2012

Role of Fyn and Lyn in IgG-mediate immune responses

Yves Falanga
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Role of Fyn and Lyn in IgG-mediate immune responses

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at Virginia Commonwealth University

By

Yves Trésor Falanga

B.S., Université the Rennes 1, 2008

Director: John J. Ryan, Professor, Department of Biology

Virginia Commonwealth University

Richmond, Virginia

July, 2012
DEDICATION AND ACKNOWLEDGEMENTS

This work is dedicated to everybody who has somewhat been discouraged by their peers, friends, family and instructors in their quest to follow their dreams; to everybody who, beside the adversity, always believed in themselves and maintained the cap to their ultimate goal; to everybody who is not afraid to shoot for the stars, hoping that, eventually it will happen some day.

I dedicate this work to my family who has always believed in me and supported me regardless of the situations we have been through. I dedicate this accomplishment to my Father, Zénon Falanga as well as my siblings, Freddy, Clara, Micheline and Benny, without whom I would not be able to achieve this challenge.

I am also very appreciative to my high school basketball coach, Sylla Matha Stone for helping me believe in myself. I thank him for teaching me not to take a shot if I am not absolutely sure to score it. I also dedicate this work to my philosophy and religion high school teacher, Father Pierre Baton for teaching me not only to question everything and think out of the box but also, and more importantly, to always seek for excellence in whatever I decide to do; to always be a better version of me for every minutes of my life. In everything!

I also dedicate this work to everybody who supported me and always believed in me at Université de Rennes 1 in France: Jacqueline Segalen, Thierry Guillaudeux, Noureddine Boujrad and Claire Picquet-Pellorce. I would not have made it this far if it was not for their mentoring.
I am very grateful to Dr. John Ryan, my Ph.D. Advisor for his patience, optimism and mentorship. He has been and will always be someone I will seek for counsels. I have been very fortunate to have the “Dream Team” in my Ph.D. Committee. I would like to thank Jennifer Stewart, Dan Conrad, Carole Oskeritzian and Robert Tombes for their mentorship, patience, availabilities and counsels. I will never have enough words to describe what these people truly mean to me. They have been more than just ordinary Committee members and advisors to me. In fact, they have been parents, big brothers and sisters, severe uncles and aunts; ... Above all, they have been my best friends. They transformed my Ph.D. training into the most amazing intellectual journey I have ever embarked for. With them, I learned how to become a better thinker and trained myself to challenge my own thoughts. With them, I felt not only that I had an important role in the society, but also that I had a goal for humanity. I sincerely believe that I will impact the history of mankind in a way I did not foresee. And for this, I owe them.

Many people have played important roles in my life, shaping the person I became today. I will dedicate this work and the forthcoming accomplishments in my life to the most amazing woman I have ever met and that I will never meet an equal to: my Mother. I dedicate my life to the memory of Micheline Marie Esther Kimpanga, the most amazing creature I was blessed to have met. She always wanted me to be the man I am now and the man I will be in the future. She always exhorted me to seek for excellence and happiness in my work and simplicity in my lifestyle. To her I promised to be an instrument of peace for the
world and people around me. She was and will always be my motivation. To her, I dedicate this work.
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LIST OF ABBREVIATIONS

+/- Heterozygous deletion of a gene

-/- Homozygous deletion of a gene

°C Temperature expressed in Celsius degrees

2.4G2 mAb recognizing the murine FcγRII and FcγRIII

a.a. Amino Acid

Ab Antibody

ADCC Antibody-dependent cellular cytotoxicity

Akt or protein kinase B, serine/threonine protein kinase

Ag Antigen

AID Activation-induced cytidine deaminase

Alum Aluminum hydroxide

AP-1 Activator protein 1

APC Antigen-presenting cell

B220 mAb recognizing the murine CD45R

B6.129 Mouse strain, cross of C57BL/6 and 129sv
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ba103</td>
<td>mAb recognizing to mouse CD200 receptor-like 3</td>
</tr>
<tr>
<td>B.C.:</td>
<td>Before Christ</td>
</tr>
<tr>
<td>B cell</td>
<td>B lymphocyte</td>
</tr>
<tr>
<td>Bcl-6</td>
<td>B cell lymphoma 6 protein</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone marrow-derived mast cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Inbred mouse strain</td>
</tr>
<tr>
<td>CBP</td>
<td>Csk binding protein</td>
</tr>
<tr>
<td>CCL2 [or MCP-1]</td>
<td>Chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>Csk</td>
<td>C-Src kinase</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>CXCL3</td>
<td>Chemokine (C-X-C motif) ligand 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<td>--------------------------------------------</td>
</tr>
<tr>
<td>CXCR5</td>
<td>Chemokine receptor type 5</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOK-1</td>
<td>Docking protein 1</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment of an immunoglobulin</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
</tr>
<tr>
<td>FcεRI</td>
<td>The high affinity IgE receptor</td>
</tr>
<tr>
<td>FcεRII</td>
<td>The low affinity IgE receptor (CD23)</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor binding to the Fc portion of IgG</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>GC B cell</td>
<td>Germinal center B cell</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CFU</td>
<td>Granulocyte monocyte-colony forming unit</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte colony-stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin-Eosin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complexes</td>
</tr>
<tr>
<td>iCOS</td>
<td>Inducible T cell costimulator</td>
</tr>
</tbody>
</table>
IFN-γ  Interferon gamma
Ig  Immunoglobulin
IL  Interleukin
IL-4Rα  Interleukin 4 receptor alpha
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibitory motif
JAK  Janus kinase
JNK  c-Jun N-terminal kinase
kDa  Kilodalton
KO  Knockout, when a gene is deleted
LAT  Linker of activated T cells
LPS  Lipopolysaccharide
LTC4  Leukotriene C4
M-CSF  Macrophage Colony-stimulating Factor
mAb  Monoclonal antibody
MACS  Magnetic cell sorting
MAPK  Mitogen-activated protein kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MAR-1</td>
<td>Monoclonal antibody recognizing the mouse FcεRI</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Mast cell protease 1</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>µl</td>
<td>Microliters</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage Inflammatory Protein 1 alpha</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cell</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NP-KLH</td>
<td>4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>ns</td>
<td>Non significant</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>p38</td>
<td>Protein of 38 kDa part of the MAPK family</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>PAFAH</td>
<td>PAF acetylhydrolase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidyl inositol 3 phosphate</td>
</tr>
<tr>
<td>PKCθ</td>
<td>Protein kinase C, theta chain</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PSA</td>
<td>Passive systemic anaphylaxis</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein Tyrosine kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SAP</td>
<td>SLAM-associated protein</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family tyrosine kinase</td>
</tr>
<tr>
<td>SH-2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic Hypermutation</td>
</tr>
<tr>
<td>SHP-2</td>
<td>SH2 domain-containing protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol 5’ phosphatase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>Siglec</td>
<td>Sialic acid binding Ig-like lectins</td>
</tr>
<tr>
<td>SLAM</td>
<td>Signaling Lymphocytic Activation Molecule</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Src</td>
<td>Proto-oncogene, also name of tyrosine kinase family</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>T cell</td>
<td>T lymphocyte</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor chain</td>
</tr>
<tr>
<td>$T_{FH}$</td>
<td>Follicular helper T cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper cells mainly secreting IL-17</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TRPC1</td>
<td>Transient receptor potential channel 1</td>
</tr>
<tr>
<td>WEHI</td>
<td>Walter and Eliza Hall Institute</td>
</tr>
<tr>
<td>Wsh -/-</td>
<td>Mast cell deficient mouse, c-kit is not transcribed</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>XL</td>
<td>Crosslinking</td>
</tr>
</tbody>
</table>
PART I

Role for Fyn and Lyn kinases in the regulation of IgG-mediated systemic anaphylaxis
ABSTRACT

Lyn but not Fyn kinase controls IgG-Mediated Systemic Anaphylaxis

By

Yves Trésor Falanga

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2012

Director: John J. Ryan, Professor, Department of Biology

Anaphylaxis is a rapid, life-threatening hypersensitivity reaction. Until recently, it was mainly attributed to histamine released by mast cells activated by allergen crosslinking (XL) of FcεRI-bound allergen-specific IgE. However, recent reports established that anaphylaxis could also be triggered by basophil, macrophage and neutrophil secretion of platelet activating factor subsequent to FcyR stimulation by IgG/Ag complexes. I have investigated the contribution of Fyn and Lyn tyrosine kinases to FcγRIIb and FcγRIII signaling in the context of IgG-mediated passive systemic anaphylaxis (PSA). I found that mast cell IgG XL induced Fyn, Lyn, Akt, Erk, p38 and JNK phosphorylation. Additionally, IgG XL of mast cells, basophils and macrophages resulted in Fyn- and Lyn-regulated mediator release in vitro. FcγR–mediated activation was enhanced in Lyn-deficient (KO) cells, but decreased in Fyn KO cells, compared to wild type cells.
More importantly, Lyn KO mice displayed significantly exacerbated PSA features while no change was observed for Fyn KO mice, compared to wild type littermates. Intriguingly, this work establishes that mast cells account for the majority of serum histamine in IgG-induced PSA. Taken together, these findings establish pivotal roles for Fyn and Lyn in the regulation of PSA and highlight their unsuspected functions in IgG-mediated pathologies.
CHAPTER 1 – INTRODUCTION

A short history of Immunology (1)

Immunology, from its latin origin “immunis” (meaning exempt), is a branch of biology and biomedical sciences focused on the immune system.

The story of immunology dates back from antiquity. In fact, ancient civilizations were based upon strong spiritual and superstitious beliefs in which the occurrence of disease or death was solely perceived as a punishment from the gods after trespass or sins. These stories are found in religious documents and tales such as the mythological story of Pandora box and Pharaoh and the Egyptian plague in the Bible. Therefore, these early societies established deities, Priests, Shamans, Taboos and sacrifices. The practice of prayers and incantations and the wearing of amulets were common ways to be granted protection from diseases and death by the gods.

