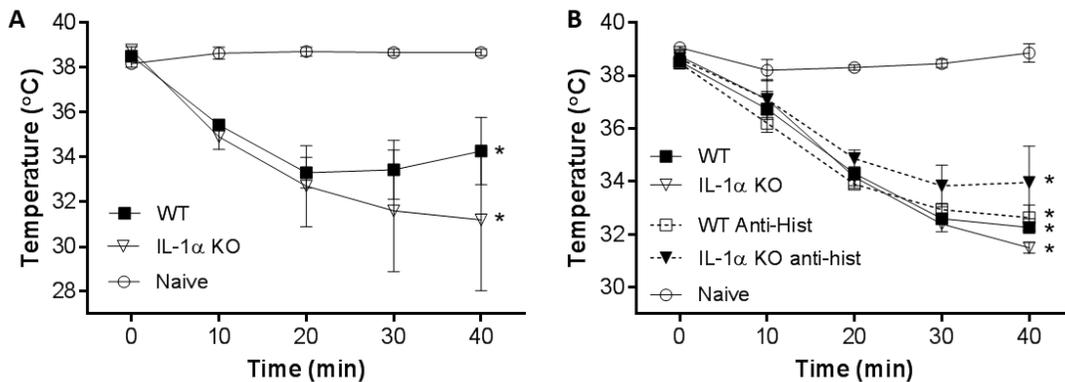
At this point, the evidence indicated that cPLA<sub>2</sub> is active and capable of facilitating prostaglandin and leukotriene production in IL-1 $\alpha$ <sup>-/-</sup> mice, both at the local and the systemic level. This suggested, by extrapolation, that PAF production in IL-1 $\alpha$ <sup>-/-</sup> mice might not be compromised. Therefore, we turned our attention to PAF degradation, as higher PAF-AH activity could explain the lack of response in the IL-1 $\alpha$ <sup>-/-</sup> mice. To test this, we used a similar experimental system as mentioned in 3.4. As shown in Figure 10, PAF-AH activity was comparable in both wild type and IL-1 $\alpha$ <sup>-/-</sup> mice indicating that differences in PAF degradation cannot account for the IL-1 $\alpha$ <sup>-/-</sup> phenotype.



**Figure 10.** Activity of PAF-AH in plasma at different time points after the challenge. here is no difference in the activity of PAF-AH in the plasma of wild type and IL-1 $\alpha^{-/-}$  mice before or after challenge. All data are represented as mean  $\pm$  SEM from two independent experiments. n=8-10 mice at each time point.

### PAF Signaling in the IL-1 $\alpha^{-/-}$ Mice

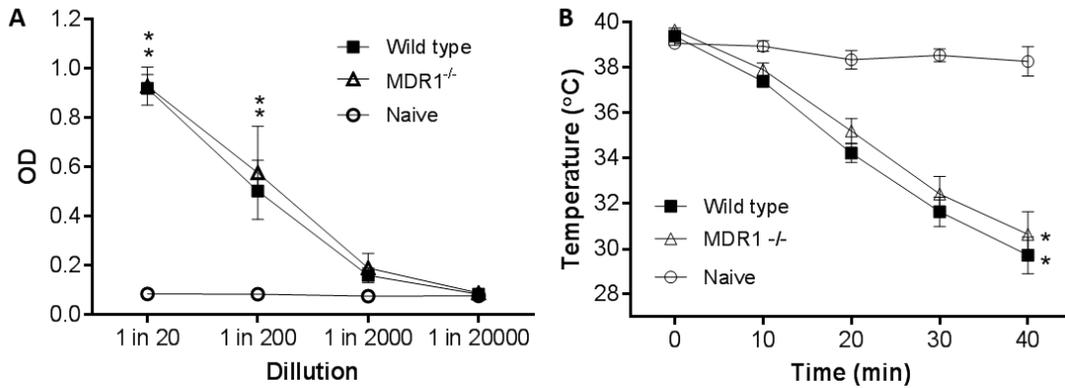
Since PAF metabolism seemed to be intact in IL-1 $\alpha^{-/-}$  mice, we considered whether PAF signalling could be defective in IL-1 $\alpha^{-/-}$  mice. To test this, we used a model of anaphylaxis elicited by the intravenous injection of PAF. The experiments shown were done using the same batch of PAF. IL-1 $\alpha^{-/-}$  mice responded to PAF in a manner similar to the wild type mice (Figure 11A). We considered the possibility of PAF acting on mast cells and via histamine release causing anaphylactic symptoms in the IL-1 $\alpha^{-/-}$  mice. To account for this possibility, we used anti-histamines prior to the PAF challenge and showed that the effect observed in IL-1 $\alpha^{-/-}$  mice upon systemic PAF challenge was histamine independent (Figure 11B)