Reports of extraordinary and exceptional individuals resistant to plague in ancient Greece, or unaffected by dangerous snake venoms in North Africa, or surviving severe pandemics have been mentioned in history. Numerous historians will agree that the main causes of mortality in ancient civilizations were related to infectious diseases and poison. Contrasting with civilizations of their times, Greek and Roman philosophers and scientists such as Socrates and Galen had a rational approach to the etiology of disease. They postulated that disease was coming from imbalance of humors (blood, phlegm, yellow bile and black bile) or presence of bad air (mal-aria). In addition to this theory, many believed that infectious diseases were caused by poison, generally known from
its Latin name “virus” (or Pharmakeia in Greek). One of the oldest pandemic cases documented is “the plague of Athens”, epidemic that decimated ancient Greece around 430 B.C., killing Pericles and about the fourth of the Greek population on its way. It was noted that individuals who survived that plague were never or at least somewhat less affected by further occurrence of this zoonotic disease. One of the fascinating stories comes from ancient Roman Emperor Mithridates VI, who decided to inoculate himself with low and increasing doses of poison in order to render himself resistant to a potential poisoning by his enemies. This later concept, nowadays known as desensitization, is clinically used to render severely allergic patients tolerant to the allergen that would in normal conditions cause critical conditions or fatalities.

It is not until the end of the 19th century that the etiology and pathogenesis of common infectious diseases were starting to be understood, essentially with Louis Pasteur’s revolutionary “germ theory”, which stipulates that infectious diseases are caused by germs. Louis Pasteur’s findings are also credited to be at the origin of the concept of acquired immunity. At that period of history, the Institut Pasteur in Paris was the epicenter of modern bacteriology. For example, Pierre-Paul Émile Roux, closest collaborator of Pasteur and co-founder of the Institut Pasteur, discovered the anti-diphtheria toxin. At the same time, after accepting an invitation from Roux, Alexander Yersin joined the Institute and characterized the bacillus responsible for the bubonic plague. This bacillus was later named on his honor (Yersinia pestis). Furthermore, DNA studies on remains from the victims of the 14th century “Black death” showed that Yersinia pestis was
in fact the pathogen that caused the deadliest pandemics in human history.

The late 19th century and early 20th century was populated with brilliant scientists who contributed to modern microbiology and immunology such as Robert Koch and his discovery of *Bacillus anthracis*, the tuberculosis bacillus and *vibrio cholerae*. During the same period at the Robert Koch Institute in Germany, Emil von Behring and Kitasato discovered antibodies to diphtheria and tetanus. They uncovered that over the course of diphtheria, the human body was producing protective substances (antitoxins) that were circulating in the blood (2, 3). More important was the evidence that the “so-called” protection could be transferred from one individual to another. This discovery saved numerous lives and allowed Behring to win the first Nobel Prize for physiology and medicine in 1901 while Koch received his in 1905 (1). Discoveries from the Koch Institute greatly contributed to shape modern microbiology and immunology, with the nascent discipline of hematology pioneered by Paul Ehrlich. Paul Ehrlich was the first to demonstrate that acquired immunity could be transmitted from the mother to the offspring (3, 4). He is credited to be the founding father of histology, hematology and chemotherapy in addition to immunology (4). He discovered mast cells and brought a cellular explanation to most of the infectious diseases that struck during that period of history. Additionally, one of the tremendous contributions of Paul Ehrlich was his study on the etiology of syphilis (*Treponema pallidum*) and his theory of a “magic bullet” cure capable of eradicating all diseases (chemotherapy). His work on syphilis granted him the Nobel Prize for physiology and medicine in 1908.
Modern immunology has come a long way, from spiritual and superstitious beliefs to a more rational and scientific approach. Over the years and centuries, countless theories were postulated to explain the mechanisms by which people were infected at first, and how the infection could spread from one individual to another [Seed theory “seminaria” from Fracastoro]. Later, brilliant scientists like Pasteur, Koch, Behring, Ehrlich Metchnikoff and Jenner not only hypothesized but clearly demonstrated that diseases are caused by germs (bacteria, virus), that these germs can be transferred from one infected subject to an uninfected one and spread to an entire community, decimating villages, cities and countries. The greatest contribution from this era in bio-medical sciences was the preliminary but pivotal discovery of the immune system: cellular immunology with the identification of mast cells and macrophages and humoral immunology with the discovery of antibodies and vaccination.

_The Immune System_

The immune system is a biological structure found in living organisms. Composed of numerous members, its primary goal is ensuring protection against any type of intrusions. All the cells of the immune system arise from a common hematopoietic stem cell (HSC) progenitor present in the bone marrow. During the early stages of differentiation, some cells migrate from the bone marrow to the thymus. Therefore, the bone marrow and the thymus are considered as the primary lymphoid organs, in contrast to secondary lymphoid organs such as the spleen and the lymph nodes. The robustness of the immune system is dictated
by the distinction between self and non-self molecules. In fact, this step is critical for the survival of the organism, usually being a matter of life or death. In mammals at least, the most commonly encountered pathogens belong to four global categories: bacteria, viruses, fungi and parasites. In order to efficiently clear the infectious pathogen, the immune system is temporally and functionally subdivided in two segments: the innate immune system and the adaptive immune system. The first part of this dissertation will focus on cells of the innate immune system, specifically mast cells, basophils and macrophages whereas the second part will cover mechanisms of the adaptive immune system and the study of B cells.

- **The innate immune system**

  The innate immune system is the first line of defense against pathogenic infections. While the cellular compartment of the innate immune system is composed of circulating (monocytes, basophils, neutrophils and eosinophils) and resident cells (macrophages and mast cells), the humoral section is mainly made out of complement molecules. Once differentiated from the bone marrow’s common myeloid progenitor (CMP), some cells of the innate immune system will always circulate in the blood stream (monocytes, basophils, neutrophils and eosinophils) while others such as mast cells and macrophages will migrate to the tissue. Tissue-resident cells generally locate at the vicinity of the external environment: under the skin, in the airway and in intestinal epithelia for example. In fact, upon recognition of pathogen associated molecular patterns by specialized receptors such as those of the Toll-like receptor family (TLR) present
on the cell surface or through immunoglobulin receptors, cells from the innate immune system secrete chemicals (cytokines, chemokines, and lipid-derived mediators) that will induce the death of the pathogen and the recruitment of other immune cells.

- The Adaptive immune system

During the initial phase of infection, specialized antigen-presenting cells (APCs) like dendritic cells (DCs) will process the antigens associated with the pathogen, migrate to the closest secondary lymphoid organ and trigger mechanisms of the adaptive immune response by presenting the processed antigen to an antigen-specific T cell. Activated T cells will then activate antigen-specific B cells, which will later undergo isotype switch and somatic hypermutation (SHM) and differentiate into antibody-producing plasma cells. Plasma cells secrete a variety of antibodies, all recognizing the antigen with different affinity. During the process of isotype switching, plasma cells produce different classes of antibodies (isotype), ranging from IgM, IgD, IgG, IgE to IgA. Antibody molecules are constituted of two parts: the antigen-binding fragment (Fab), recognizing the antigen and the Fc fragment responsible for the effector function by binding to Fc receptors (Figure 1). For example, the high affinity IgE receptor FcεRI present on mast cells will bind to the Fc portion of IgE molecules while FcγRIII expressed by macrophages will bind to IgG molecules.
Regulation of the Immune system via the Fc receptors

As mentioned earlier, some cells of the immune system express Fc receptors and have the capability to recognize Fc portions of antibodies. In fact, ligation of antigen-bound antibodies to the receptor will trigger an intracellular cascade of events culminating in calcium mobilization, transcription factor activation and the release of mediators. These mechanisms are subject to fine regulation by the cellular machinery. Subsequently, any defects in the control of cell activation will generally result in pathological conditions such as allergies, asthma and systemic anaphylaxis. While the initiation of an allergic response is commonly attributed to mast cell activation via FcεRI (5, 6), scientists agree that it is the combination of many different factors that lead to the onset of this condition. Furthermore, in some cases, the exposure to IgE-bound allergen causes rapid and exacerbated hypersensitivity reactions called anaphylaxis.
Figure 1

Immunoglobulin structure.

Immunoglobulins or antibodies are proteins made up of two light chains (red) and two heavy chains (blue). Each chain contains both the variable region (responsible of antigen recognition) and the constant region. The two heavy chains are linked to each other and to the light chain by disulfide bonds.
Consequently, the role of mast cells in allergy, inflammatory diseases and immune homeostasis has been most extensively studied downstream of IgE receptor signaling (7-13), although interesting studies reported that mast cells can also be activated by their IgG receptor (14) (Figure 2). Data generated from murine models demonstrate that an anaphylactic reaction can be induced through the IgG-FcγR pathway (15-18). In this “alternative” pathway of anaphylaxis, FcγR activation of basophils, macrophages, and neutrophils elicits anaphylaxis symptoms, including a drop in core body temperature.

**Lyn kinase**

FcεRI activation involves a plethora of signaling molecules, including Fyn and Lyn kinases. Lyn kinase, which is expressed as 53 and 56 kDa isoforms due to alternative splicing (19-21), is expressed in nearly all immune cells, except the T cell lineage. For example, the association of Lyn kinase with the B cell antigen receptor (BCR) (22) and the high affinity IgE receptor, FcεRI (23), places Lyn in a critical position to control inflammation. Because of the known stimulatory roles for Src family kinases, it was assumed that Lyn possessed pro-stimulatory roles in signaling. Consistent with this, Lyn knockout (KO) mast cells were found to have poor calcium flux responses after IgE-mediated activation (24). However, further study – not accounting for the increased IgE in Lyn KO – showed that Lyn deficiency exacerbated IgE-mediated mast cell activation and anaphylaxis (25). These apparently contradictory data were explained by the ability of Lyn to recruit several inhibitory proteins, including SHIP-1, SHP-2, DOK-1, and CBP.
(26-29). These negative regulators reduce signaling through other Src family members, including PI-3 kinase and the Ras-MAPK cascade. Hence, Lyn appears to be a negative regulator of the mast cell response.
Mast cells in innate and adaptive immunity. Mast cells are generally located underneath the epithelial membranes such as the skin, the gastrointestinal track and the upper respiratory ways. They express the high affinity IgE receptor FcεRI, capable of recognizing the Fc portion of IgE molecules. IgE-bound antigen aggregation is required for FcεRI activation. Upon FcεRI activation, mast cells undergo a rapid release of preformed mediators such as histamine and leukotrienes, two potent vasoactive mediators causing vasodilation and smooth muscle cell contraction. In the lungs, they are responsible for bronchial constriction, generally associated with asthma. Vasodilation in the site of infection is critical to the clearance of the pathogen because it facilitates the recruitment of other specialized immune cells from the blood vessels to the site of infection. Following the immediate degranulation phase, mast cells will secrete chemokines and cytokines (even though some of them can be preformed and released during the early phase). This second wave of mediators takes longer, due to de novo biosynthesis of molecules in response to stimuli. In fact, mast cells secrete a plethora of pro- and anti-inflammatory cytokines both promoting and inhibiting mast cell activation respectively. For example, mast cell-secreted IL-4 and eotaxin triggers mucus production and eosinophil recruitment in the lungs, while it induces T cell production of IL-4 and IL-5. These latter cytokines are necessary to maintain B cell survival and isotype switch to IgG1 and IgE antibodies and important for eosinophils recruitment respectively. Furthermore, mast cell activation can lead to the production of anti-inflammatory molecules.
such as IL-10 and TGF-β acting locally by decreasing the surface expression of FcεRI and affecting other immune cells in paracrine mechanisms of action.
**Fyn kinase**

Fyn on the other hand is a 59 kDa Src-family protein tyrosine kinase involved in mast cell degranulation and cytokine release (30, 31). Once phosphorylated, Fyn activates the adaptor protein Gab2, initiating the phosphatidylinositol-3’ kinase (PI3K) pathway. This enhances calcium flux through a TRPC1-mediated process leading to cortical F-actin depolymerization (32). In addition, Fyn enhances transcription factor activity, degranulation and cytokine release (6, 25). Fyn activation is currently viewed as a positive regulator of IgE-mediated inflammatory signals, similar to the well-documented Syk kinase pathway.

**Regulation of the immune response by FcγRs**

In addition to FcεRI, mast cells express the IgG receptor family (FcγR). The high-affinity IgG receptors include FcγRI in humans and mice, and FcγRIV in mice only, while the low affinity IgG receptors include FcγRII and FcγRIII, in humans and mice. Humans have three FcγRII (A, B, and C) and two FcγRIII (A and B) whereas mice have one receptor of each type (FcγRIIB and FcγRIIIA) (33). Activating FcγR on mouse cells consist of a ligand-binding alpha-chain (α) and a signal-transducing gamma-chain (γ) bearing immunoreceptor tyrosine-based activation motif (ITAMs), which once phosphorylated upon receptor crosslinking, act as a docking site for signaling molecules such as Syk kinase, resulting in the initiation of activatory signaling pathways. In contrast, ligand binding can also result in downregulation of antigen-dependent mediator release
due to the existence of the immunoreceptor tyrosine-based inhibitory motif (ITIMs) on the cytosolic portion of FcγRIIB α-chain. Subsequent phosphorylation of ITIM upon FcγRIIB ligation induces the activation of Lyn kinase, which will recruit and activate phosphatases like SHIP in addition to directly activating ITIM, leading to decreased mediator release (34). FcγRIIB inhibits several aspects of mast cell and basophil functions such as IgE-dependent degranulation and IL-4 production (35, 36). Additionally, beside mast cells, FcγRIIB is also expressed by other immune cells including dendritic cells, macrophages, activated neutrophils, basophils and B cells. When expressed by these cells, FcγRIIB inhibits the functions of activating FcγRs, such as phagocytosis and pro-inflammatory cytokine release (33, 35, 36).

**Similarities between IgE and IgG receptors**

IgG receptors (FcγR) share a common gamma subunit with FcεRI (Figure 3), and share similar signaling pathways. Although the importance of Syk kinase in IgG signaling has been demonstrated (37, 38) little is known about Src family kinase functions. A major part of this study will focus on understanding the importance of Fyn and Lyn kinases in FcγR signaling in mast cells, basophils, and macrophages as well as the involvement of these two kinases in pathophysiological events such as anaphylaxis.
FcεRI
- Mast cell
- Basophil
- Eosinophil (induced)
- Monomeric IgE

- High Affinity
- Hypersensitivity
- Asthma

FcγRI
- Macrophage
- Dendritic cell

- High Affinity

FcγRIII
- Monocyte
- Macrophage
- Neutrophil
- Mast cell
- Basophil
- Dendritic Cell

- Low Affinity
- Phagocytosis
- ADCC
- NK activation

FcγRIV
- Neutrophil
- Dendritic cell
- Monocyte
- Macrophage

- Int. Affinity
- Anaphylaxis

FcγRIIB
- Monocyte
- Macrophage
- Neutrophil
- Mast cell
- Basophil
- Dendritic Cell

- Low Affinity
- Aggregated immune complexes
- ADCC
**Figure 3**

**Similarities between FcεRI and the Fcγ receptors family.**

The high affinity IgE receptor FcεRI is constitutively expressed on mast cells and basophils surface while in some conditions, monomeric IgE can trigger its expression on eosinophils and human macrophages. FcεRI is constituted of one alpha chain, one beta chain and two gamma chains linked by disulfide bonds. IgE and IgG molecules have similar structures, as do their receptors. In fact, the IgG receptor family comprises activating receptors bearing intracellular ITAMs (FcγRI, FcγRIII and FcγRIV in mice, and FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA in humans) and inhibitory receptors with intracellular ITIMs (FcγRIIB) in mice and humans. Activating IgG receptors have similar structures, related to FcεRI. They are formed of an alpha chain and two gamma chains while the inhibitory FcγRIIB is constituted by a unique alpha chain. In addition to triggering cell activation and inhibition, these receptors are also involved in phagocytosis and anaphylaxis. They are expressed on macrophage, DC, mast cell, neutrophil and eosinophil. They bind to specific subclasses of IgG molecules with different specificities. Malfunction or deficiency in these receptors is linked to recurrent bacterial and parasitic infections and autoimmune diseases. [Cell types in bold font represent the cell population that has the highest expression level].
When things go wrong

In pathologic and particular conditions, mast cell activation with antigen-specific IgE can lead to anaphylaxis, a rapid and life-threatening hypersensitivity reaction. The onset and regulation of anaphylaxis has been exclusively attributed to mast cells, delineating the classical pathway of anaphylaxis involving IgE-mediated FcεRI mast cell activation and subsequent histamine release (5). However, elegantly conducted research on murine models demonstrates that an anaphylactic reaction can be induced through the IgG-FcγR pathway (15-18). In this novel pathway of anaphylaxis, activation of basophils and macrophages (FcγRI and FcγRIII) (15-17), and recently FcγRIV on neutrophils (18) elicits anaphylaxis signs, including a drop in core body temperature. Additionally, mast cells have recently been reported to be responsible for FcγRIIA-dependent cutaneous anaphylaxis whereas monocyte/macrophages and neutrophils were found to be involved in FcγRIIA-dependent systemic anaphylaxis (39).

Our findings show that Fyn, Lyn, Akt, Erk, p38 and JNK are activated upon FcγR stimulation, and that Fyn and Lyn regulate FcγR-mediated degranulation, cytokine and chemokine release not only in mast cells, but also basophils and macrophages. Furthermore, we demonstrate that mast cells account for the majority of circulating histamine during FcγR-induced passive systemic anaphylaxis (PSA), which is regulated by Fyn and Lyn. Moreover, we show that Lyn, but not Fyn kinase, is a major regulator of IgG-mediated PSA. These results bring new insights to the function of Fyn and Lyn kinases downstream of FcγR stimulation.
CHAPTER 2 – DISSERTATION OBJECTIVE

Numerous studies have demonstrated the importance of Src family kinases in the regulation of the immune response. As mentioned earlier, Juan Rivera’s group was able to demonstrate that Fyn and Lyn kinases have antagonistic functions in mouse mast cell IgE receptor stimulation. Fyn -/- mast cells showed decreased mediator release while Lyn -/- cells displayed an exacerbation. These findings led us to consider the following hypotheses:

- Primarily, we wanted to know whether these two kinases were activated upon IgG receptor stimulation. If they are, what are their functions in mast cells? This hypothesis is of significant relevance given that IgG molecules are the most abundant Ig in the serum and that mast cells express three isoforms of the IgG receptor (FcγRI, FcγRIIB and FcγRIII).

- Additionally, like mast cells, basophils and macrophages also express FcγRI, FcγRIIB and FcγRIII. We consequently wanted to test the hypothesis that Fyn and Lyn kinases have regulatory roles in IgG-mediated cell activation in these cells.

- The regulation of the immune system includes a diversity of cell lineages and a plethora of molecules. We therefore sought to investigate the regulatory roles of Fyn and Lyn kinases in the context of IgG-mediated passive systemic anaphylaxis, using a mouse model.

- The second part of this dissertation focuses on investigating the role of Fyn kinase in antibody production. While IL-4 is a vital cytokine for B cell survival and isotype switching, recent studies reported that Fyn kinase is
required for T cell IL-4 production. Consequently, we wanted to test the hypothesis that Fyn kinase has a role in antibody production.
CHAPTER 3 – MATERIAL AND METHODS

Animals

C57BL/6x129sv wild type (WT), C57BL/6x129sv Fyn-deficient (KO), 129sv WT and 129sv Lyn KO inbred strains were described previously (25, 30). Mast cell-deficient Wsh-/- and their C57Bl/6 control mice were purchased from the Jackson Laboratory. All mice were used at a minimum of 9 weeks of age, and all experiments received approval from the Virginia Commonwealth University institutional animal care and use committee (IACUC).