**Figure 11.** Changes in core body temperature after an intravenous PAF challenge A, PAF challenge causes a severe anaphylaxis in the IL-1 $\alpha^{-/-}$  mice comparable to the wild type controls. B, Anti-histamines given thirty minutes prior the PAF challenge failed to cause any effect in either the wild type or the IL-1 $\alpha^{-/-}$  mice. n=5 for both sets of experiments. Data is represented as mean  $\pm$  SEM from two independent experiments. n=10 mice. \*p<0.005 compared to the naïve controls.

### ***MDR-1 and PAF Secretion***

The evidence to this point indicated that PAF metabolism and signaling were intact in IL-1 $\alpha$ <sup>-/-</sup> mice. Thus, we next proceeded to investigate PAF secretion. In a study using mesangial cells, it was shown that PAF cannot translocate across the cell membrane by itself and requires a specific transporter called MDR1.<sup>50</sup> As shown in Figure 12A, MDR1<sup>-/-</sup> mice became sensitized, as indicated by the elevated levels of PN-specific IgG1, and underwent full anaphylaxis upon challenge, comparably to the wild type controls, as reflected by both the drop in core body temperature and the increase in hematocrit (Figure 12).



**Figure 12.** Role of MDR1<sup>-/-</sup> in IgG1 mediated anaphylaxis. A, MDR1<sup>-/-</sup> were sensitized to peanut using the epicutaneous model. B, The anaphylactic response was identical to the wild type controls upon challenge indicating a redundancy for MDR-1. The data are represented as mean  $\pm$  SEM. n=5 mice. \*p<0.005 compared to the naïve controls.

## 4. DISCUSSION

### **IgE and its Role in Peanut Induced Anaphylaxis**

The molecular basis of PIA remains controversial. Traditionally, mast cells and PN IgE in the serum have been considered the principal elements of PIA. However, there are PN allergic individuals who do not have any detectable levels of PN IgE in the serum.<sup>64</sup> Furthermore, a clinical study using an anti-IgE antibody showed that, while a substantial number of patients were able to tolerate 6-8 peanuts on an oral food challenge after a subcutaneous dosage of the antibody every four weeks for a total of four doses, nearly 25% of patients did not respond to the treatment or had significant allergic reactions.<sup>65</sup> Experimentally, both IgE<sup>-/-</sup> mice and mast cell deficient mice (*Kit<sup>W</sup>/Kit<sup>W-v</sup>*) have been shown to undergo severe anaphylaxis.<sup>66, 67</sup> Additionally, there is a poor correlation between mast cell products such as histamine and tryptase and anaphylaxis.<sup>35</sup> These studies show that there are anaphylactic pathways that are not dependent on IgE and mast cells.