Cytokines and reagents

Cytokines and ELISA assay kits were purchased from PeproTech (Rocky Hill, NJ). Bio-plex Pro cytokine assay kits were purchased from BioRad (Hercules, CA). Histamine and leukotriene C₄ (LTC₄) ELISA kits were purchased from Cayman Chemicals (Ann Arbor, MI) and Neogen Corporation (Lansing, MI). Cytokine measurements were performed as per the manufacturer’s directions. Antibodies specific for tyrosine-phosphorylated (pY) Lyn and total Lyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for the tyrosine-phosphorylated form of the Src kinase activation loop (pY416), phospho Erk (p44/p42 MAPK Thr 202/Tyr 204), Erk, phospho Akt (Thr 308), Akt, phospho p38, p38, phospho JNK (Thr 183/Tyr 185), JNK and for Fyn were purchased from Cell Signaling Technologies (Danvers, MA). Anti-mouse FcεRIα was purchased from eBioscience (San Diego, CA). Rat anti-mouse FcRγII/RIII (2.4G2) were a kind donation of Professor Fred Finkelman from
Cincinnati Children Hospital (Cincinnati, OH). Purified mouse IgE, rat anti-mouse IgE, rat IgG isotype control, and rat IgG anti-c-kit (CD117) were purchased from BD Pharmingen (San Diego, CA).

**Cells**

Mouse bone marrow-derived mast cells (BMMCs) were derived by culture in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1mM sodium pyruvate, and 1 mM HEPES (all from Biofluids, Rockville, MD), supplemented with IL-3–containing supernatant from WEHI-3 cells and stem cell factor (SCF)-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF was adjusted to 1 ng/ml and 10 ng/ml, respectively, as measured by ELISA. Mouse bone marrow-derived basophils were obtained by culturing bone marrow cells for six days in cRPMI 1640 medium supplemented with 5 ng/ml recombinant IL-3 (PeproTech, Rocky Hill, NJ). At day 6, they were analyzed and sorted by flow cytometry on the basis of FcεRI-positive and Kit-negative characteristics. Mouse bone marrow-derived macrophages were obtained by culturing bone marrow cells for eight days in cRPMI supplemented with 50 ng/ml MCSF (PeproTech).

**In vitro activation of antibody production**

Naïve B cells were isolated using magnetic beads following manufacture’s protocol (Miltenyi Biotec). Briefly, single cell suspension was generated and cells were resuspended in MACS buffer (PBS pH 7.2, 0.5% BSA and 2mM EDTA) and
incubated with anti-CD43 magnetic beads for 15 minutes at 4°C. Cells were then washed and passed through a magnetic column. Flow-through was collected. For IgG1 and IgE production, B cells were cultured with 1µg/mL of stimulating anti-CD40 antibody (Invitrogen) and IL-4 (10,000U/mL) for seven days. Supernatants were then harvested and analyzed for IgG1 and IgE production by ELISA. For western blots, B cells were stimulated with 30ng of IL-4 (Peprotech).

Quantification of antibody titers by ELISA

For total IgM and IgG1 ELISAs, samples were serially diluted and added to 96-well plates (50µL/well) pre-coated with 5 µg/mL of goat-anti IgM and IgG, respectively (Southern Biotech). For standard curve, normal mouse IgM and IgG1 (Southern Biotech) were used. After incubation at 37°C for 1 h, bound Abs were revealed by goat-anti-IgM-AP and goat-anti-IgG1-AP, respectively (Southern Biotech). Total IgE ELISA was carried out as previously described [34,35]. For antigen-specific ELISAs, ELISA was carried out as described with minor modifications [34]. Plates were coated with NP14BSA (Biosearch Technologies)(15 µg/mL in PBS) for samples and with 5 µg/mL of goat-anti Ig (Southern Biotech) in PBS for standard. Standard curves were performed by coating with anti- IgM, anti-IgG1, anti-IgG2a or IgG2b and adding known amounts of IgM, IgG1, IgG2a or IgG2b, respectively. The values for experimental samples are reflected as relative units (RU) because serum antigen-specific Abs were captured with antigen. The remaining steps were carried out as described.
**Proliferation**

To determine proliferation, cells were isolated and stimulated as described (see above). After 96 hours in culture, cells were pulsed with 1 µCi/well of tritiated ([3H]) thymidine (Perkin Elmer) for 24 hrs. Plates were then harvested onto GFC plates using a filtermate cell harvester. Plates were dried for at least 2 hours, 25µL of scintillation fluid was added, and counts were determined using the Topcount Plate Counter (Perkin Elmer, Waltham, MA). Proliferation was reported as counts per minute (CPM).

**Flow cytometry**

Mice were bled via tail vein nick and spleens were isolated. Cells were labeled following RBC lysis and filtration through 40µm cell strainers. Antibodies included unlabeled CD16/32 (2.4G2), FITC-conjugated anti-mouse c-Kit (2B8) and Gr-1 (RB6-6B2); PE-conjugated anti-FcεRIα (MAR-1) and Gr-1 (RB6-8C5); Alexa 647-conjugated anti-mouse CD49b (DX5) and B220 (RA3-6B2); PE-Cy7 conjugated anti-mouse CD3 (145-2C11) and CD11b (M1/70) from BioLegend; and PE-conjugated anti-Siglec-F (E50-2440) from BD Biosciences. Flow cytometric analysis was performed using a Canto (BD Biosciences) and data analysis was conducted with FlowJo software (Tree Star). B cell isolation and labeling was conducted as described previously [34,36]. Cells were labeled following RBC lysis and filtration through 40 µm cell strainers. Abs included anti-mouse unlabeled 2.4G2, biotinylated CXCR5 (RF8B2); PE-conjugated Fas (Jo2); APC-conjugated GL7 (GL7), PE-Cy7 conjugated-streptavidin from BD Bioscience;
FITC-conjugated B220 (RA3-6B2), CD4 (RM4-5) and PerCP-Cy5.5 conjugated PD-1 (29F.1A12) from Biolegend. Lymphocytes were gated based on FSC vs. SSC. Cell subsets were defined as follows: Plasma cells: B220^{lo/-}\text{CD138}^{+} [36]; germinal center B cells: B220^{+}\text{GL7}^{+}\text{Fas}^{hi} [37] and follicular helper T cells: CD4^{+}\text{CXCR5}^{+}\text{PD-1}^{hi} [38] Flow cytometric analysis was performed using a Canto or AriAll (BD Biosciences), and data analysis was conducted with FlowJo (Tree Star).

**Western blot**

Cells were dissociated in RIPA (Radio-Immunoprecipitation Assay) buffer and Western blotting was performed using 50 μg total cell lysate per sample. Proteins were loaded and separated over an 8–16% or 4–20% gradient SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membranes (Pall Corporation, Ann Arbor, MI), and blocked for 60 minutes in Blotto B buffer (Rockland Immunochemicals, Gilbertsville, PA) plus 0.1% Tween-20. Blots were incubated in a solution of TBS supplemented with 0.1% Tween-20 and 5% BSA (TBST), with the indicated antibodies overnight at 4°C with gentle rocking. Blots were washed six times for 10 minutes each in TBST, followed by incubation in Blotto B containing a 1:5,000 dilution of HRP linked anti-IgG matched to the relevant species, from Cell Signaling (Danvers, MA). Size estimates for proteins were obtained using molecular weight standards from Bio-Rad (Hercules, CA).
**Microscopy**

Cells were sensitized with 10 µg/ml of 2.4G2 for 60 minutes at 4°C and washed 3x with phosphate buffered saline (PBS). Alexa Fluor 488-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) was added to the cell suspension at the concentration of 10 µg/ml and the cells were incubated at 37°C 5% CO2. At appropriate time points, 5 x 10^4 cells were centrifuged at 1500 g for 5 min at 4°C. The cells were then cytocentrifuged onto glass slides at 1000 g for 3 min in a Cytospin 4 centrifuge (Thermo Electron, Pittsburgh, PA) and fixed in methanol for 4 min. Slides were stained for immunofluorescence microscopy as previously described (40). Primary antibodies were Fyn and Lyn (Santa Cruz Biotechnologies, Santa Cruz, CA), both at dilutions of 1:50, followed by Rhodamine Red (Jackson ImmunoResearch, West Grove, PA), at a dilution of 1:50. Slides were mounted with the Prolong gold antifade reagent with DAPI (Invitrogen). Images were acquired by spinning disc confocal microscopy using a BX51 microscope (Olympus, Center Valley, PA) affixed with an Olympus disk spinning unit and an Ocra-R2 CCD camera (Hammamatsu, Japan). Images were processed using the Slidebook software package (Intelligent Imaging Innovations, Denver, CO).

**Passive Systemic Anaphylaxis**

Mice were injected intravenously with 200 µl of PBS containing 5mg of histamine, 700 ng of PAF or 500 µg of rat anti-mouse CD16/CD32, clone 2.4G2 (17, 41).
The core body temperature of each animal was measured using a rectal microprobe (Physitemp Instruments). Mice were then euthanized, and blood was collected by cardiac puncture to prepare serum.

*Lungs inflation*

Two hours after the induction of PSA, mice were sacrificed and lungs were inflated with Agarose (Invitrogen) pre-warmed at 60ºC, administered through intra-tracheal incision. Animals with inflated lungs were then directly placed on ice. Later the lungs were resected from the animal and stored into 40 ml of 10 % Neutral Buffered formalin. Lung sections, mounts and Hematoxylin-Eosin (H&E) staining were performed by the Virginia Commonwealth University’s Department of Pathology (Richmond, VA). Lung sections were imaged with a Nikon E-600 compound microscope using a 20× objective.