### **Epicutaneous Sensitization to Foods**

Although the gastrointestinal tract has been typically considered the site of PN sensitization, increasing evidence points at the skin as an initiating site for PN allergy. With this in mind, we developed a model of epicutaneous sensitization. Our data showed that anaphylaxis in this model is primarily dependent on IgG1 and macrophages. In contrast, Bartnikas *et al* (2013), recently showed that skin sensitization lead to IgE and mast cell-dependent anaphylaxis.<sup>68</sup> The key differences between the two models is the method of sensitization. Their protocol relies on the application of an OVA-containing patch after tape stripping the skin; the mice are exposed to OVA for a total of 21 days, 7 days at a time with a 2-week break between each continuous exposure. In contrast, our protocol involves discrete applications of PN after tape stripping the skin for 10 consecutive days. It is known that a long and sustained Ag exposure extends the germinal center reaction which may result in an increased number of IgG<sup>+</sup> B cells class switching to IgE.<sup>69, 70</sup> Ultimately, this would increase the levels of Ag specific IgE and could explain why anaphylaxis is IgE-dependent in their system. The argument of which model might be more relevant is spurious. The model we established is a tool to study IgE and mast cell-independent pathways of anaphylaxis.

### **Role of PAF in Peanut Induced Anaphylaxis**

Previously, we had shown an important but partial role for PAF in PIA in a model of oral sensitization where anaphylaxis is primarily driven by mast cells and IgE *i.e.*, the conventional pathway.<sup>32</sup> Here, we have discovered that PAF is the key mediator in a model where the alternative pathway is predominant. However, the evidence for this is indirect *i.e.* from blocking the action of PAF, either pharmacologically or genetically. Demonstrating the presence of PAF during the anaphylactic reaction was an important goal of this study and we explored a number of options. First, the only commercially available ELISA for PAF was unable to produce a linear response when different sample dilutions were used, indicating the presence of non-specific binding. We speculate that the antibody provided was not specific such that the assay detected binding of contaminating lipids that are structurally similar to PAF. Second, there is a bioassay to measure PAF that exploits its

platelet aggregating effect. However, the assay had a high range of variability that made it unreliable. Third, an apparently reliable kit based on radioimmunoassay that has been used by other groups is now commercially unavailable. In light of these limitations, mass spectrometry has now become the preferred method to measure PAF. Thus, we embarked on a collaboration with Dr. Philip Britz-McKibbin (McMaster University, Hamilton, Ontario). Overall, we generated one hundred and fifty samples from wild type and IL-1 $\alpha$ <sup>-/-</sup> mice that were challenged and then sacrificed at different time points to collect plasma. PAF could not be detected in the plasma upon challenge even in the wild type mice. Our data with PAF signaling blockade shows that PAF is a critical mediator for anaphylaxis which means that it should be present in the system. Since PAF is metabolized rapidly *in vivo*, it could explain its low abundance in the blood.<sup>45</sup> Thus, it is very likely that PAF is present at levels below our detection limit. We must also consider the fact that the human studies that measured PAF used a radioimmunoassay which ultimately relies on PAF specific antibodies and as we saw previously with our own ELISA data, antibodies for PAF can pick up structurally similar molecules leading to unreliable data. Therefore, it might be the case that clinical studies measuring “PAF” are actually measuring molecules that are extremely similar to PAF explaining why their reported levels are higher than our detection limit.

### **Role of IL-1 $\alpha$ in Peanut Induced Anaphylaxis**

Despite the vast literature on the biological roles of IL-1 $\alpha$ , it has never been implicated in anaphylaxis. The finding that IL-1 $\alpha$  is critically required in IgG1-dependent anaphylaxis is novel. Since IL-1 $\alpha$  is traditionally described as an extracellular cytokine, we expected this to be the case in this model as well. However, IL-1 $\alpha$  neutralizing antibodies and IL-1R<sup>-/-</sup> mice showed that the role of IL-1 $\alpha$  in this model is intracellular. The dramatic reduction of PIA in skin-sensitized IL-1 $\alpha$ <sup>-/-</sup> mice may be related to PAF release from macrophages. To demonstrate this conclusively, measurement of PAF is essential and we would expect that IL-1 $\alpha$ <sup>-/-</sup> mice are impaired in their ability to produce PAF upon challenge. Given the inability to measure PAF to date, we decided to explore other pathways connected to PAF metabolism in IL-1 $\alpha$ <sup>-/-</sup> mice.