*PAF acetyl hydrolase (AH) Activity Assays*

Substrate (50µM 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine with 0.05 µCi of hexadecyl-2-acetyl-sn-glyceryl-3-phosphocholine, 1-O-[acetyl-(N)-3H] (NEN/PerkinElmer; 13.5 Ci/mmol) added as a tracer), was combined with Serum and incubated for 30 minutes at 37º C to determine PAFAH enzymatic activity. PAFAH activity was expressed as release of [3H] acetate, using a method that we described previously (42). 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) was purchased from Avanti Polar Lipids (Alabaster, AL).
Statistical Analysis

Data are presented as the mean plus or minus SEM of at least 3 independent experiments. Comparisons were made by the two-tailed Student t test for independent samples. $P$ values less than 0.05 were considered statistically significant. Analysis was performed with GraphPad Prism software.
CHAPTER 4 – RESULTS

Regulation of IgG receptor signaling by Fyn and Lyn kinases

- FcγR stimulation induces rapid Fyn, Lyn, Akt, Erk, p38 and JNK activation in bone marrow-derived mast cells.

We first observed that only Lyn kinase deficiency affected mast cell surface expression of FcγRIIB and FcγRIII (2.4G2 staining) (Figure 4). Furthermore, Lyn kinase deficiency appeared to have a dominant phenotype in Fyn/Lyn double KO mast cells. In order to compare the effect of IgE versus IgG receptor stimulation on the phosphorylation state of Fyn kinase, bone marrow-derived mast cells were stimulated in vitro with IgE plus antigen or 2.4G2 plus anti-IgG (referred to as IgE or 2.4G2 crosslinking (XL) respectively). Fyn kinase was then immunoprecipitated and proteins separated by SDS-PAGE. The phosphorylated form of Fyn was detected by immunoblotting for the activation loop of the Src kinase family on residue 416 (pY416) (Figure 5a). Our results show that Fyn kinase (59kDa) is rapidly activated downstream of both the IgE and the IgG receptors. Quantification of western blot signal intensity by densitometry after adjusting for loading showed that FcεR and FcγRI activate Fyn to a similar extent.

Comparable to Fyn, we found that both Lyn kinase isoforms (53 and 56 kD) (21) were also activated following 2.4G2 XL (Figure 5b). FcγR-mediated Lyn activation was nearly as strong as IgE-mediated effects as attested by
densitometry analysis of band intensity. Furthermore, staining of Fyn or Lyn by immunofluorescence after 2.4G2 XL in BMMC revealed a diffuse cytoplasmic location of these two kinases, not only at the proximity of FcγRIIB and FcγRIII (Figures 5c, 5d and Figure 6)
Figure 4

Lyn deficiency affects FcγRII/RIII surface expression on mast cells.

Bone marrow-derived mast cells were cultured in IL-3 and SCF as described in the material and methods section. Surface expression of FcγRII/RIII (CD32/CD16) levels were assessed with a fluorescently labeled rat IgG2b molecule (2.4G2 clone) recognizing both FcγRII/RIII (CD32/CD16). The graph summarizes 3 independent experiments. Data shown are mean ± SEM.
Figure 5

Fyn and Lyn kinases are activated upon FcγRIIB/RIII stimulation of bone marrow-derived mast cells. BMMCs were sensitized with either anti-DNP IgE (10 µg/ml) or rat anti-mouse FcγRII/III 2.4G2 (10 µg/ml) and stimulated with DNP-HSA (100ng/ml) (IgE XL) or goat anti-rat IgG (10 µg/ml) (2.4G2 XL) respectively for the indicated times. (a) Fyn immunoprecipitation and western blotting with anti-Src kinase activation loop antibody (pY416). Total Fyn expression is shown as a loading control. The image is representative of 3 independent experiments. Data shown are mean ± SEM. The fold induction was quantified by densitometry analysis of band intensity with Image J software. The mean fold change is relative to the control cells receiving media alone as stimulus. (b) Western blot for activated Lyn, detected with anti-phosphotyrosyl 396 Lyn antibody. Total Lyn expression represents the loading control. The image is representative of 3 independent experiments. (c, d) BMMCs were sensitized with 10 µg/ml of 2.4G2 for 60 minutes at 4°C and cross-linked with 10 µg/ml of Alexa Fluor 488-conjugated goat anti-rat IgG [Green] for indicated time-points before being cytocentrifuged onto glass slides for microscopy analysis (Refer to the material and methods section). Fyn and Lyn are visualized by rhodamine X-conjugated secondary antibodies [Red] recognizing anti-Fyn and anti-Lyn primary antibodies. Cell nuclei are visualized by DAPI staining [Blue]. Images were acquired by spinning disc confocal microscopy using a BX51 microscope (Olympus, Center Valley, PA) affixed with an Olympus disk spinning unit and an Ocra-R2 CCD camera (Hammamatsu, Japan). Images were processed using the Slidebook.
software package (Intelligent Imaging Innovations, Denver, CO). Representative images of at least 3 independent experiments.

**STEP 1 - ACTIVATION**
- Sensitization with 2.4G2
- Activation/crosslinking with a fluorescently labeled Goat anti-rat IgG (Alexa 488)
- Cold washes to remove the excess IgG
- Permeabilization

**STEP 2 - INTRACELLULAR STAINING**
- Staining of Fyn/Lyn with a rabbit primary antibody
- Washes
- Detection with a secondary Goat anti-rabbit IgG (mouse, rat, human adsorbed) coupled to either Rhodamine Red X (or) Cy3
Figure 6

Immunofluorescence strategy for the dynamic study of Fyn, Lyn, FcγRIIB and FcγRIII
Intracellular signaling events occurring in mast cells after FcεRI activation of have been intensively studied and are well established. Since our results show that comparable activation levels of Fyn and Lyn can be achieved through FcεR or FcγRI stimulation, we next decided to investigate the dynamics of phosphorylation events subsequent to Fyn and Lyn activation. We found that Fyn activation after 2.4G2 crosslinking occurred rapidly, with maximal phosphorylation observed within 3 minutes (Figure 7a). In addition, FcγR activation of mast cells triggered the activation of Akt, Erk, p38 and JNK (Figure 7b). A representative densitometry analysis of band intensity shows the phosphorylation dynamics of these signaling molecules (Figure 7c), with ERK phosphorylation being the strongest, reaching about 70-fold increase in less than a minute after the initiation of 2.4G2 XL. Our results showed parallel phosphorylation for Fyn, Akt and Erk during the early time points of FcγR activation of mast cells, with only Lyn displaying sustained phosphorylation over about 30 minutes. Collectively, our results show that comparable Fyn and Lyn activation can be achieved through FcεR or FcγRI stimulation. In addition, FcγR-mediated signals activated similar downstream targets to those previously shown for FcεRI signaling (30, 43-49), especially the robust and early Akt and Erk signaling.
Figure 7

FcγR stimulation activates Fyn, Lyn, Akt, Erk, p38 and JNK in BMMCs. BMMCs were sensitized with rat anti-mouse FcγRII/III 2.4G2 (10 µg/ml) and stimulated with goat anti-rat IgG (10 µg/ml) (2.4G2 XL) respectively for the indicated times. (a) Fyn was immunoprecipitated and activated Fyn was detected by an anti-Src kinase activation loop antibody (pY416) after 2.4G2 XL. Total Fyn expression is shown as a loading control. (b) Representative immunoblot of the phosphorylation kinetics of Lyn, Akt, Erk, p38 and JNK after 2.4G2 XL. Total protein expression is shown as loading control. (c) Phosphorylation time course of activated Lyn (pY396), Akt (pT308), Erk (pT202/pY204), p38 (pT180/pT182) and JNK (pT183/pY185) upon 2.4G2 XL. The densitometry analysis of band intensity was calculated using the Image J software. Fold change is relative to the control cells incubated in media alone. The graph summarizes 3 independent experiments. Data shown are mean ± SEM.
•  **Effects of Fyn or Lyn deficiency downstream of mast cell IgG receptor signaling**

Fyn and Lyn kinases act as opposite regulators of IgE-induced mast cell activation (25), (30, 31). We therefore investigated their roles in IgG-mediated BMMC activation. In order to fairly compare how downstream signaling molecules are affected by Fyn or Lyn deficiency, we depleted these kinases with specific siRNA on C57BL6 mast cells, given that Fyn KO and Lyn KO were generated on different backgrounds (B6.129sv and 129sv, respectively). Fyn KO BMMCs displayed increased phospho-JNK while no change was observed for phospho-Akt and phospho-p38 subsequent to IgG XL (Figure 8a, 8b). On the other hand, Lyn-deficient BMMCs displayed significantly high phospho-Akt while there was no change in JNK or p38 phosphorylation status in comparison to WT BMMCs. Complementing the data in Figure 6, these data demonstrate that Fyn and Lyn regulate particular signaling pathways upon IgG XL in mast cells by targeting different downstream signaling molecules.

•  **Fyn kinase deficiency diminishes FcγR-mediated activation of mast cells, basophils, and macrophages**

To determine the functional importance of Fyn kinase downstream of FcγR signaling, BMMCs derived from WT or Fyn KO bone marrow progenitors were stimulated by 2.4G2 crosslinking, and cell culture supernatants were collected to measure degranulation and cytokine release. Fyn deficiency significantly decreased histamine release (Figure 9a), and profoundly reduced IL-
6 (Figure 9b) and IL-13 (Figure 9c) levels. There were no striking differences in leukotriene C4 (LTC4), MIP-1 alpha or TNF-alpha secretion (data not shown).
Figure 8

Effects of Fyn or Lyn deficiency on mast cell FcγR stimulation.

Fyn or Lyn kinases were knocked down by siRNA in BMMCs, which were then stimulated with 2.4G2 XL for the indicated times. (a) Representative image of Lyn, Fyn, phospho-Akt, phospho-JNK, phospho-p38 immunoblot after SDS-page western blot. Total Fyn, Lyn, Akt (pT308), JNK (pT183/pY185), p38 (pT180/pT182) and GAPDH lanes are shown as loading controls. (b) Phosphorylation kinetics of activated Akt, p38 and JNK upon 2.4G2 XL. The densitometry analysis of band intensity was calculated using Image J software. Fold change is relative to the control cells incubated in media alone.
We used the calcium ionophore ionomycin as a positive control for all of our cytokine and mediator release measurements in this study and observed that the deficiency in either Fyn or Lyn did not significantly impact the amount of mediators secreted (data not shown). These data mirror previous reports of the importance for Fyn kinase in IgE-mediated stimulation (25, 30), expanding its significance to FcγR stimulation.

Basophils and macrophages share FcγRIIB/III expression with mast cells, and are also involved in innate immunity. In macrophages, FcγRs receptors regulate a variety of functions, such as macrophage activation or inhibition as well as opsonization of antibody-neutralized pathogens (50-54). Recent studies have identified the pivotal importance of basophils and macrophages in IgG-related allergic and anaphylactic reactions (17, 55-61). To determine the functional relevance of Fyn kinase downstream of FcγR signaling in basophils and macrophages, these cells were derived from WT and Fyn KO bone marrow as described in Materials and Methods. Interestingly, after 2.4G2 crosslinking, we observed that Fyn KO basophils and macrophages displayed a significant decrease in secreted cytokines and chemokines in comparison to the WT cultures (Figures 9d-h). These data demonstrate that Fyn kinase is required for optimal FcγR-mediated activation basophils and macrophages in addition to mast cells.
Figure 9

Fyn kinase deficiency diminishes mast cell, basophil and macrophage FcγR-mediated activation.

(a, b, c) WT and Fyn KO BMMC were activated with 2.4G2 XL as described in Figure 5 for 1 hour to measure early phase mediators (Histamine) or 18 hours for cytokine release, analyzed by ELISA. (d, e) WT and Fyn KO bone marrow-derived macrophages were activated for 18 hours, and cell supernatant was analyzed by ELISA. (f, g, h) WT and Fyn KO bone marrow-derived basophils were activated for 18 hours and cell supernatant analyzed by ELISA. Data shown are mean ± SEM of at least 3 independent experiments done in triplicate. *p<0.05; **p<0.001; ***p<0.0001 based on Student’s t test Fyn WT to Fyn KO cells.
Lyn kinase deficiency enhances FcγR-mediated activation of mast cells, basophils and macrophages

To determine the role of Lyn in FcγR signaling, wild type and Lyn KO mast cells were stimulated by 2.4G2 crosslinking. We found that Lyn KO BMMCs displayed a significant increase in the secretion of early phase mediators such as Leukotriene C4 (LTC₄) (Figure 10a). Additionally, the amount of IL-13 and MIP-1α released was significantly increased in Lyn KO BMMCs compared to the control cells (Figures 10b, 10c). However, we did not observe a significant increase in histamine and other cytokines including TNF-alpha (data not shown). These results support the idea that Lyn kinase selectively regulates the amount of mediators released upon FcγR stimulation in BMMCs. Similar results were observed in macrophages (Figures 10d, 10e) and basophils (Figures 10f-h), where Lyn deficiency also selectively enhanced FcγR-mediated cytokine/chemokine release. Taken together, our findings support that Fyn and Lyn kinases are key opposing regulators of FcγR-dependent mast cell, basophil and macrophage activation.
Figure 10

Lyn kinase deficiency enhances FcγR-mediated mast cell, basophil, and macrophage activation.

(a, b, c) WT and Lyn KO BMMC were activated with 2.4G2 XL as described in Figure 5 for 1 hour to measure early phase mediators (Leukotriene C4) or 18 hours for cytokine release, and analyzed by ELISA. (d, e) WT and Lyn KO bone marrow-derived macrophages were activated for 18 hours, and cell supernatant was analyzed by ELISA. (f, h) WT and Lyn KO bone marrow-derived basophils were activated for 18 hours as described above, and cell supernatant was analyzed by ELISA. Data shown are mean ± SEM of at least 3 independent experiments done in triplicate. *p<0.05; **p<0.001; ***p<0.0001 based on Student’s t test WT to Lyn KO cells.
Regulatory functions of Fyn and Lyn kinases during FcγR-induced passive systemic anaphylaxis (PSA)

- Lyn but not Fyn kinase regulates IgG-induced PSA

The importance of Fyn and Lyn in FcγR signaling prompted us to investigate their regulatory roles in vivo, in the context of passive systemic anaphylaxis (PSA). We performed an IgG-mediated PSA assay using intravenously injected 2.4G2 (41). Recent studies have reported that mast cells are not required for IgG-mediated PSA, and identified basophils, macrophages and neutrophils as the key players (15, 17, 18). Both Fyn KO age and gender-matched littermate mice developed anaphylaxis as assessed by decreased core body temperature, followed by a recovery period of approximately 2 hours. Unlike in vitro FcγR stimulation (Figure 9), Fyn deficiency did not convey protection from anaphylaxis severity (Figure 11a).

In contrast, 2.4G2-induced PSA was exacerbated in Lyn KO mice compared to their wild type littermates (Figure 11b), correlating with our in vitro observations with mast cells, basophils and macrophages (observed in Figure 10). Despite a dramatic (11°C) drop in body temperature, none of the Lyn KO mice died in this experiment. Furthermore, the comparison of Fyn KO and Lyn KO mice lungs two hours after PSA did not reveal any drastic changes in airway inflammation features such as perivascular edema or immune cell infiltration (Figure 11c). Surprisingly, we noticed that Lyn kinase deficiency not only exacerbated the severity of IgG-induced PSA but also, significantly extended the recovery time after the onset of anaphylaxis (the data will be discussed later in
this dissertation). Additionally, we did not observe any changes in circulating cytokines (Bioplex for GM-CSF, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17α, MCP-1, MIP-1α, MIP-1β and TNF) in the sera of mice 2 hours after 2.4G2-induced PSA (Figure 12). In general, 129Sv mice (Lyn WT and KO) displayed a lower nadir than their Fyn WT and KO counterparts. This could be due to higher numbers of blood basophils, monocytes and neutrophils on this genetic background. Furthermore, we noticed that Fyn KO mice had significantly low monocyte numbers compared to their WT littermates. This observation only partly contributes to understanding the observed phenotype. Also, we found that Fyn or Lyn deficiency did not affect circulating B cell, T cell or eosinophil percentages (Figure 13).

- **Fyn and Lyn kinases regulate serum histamine during PSA**

Histamine and platelet-activating factor (PAF) are very potent mediators causing bronchoconstriction, vascular leak and vasodilation, three features observed during human and mouse systemic anaphylaxis (17, 41, 59, 62, 63). Due to a paucity of sensitive assays for measuring PAF, and given that both PAF and histamine cause anaphylaxis, we next investigated the effects of 2.4G2-induced PSA on circulating histamine levels in the context of Fyn or Lyn deficiency. Our results showed that the serum of Fyn KO mice contained significantly lower histamine levels than their wild type counterparts (Figure 14a). In contrast, Lyn KO mice displayed elevated serum histamine compared to their littermates, in agreement with their exacerbated PSA phenotype (Figure 14b).
Lyn but not Fyn kinase deficiency is pivotal to IgG-induced passive systemic anaphylaxis.

(a,b) PSA was induced with intravenous injection of 500µg of 2.4G2. Changes in the core body temperature were measured by rectal micro-probe [Fyn WT (PBS, n=4; 2.4G2, n=6), Fyn KO (PBS, n=3; 2.4G2, n=5), Lyn WT (PBS, n=3; 2.4G2, n=3), Lyn KO (PBS, n=3; 2.4G2, n=4)]. (c) Two hours after the induction of PSA, mice were sacrificed and their lungs were inflated with Agarose (in 10 % formalin) pre-warmed at 60°C, later placed on ice and stored into 40 ml of 10% Neutral Buffered formalin. Hematoxylin-Eosin (H&E) staining on lung sections was imaged with a Nikon E-600 compound microscope using a 20× objective. The black arrows indicate perivascular edema. Data shown represent the mean ± SE.
Circulating cytokine levels in the serum after 2.4G2-induced PSA
Figure 12

Serum levels of circulating cytokines and chemokines following IgG-induced passive systemic anaphylaxis

PSA was induced with intravenous injection of 500µg of 2.4G2. Two hours later, blood was collected by cardiac puncture and serum level of circulating mediators was assessed by micro-beads Bioplex Assay. Fyn WT (PBS, n=4; 2.4G2, n=6), Fyn KO (PBS, n=3; 2.4G2, n=5), Lyn WT (PBS, n=3; 2.4G2, n=3), Lyn KO (PBS, n=3; 2.4G2, n=4). Data shown represent the mean ± SE.
Figure 13

Effects of Fyn and Lyn deficiency on peripheral blood mononuclear cells

(a,b) Blood from Fyn KO and Lyn KO mice as well as their littermate controls (n = 8 per group) was collected and the peripheral blood mononuclear cells (PBMCs) numbers were assessed by flow cytometry analysis. B cells (CD19+), T cells (CD4+), monocytes (CD11b+Gr1–), neutrophils (CD11b+Gr1+), basophils (IgE+CD49b+), eosinophils (Gr1+SiglecF+), and mast cells (IgE+CD117+). (c, d, e) Comparison of basophils, monocytes and neutrophils percentages between Fyn KO and Lyn KO mice as well as their littermates control. Data shown represent the mean ± SE.
Mast cells account for the amount of serum histamine released in IgG-induced PSA

Mast cells and basophils degranulate and release histamine once activated through their FcγR. Our results show that the amount of histamine released in the serum during IgG-induced PSA is regulated in a Fyn and Lyn dependent-manner (Figures 14a, 14b). However, the cell population accounting for the amount of released histamine remained unclear. To address this question, we induced PSA by 2.4G2 intravenous injection into wild type or mast cell-deficient mice (Wsh-/−) mice, and monitored the drop in core body temperature. As seen in Figure 15a, Wsh-/− mice developed PSA to a similar extent as their WT controls (C57BL6). Surprisingly, mast cell-deficient mice had little serum histamine two hours after the induction of PSA in comparison to their controls (Figure 15b). Our data demonstrate that mast cells account for the majority of histamine released during IgG-induced PSA.