Our research strategy investigated three potential mechanisms: PAF metabolism, PAF secretion and PAF signaling. In terms of production, our data showed that cPLA<sub>2</sub> was functional in IL-1 $\alpha$ <sup>-/-</sup> macrophages since they were capable of producing PGE<sub>2</sub> and Cys-LTs, mediators downstream of cPLA<sub>2</sub> activation, upon stimulation with ICs. However, PAF production requires an additional step that is distinct from other eicosanoids produced by macrophages namely the addition of the acetyl group on lyso-PAF by PAF-acetyltransferase.<sup>38</sup> It is possible that IL-1 $\alpha$  regulates the activity of this enzyme. There are no commercially available assays to measure the activity of PAF-acetyltransferase but there are radiolabeling methods that have been used in the past.<sup>71</sup> We also considered degradation of PAF, as it could be that PAF was being degraded more rapidly in IL-1 $\alpha$ <sup>-/-</sup> mice. We measured the activity of PAF-AH, the enzyme that degrades PAF, and showed that it was similar in both IL-1 $\alpha$ <sup>-/-</sup> and wild type mice. This indicated that excess degradation of PAF was not responsible for the lack of anaphylaxis in skin sensitized IL-1 $\alpha$ <sup>-/-</sup> mice.

In order to investigate the integrity of PAF signaling, we injected PAF intravenously into wild type and IL-1 $\alpha$ <sup>-/-</sup> mice to cause anaphylaxis. Interestingly, even the mice that did not undergo very severe hypothermia still had very high hematocrit indicating that role of PAF was more associated with vascular leakage. In terms of our experimental objective, these data showed that PAF-PAFR signaling pathway was intact in the IL-1 $\alpha$ <sup>-/-</sup> mice.

Then, we focused on PAF secretion as PAF is a large lipid molecule and cannot directly diffuse through the cell membrane. In this context, MDR-1 is the only transporter that has been shown to transport PAF out of the cell. In addition to the transport of PAF, MDR-1 actively maintains the blood-brain barrier and is responsible for transporting chemotherapy drugs out of the cancer cells providing them with drug resistance.<sup>51</sup> We tested the involvement of MDR-1 in the model of epicutaneous sensitization by using MDR-1<sup>-/-</sup> mice. The data showed that MDR-1 was redundant during anaphylaxis indicating that either PAF secretion was not essential to anaphylaxis or that MDR-1 was not responsible for PAF secretion. It should be noted that the data showing the ability of MDR-1 to transport PAF was generated in *in vitro* systems which does not always translate well into *in vivo* models. It is possible that novel transporters contribute to the secretion of PAF within the context of an IgG1-macrophage mediated anaphylactic response.

### **Future Directions**

At this juncture, the central issue is the quantification of PAF in murine samples. We have now established a collaboration with Dr. Michael Thomas (Medical School of Wisconsin, Milwaukee, Wisconsin) who published the original paper on using mass spectrometry to measure PAF.<sup>72</sup> They use a much more sensitive triple-quadruple mass spectrometer which could account for lack of sensitivity in our method; the time of flight mass spectrometers which we used are faster than but not as sensitive.<sup>72</sup> It is also interesting that all the studies that have successfully measured PAF using mass spectrometry have used *in vitro* samples from treated cells. This might be because it is easier to concentrate PAF in the cell culture supernatants. As such, we would also analyze cell culture supernatants from wild type and IL-1 $\alpha$ <sup>-/-</sup> macrophages under different stimulations.