Lyn kinase deficiency induces increased vascular sensibility to histamine

In vivo vascular sensibility to histamine depends on the amount of histamine administered, the number of functionally available histamine receptors and the contribution of signaling molecules downstream of the histamine receptor. In order to assess the impact of Fyn and Lyn kinase deficiency in the vascular sensitivity to histamine, we intravenously injected 5 mg of histamine into Fyn KO, Lyn KO and their wild type counterparts (Figure 14c). While Fyn KO mice exhibited anaphylactic responses mirroring their littermates, Lyn KO mice
were more responsive than controls when given the same amount of histamine, suggesting that Lyn deficiency might also affect the vasculature in addition to regulating the amount of released histamine.
Figure 14

Fyn and Lyn kinases regulate the amount of serum histamine during IgG-induced PSA

(a, b) Circulating histamine levels 2 hours after PSA induction [Fyn WT (PBS, n=4; 2.4G2, n=6), Fyn KO (PBS, n=3; 2.4G2, n=5), Lyn WT (PBS, n=3; 2.4G2, n=3), Lyn KO (PBS, n=3; 2.4G2, n=4)]. Data shown represent the mean ± SE. (c) Fyn KO (n=4) and Lyn KO (n=4) mice or control littermates (Fyn, n=4; Lyn, n=4) were injected i.v. with 5 mg of histamine and core body temperature was measured by rectal microprobe. Data shown represent the mean ± SEM.
Mast cells account for the majority of serum histamine released during IgG-induced PSA

(a) PSA was induced in mast cell-deficient mice (Wsh-/-, n=4) as well as their WT controls (C57BL6, n=5) by intravenous injection of 500µg of 2.4G2. The core body temperature was monitored by rectal microprobe. Data shown are the mean ± SEM. Two hours after the induction of PSA in (a), blood was collected from mice, serum prepared and the amount of circulating histamine was assessed by ELISA (b). Data shown are the mean ± SE.
PAF injection recapitulates IgG-mediated PSA in Fyn and Lyn KO mice while the deficiency in neither Fyn nor Lyn affects PAF acylhydrolase (PAFAH).

PAF is known to be the main mediator regulating IgG-induced PSA (15-18). To assess its importance in our model, we intravenously injected PAF (700ng) into Fyn KO, Lyn KO, and matched wild type mice (Figure 16a). PAF administration recapitulated IgG-mediated PSA kinetics in Fyn and Lyn KO (Figures 11a, 11b). Since PAF is the key factor in IgG-mediated anaphylaxis, we assessed serum activity levels of its inactivating enzyme, PAF acetylhydrolase (PAFAH). As shown in Figure 16b, steady-state PAFAH serum enzymatic activity was unaffected by Lyn or Fyn deficiency. Furthermore, we observed that Lyn deficiency not only worsened anaphylaxis severity (Figures 11b, 14c, 16a), but also lengthened the recovery time to approximately 5 hours, instead of the 2 hours observed with littermate controls (Figure 17). These findings could be explained by increased leakage from endothelial cells, or altered decay rates for these anaphylactic mediators. Collectively, these data suggest that Lyn deficiency exacerbates responses to both histamine and PAF. Paired with enhanced macrophage and basophil responsiveness to IgG, the loss absence of Lyn kinase appears to exaggerate the severity and recovery time of anaphylaxis.
Figure 16

Intravenous administration of PAF recapitulates IgG-induced PSA kinetics while the deficiency in neither Fyn nor Lyn affects PAF acetylhydrolase (PAFAH)

(a) PSA was induced by intravenous delivery of 700 ng PAF in Fyn KO (n=3) and Lyn KO (n=4) as well as their littermate controls (Fyn, n=4; Lyn, n=4). Core body temperature was measured by rectal microprobe. Data shown represent the mean ± SE. (b) Enzymatic activity of serum levels of PAF acetylhydrolase (PAFAH) in Fyn and Lyn KO mice and their control littermates (n≥ 9 per group). Data shown represent the mean ± SEM.
Figure 17

Lyn KO mice have exacerbated anaphylaxis and extended recovery time

Anaphylaxis was induced in Lyn KO mice (n = 4 per treatment groups) by intravenous injection of either 5 mg of histamine, 700 ng of PAF or 0.5 mg of 2.4G2. The severity of anaphylaxis was assessed by core body temperature measurements using a rectal microprobe. Mice were monitored over 5 hours until recovery. Data shown represent the mean ± SEM.
**Fyn KO**

- **Mast cell**
  - ↑ Peritoneum
  - ↓ Degranulation
  - ↓ Cytokine release

- **Basophil**
  - FcyRI/RIIb
  - FcyRIIb

- **Macrophage**
  - FcyRI/RIIb

**Lyn KO**

- **Mast cell**
  - ↑ Peritoneum
  - ↑ Degranulation
  - ↑ Cytokine release

- **Basophil**
  - FcyRI/RIIb

- **Macrophage**
  - FcyRI/RIIb

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* ns = non significant
Figure 18

Roles of Fyn and Lyn kinase in the regulation of Fcγ receptors in mice.
**Figure 29**

**Fyn and Lyn kinases are activated in both the IgE and IgG receptor pathways.** FcεRI and FcγRIIB/RIII activation by antigen-bound IgE and IgG respectively elicit intracellular activation of Fyn and Lyn kinases. Phosphorylation of Fyn will lead to the activation of Syk, which will later dock to LAT and trigger the activation of the Ras/MAPK, PKC, AP-1 and NFAT pathways. In addition of being a positive regulator of receptor signaling, Lyn kinase activation can also lead to Csk recruitment through the SH2 domains of Cbp/PAG. Csk is a phosphatase which once activated removes phosphate residues from its target (such as Fyn kinase), resulting in signal inhibition. In addition, recent data suggest that Fyn kinase activation is required for STAT5 transcription factor phosphorylation and translocation to the nucleus (64), expanding our understanding of the role of this kinase. Receptor activation generally leads to a mixture of positive (activating) and negative (inhibiting) signals and corresponding mediators. For example, mast cell activation triggers the release of a plethora of mediators including IL-10 and TGF-β, two anti-inflammatory cytokines. These cytokines can act in an autocrine manner and dampens further release of mediators. One plausible hypothesis is that these cytokines trigger the synthesis of specific micro-RNAs that will target Fyn and STAT5.
CHAPTER 5 – DISCUSSION

Recently, the Src family kinases Lyn and Fyn have been found to exert opposing effects on IgE-mediated mast cell activation (25, 30, 65), but little is known about the role of these enzymes in IgG signals. Our findings show that FcγR triggers Fyn and Lyn kinase activation similar to FcεRI crosslinkage in mast cells (Figures 5, 7), which might be explained by the fact that these two receptor families share the common γ chain (33). Furthermore, Fyn and Lyn appear important for the regulation of IgG-induced degranulation and cytokine production, suggesting an important role in mast cell functions. Here we show that Lyn deficiency significantly increases Akt phosphorylation subsequent to mast cell IgG receptor stimulation. These data mirror previous observations of FcεRI-mediated mast cell activation from Kitaura et al. (43). In that report, IgE XL of Lyn KO mast cells induced increased Akt phosphorylation, correlating with enhanced NF-κB, NF-AT and AP-1 transcriptional activation and subsequently elevated IL-2 and TNF production. Taken together, these data demonstrate that Lyn and Fyn kinase play important and similar roles in FcεRI and FcγRIII activation of mast cells. The fact that Fyn and Lyn deficiency similarly altered FcγR signaling in basophils and macrophages (Figures 9, 10) supports the importance of these antagonistically-paired Src-family kinases in IgG-mediated immune responses.

The onset and the regulation of anaphylaxis has been exclusively attributed to mast cells, delineating the generally accepted classical pathway of anaphylaxis involving IgE-mediated FcεRI mast cell activation and subsequent
histamine release (5). In addition to this well-characterized pathway, recent reports in the literature demonstrate that in a murine model, the anaphylactic reaction can be triggered by an IgG-FcγR pathway. Basophils, macrophages and recently neutrophils (18) – but not mast cells – have been identified as crucial players in this “alternative pathway of anaphylaxis”, since mast cell-deficient mice demonstrated IgG-induced anaphylaxis. In contrast, in vivo depletion of basophils with the monoclonal antibody Ba103 (16, 17), or inhibiting macrophage function via gadolinium injection (15) markedly decreased the severity of FcγRIII-mediated anaphylaxis. A recent report further showed that FcγRIV-neutrophil-induced anaphylaxis also occurs, as mice deficient in FcγRI/FcγRIIB/FcγRIIA/FcεRI/FcεRII−/− (5KO mice) still developed IgG-mediated anaphylaxis (18). In this alternative form of anaphylaxis, platelet-activating factor (PAF), rather than histamine, has been indicated as a pivotal mediator. While PAF serum levels are quite challenging to measure, the in vivo administration of a PAF antagonist protected mice from developing IgG- but not IgE-mediated anaphylaxis (17). Thus it is thought that IgG-induced anaphylaxis operates through FcγR−mediated activation of basophils, neutrophils and macrophages, with subsequent PAF release triggering vasodilation, vascular fluid leak, and loss of core body temperature (15, 17, 18, 58).

Our data support and extend the understanding of IgG-mediated anaphylaxis at the molecular signaling level. First, we found that Fyn or Lyn deletion has opposing effects on histamine release and cytokine secretion during IgG activation of mast cells, basophils, and macrophages in vitro. Interestingly,
although the effects on histamine release were consistent in vivo, they did not predict the severity of FcγR-induced PSA. While Lyn KO mice showed increased histamine and worsened hypothermia, Fyn KO had diminished histamine but no change in hypothermia versus respective WT mice. Additionally, although histamine and PAF are responsible for anaphylaxis, we found that mast cells were mainly responsible for the amount of circulating histamine present in the serum during the course of FcγR-induced PSA (Figure 15), extending the involvement of mast cells in IgG-related pathologies. It is interesting to note that a recent study found histamine produced by non-mast cell sources in a model of contact hypersensitivity (66), suggesting that in vivo histamine production varies with the eliciting stimulus. Based on previous reports (15-18), the drop in body temperature observed in mast cell-deficient animals over the course of anaphylaxis should be attributed to PAF released by basophils, macrophages and neutrophils after FcγRIII activation. Furthermore, we did not observe any significant difference in perivascular cell infiltration and pulmonary edema between Fyn KO, Lyn KO and their controls subsequent to 2.4G2-induced PSA (data not shown).