The fact that PAF challenge causes severe anaphylaxis in IL-1 $\alpha$ <sup>-/-</sup> mice indicates that the endothelial cells are most likely not affected. However, we do not have any direct evidence for this. One of the ways we could show this is by using wild type/IL-1 $\alpha$ <sup>-/-</sup> bone marrow chimeras. In a chimera, the recipient mice are lethally irradiated followed by an injection of bone marrow cells from donor mice. The recipient mice keep their structural cells but their hematopoietic cells are of the donor origin. For example, in a wild type to IL-1 $\alpha$ <sup>-/-</sup> chimera, all the structural cells will lack IL-1 $\alpha$  while the hematopoietic cells will not; the reverse will be true for an IL-1 $\alpha$ <sup>-/-</sup> to wild type chimera. By separating the depletion of IL-1 $\alpha$  between the structural and the hematopoietic compartment (*i.e.* the leukocytes), we could definitively show that the defect in the IL-1 $\alpha$ <sup>-/-</sup> mice is absent in the structural compartment providing further justification to focus on macrophages.

It is known that IL-1 $\alpha$  can cause a variety of intracellular effects including NF- $\kappa$ B activation and IL-8 induction.<sup>55, 73</sup> It has a nuclear translocation signal and has been shown to interact with histone acetyltransferases (HATs).<sup>56</sup> HATs acetylate conserved lysine residues on

histone proteins and generally act to increase gene expression by unwrapping the DNA from around the histone protein.<sup>74</sup> A lack of IL-1 $\alpha$  could conceivably cause dramatic changes in gene expression *in vivo*. An unbiased and untargeted approach such as a microarray might be useful to identify the genes that are impacted by the deficit of IL-1 $\alpha$ . Initially, we would use peritoneal macrophages because they are the prominent cell type modulating the anaphylactic response in this system. However, we could use the data from the chimeric experiment to determine whether the structural cells need to be targeted as well. Macrophages would be analyzed in both their resting and stimulated state because expression differences might only become apparent when the macrophage is active. The analysis could then be focused on genes that are associated with PAF making the search more focused.

Besides the nuclear effects IL-1 $\alpha$  might be interacting with other unknown proteins in the cytoplasm. We could identify any such proteins using co-immunoprecipitation (Co-IP) followed by mass spectrometry. Traditionally, the product of the Co-IP is probed for a known protein to show its interaction with the protein of interest. However, the use of mass spectrometry allows for a broader search which is useful because very little is known about the proteins that interact with IL-1 $\alpha$ . The lack of such protein-protein interactions in the IL-1 $\alpha$ <sup>-/-</sup> might explain the abrogated anaphylactic response seen in our model.

### **Conclusion**

The first part of my MSc project focused on the cellular and molecular characterization of a model of epicutaneous sensitization previously established in our lab. The data show that anaphylaxis in this model is primarily driven by IgG1, macrophages and PAF. Additionally, we also show that IL-1 $\alpha$  is critical for anaphylaxis when it relies on the three aforementioned factors. We further show that the role of IL-1 $\alpha$  is not extracellular. This led to studies to investigate the mechanisms underlying the intracellular role of IL-1 $\alpha$ . In this second part, we focused on three aspects of PAF metabolism: PAF production/degradation, PAF signalling and PAF secretion. First, the data show that IL-1 $\alpha$ <sup>-/-</sup> macrophages as well as IL-1 $\alpha$ <sup>-/-</sup> mice can produce eicosanoids in a manner similar to the wild type controls. Since we are unable to measure PAF currently, these data act as a proxy biomarker for the integrity of the PAF-producing machinery. The data also show that PAF degradation is unaffected in IL-1 $\alpha$ <sup>-/-</sup> mice. Second, we show that PAF can cause anaphylaxis in IL-1 $\alpha$ <sup>-/-</sup> mice indicating that PAF signaling is most likely unaffected. Third, we show that the only known transporter of PAF is redundant for anaphylaxis in this model suggesting the presence of other unknown secretion mechanisms. Overall, our data have ruled out several important lines of inquiry and, as such, informs focus future research directions on the basic mechanisms on the role of IL-1  $\alpha$  on anaphylaxis.

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