The exacerbated response of Lyn KO mice could be ascribed to many factors: increased PAF production (and/or secretion), decreased PAF metabolism, or enhanced PAF signaling in vascular endothelium. We found no changes in PAFAH activity, suggesting that reduced PAF catabolism is not an explanation. Furthermore, we found no overt changes in circulating basophils, monocyte, or neutrophils in Lyn KO mice. It is also possible that Lyn KO
endothelial cells, just like mast cells, basophils and macrophages, are hyper-responsive to any stimuli involving Lyn kinase. In fact, Kuruvilla et al. have demonstrated that Fyn and Lyn are phosphorylated upon PAF receptor activation, leading to phosphorylation of the p85 regulatory subunit of PI3K (67). In addition, Yu et al. showed that phospho-tyrosine activated Lyn kinase co-localized with PI3K in the lipid body fraction of PMN leukocytes subsequent to PAF receptor stimulation (68). Taken together, and in line with our findings, we therefore speculate that Lyn KO endothelial cells are hyper-responsive to PAF stimulation, triggering a longer and more severe vasodilation episode in comparison to Lyn-sufficient cells.

Based on these data, we can hypothesize that patients with altered Lyn function or expression could be hyperresponsive to IgE- and IgG-mediated pathology. There is precedence for this, as the majority of systemic lupus erythematosus (SLE) patients have been shown to have reduced Lyn expression in two clinical studies (69, 70). While SLE is regarded as an IgG immune complex-mediated disease, Charles et al. recently showed that basophils activated by IgE may participate in the onset or the exacerbation of lupus-like symptoms in a mouse model (71). In the same report, this group demonstrated that aged Lyn KO mice displayed increased basophil numbers in the lymph nodes, blood and spleen. Furthermore, Lyn KO mice display significantly elevated circulating autoantibodies which, in conjunction with many other factors, lead to the development of SLE-like symptoms and contribute to the elevated mortality in this population (72-74). Combined with our data, Lyn deficiency is
postulated to worsen either IgG- or IgE-mediated inflammation, which could contribute to the development of SLE in aged mice.

Several studies have reported the presence of allergen-specific IgG in allergic individuals. The involvement of this immunoglobulin isotype in the onset and the development of the allergic reaction remain poorly understood (75, 76). However, Bandukwala et al. used a murine model to demonstrate the importance of FcγRI and FcγRIII in airway inflammation and hyperresponsiveness. This study showed that C57BL/6 mice that were sensitized with noninfectious parasitic Schistosoma mansoni eggs and challenged with soluble egg antigen displayed airway inflammation, including eosinophil infiltration and severe peribronchial and perivascular inflammation. This response was greatly decreased in FcγRI<sup>−/−</sup> or FcγRIII<sup>−/−</sup> mice (77). This group also demonstrated that deficiency of FcγRIII, but not FcγRI, reduced lung resistance upon methacholine challenge, compared to littermate controls. Thus, in addition to the well-established role of IgE-FcεRI in the onset and the development of allergy and hypersensitivity, this study provides evidence that FcγRIII activation can participate in airway disease pathogenesis in a murine model. Combined with our data, these findings support the theory that the exacerbated 2.4G2-mediated PSA we noted in Lyn KO mice is elicited by FcγRIII, as previously supported by Ravetch et al. as well as Daeron et al. (78-81).

The high affinity (FcγRI) and low affinity (FcγRIII) IgG receptors are known to be important in the activation of numerous cell types of the immune system and in the phagocytosis of opsonized microbes. In contrast, one of the members
of the FcγR family, FcγRIIB, has emerged as an inhibitory receptor (41, 82, 83). Using a mouse model of allergic asthma, Dharajiya et al. demonstrated that in addition to upregulating the expression of FcγRIIB in CD14⁺MHCII⁺ mononuclear as well as in CD11b⁺ cells in the lungs, ragweed extract (RWE) challenge also led to signs of severe allergic asthma-like symptoms in FcγRIIB-deficient mice, delineated by increased airway eosinophilia, mucin production and allergen-specific IgE. Furthermore, a tremendous increase in macrophage, eosinophil and lymphocyte recruitment was observed in the broncho-alveolar lavage fluid of RWE-challenged FcγRIIB KO mice in comparison to wild type controls (84). These data, as well as many others, consolidate the idea that IgG and Fcγ receptors play important roles in the regulation of immune system homeostasis. Our data support the hypothesis that Fyn and Lyn kinases are pivotal antagonistic regulators of this paired IgG receptor system.

The data presented demonstrate that Fyn and Lyn kinases are activated during IgG-mediated signaling and have opposing regulatory functions in mast cells, basophils and macrophages. In addition to Fyn and Lyn activation, I found that FcγR stimulation also led to the phosphorylation of Akt, Erk, p38 and JNK. Further, I also uncover the contribution of mast cells as major producers of serum histamine during IgG-induced PSA, regulated in a Fyn- and Lyn-dependent manner. More importantly, the data show that overall Lyn but not Fyn kinase regulates the severity of IgG-induced passive systemic anaphylaxis, by enhancing the amount of vasoactive mediators secreted and exacerbating endothelial cell responsiveness to PAF and histamine. In line with previous
studies, these findings extend the understanding of IgG-related pathologies and demonstrate the pivotal role of Lyn kinase as the key regulator of IgG-mediated inflammation.
PART II

Fyn kinase is required for optimal antibody production
ABSTRACT

The generation of antigen-specific antibodies and the development of immunological memory require collaboration between B and T cells. T cell-secreted IL-4 is important for B cell survival, isotype switching to IgG1 and IgE, affinity maturation and the development of germinal centers (GC). Fyn-deficient (KO) T cells have reduced IL-4 production after anti-CD3/anti-CD28 stimulation. I observed that naïve Fyn KO animals had significantly low basal serum titers of IgG1 and IgG2a but not IgG2b and IgM. Further, the data show that Fyn KO mice fail to develop robust humoral immunity following a T-dependent antigen immunization (NP-KLH), as significantly low titers of NP-specific IgG2a were observed in the serum up to 28 days post-immunization. These results correlated with significantly low numbers of germinal center (GC) B cells, Follicular Helper T cells (T\text{FH}) and spleen plasma cells. Interestingly, Fyn kinase deficiency did not affect activation-induced cytidine deaminase (AID) nor B cell proliferation when cultured with anti-CD40 and IL-4. Furthermore, Fyn KO B cells displayed significantly impaired STAT3 and STAT5 activation upon IL-4 stimulation. Taken together, these data suggest that Fyn kinase has a role in the development of humoral immunity, affecting both B cells and T cells.
CHAPTER 1 – INTRODUCTION

The development of immunological memory is a key hallmark of humoral responses. Germinal centers (GCs) are critical for humoral immunity. Within GC, B cells undergo class switch recombination (CSR) and somatic hypermutation (SHM) – both of these processes are mediated by activation induced cytosine deaminase (AID). CSR and SHM lead to the development of plasma cells that secrete high affinity, class switched antibodies. GC formation is dependent on the interactions between antigen-specific B cells, T cells and follicular dendritic cells (FDCs). Antigen-activated B cells upregulate the chemokine receptor CCR7 expression and migrate toward the T cell zone, while some activated T cells upregulate CXCR5 and migrate toward the B cell follicles. It is within the B-T border that Follicular helper T cells (T_FH) interact with activated B cells presenting cognate antigen. This interaction is stable and can last for several minutes. During this interaction, T_FH cells provide B cell help via CD40L and cytokines, such as IL-21 and IL-4. Both of these cytokines are important for germinal center formation. Even prior to the discovery of T_FH cells, it was demonstrated that optimal germinal centers did not form in IL-4Rα or IL-4 deficient mice. IL-4 signaling is not only required for germinal center formation, but also for class switch recombination and somatic hypermutation.

Src family tyrosine kinases (SFKs) are involved in diverse regulatory mechanisms of B and T cell homeostasis (85-92). Fyn kinase (59 kDa) for example, is known to have critical roles in proximal T cell receptor (TCR)
activation (86). It is well documented that in the absence of T_{FH}, GCs fail to develop (93-96). Therefore, understanding the factors that regulate T_{FH} development has been of great interest. Recent studies demonstrated that SLAM-associated protein (SAP) is essential for T_{FH} development, germinal center formation and the generation of memory B cells (97-101). In fact, SAP recruits Fyn kinase to SLAM by direct binding “via an unconventional SH3 domain binding surface” that requires arginine residue 78 on the backside of SAP SH2 domain (102-104). This recruitment and activation of Fyn is known to be essential for CD4 T cell differentiation to Th2 cells (105, 106), NKT cell development (107, 108) and controlling IFN-gamma production (102, 105). SLAM deficient mice have normal GC formation following viral infection (98). Experiments also showed that mutating arginine 78 in SAP-protein impairs SAP-Fyn interaction without affecting T_{FH} development, suggesting that SAP mediates T_{FH} development independently of Fyn (99, 100). These experiments however, do not address Fyn’s potential role in T_{FH} development and GC formation, independently of SAP signaling. Experiments with Fyn-deficient mice showed that they form GCs, although how GC B cell numbers compare to that of wild type cannot be deduced from these studies, since Fyn-deficient mice are in a B6.129 background and no B6.129 wild type mice were used in that experiment.

I observed that Fyn KO mice have significantly low basal IgG1 and IgG2a. Furthermore, following a T-dependent immunization protocol (Figure 20), these mice displayed delayed Ag-specific IgG1 and significantly low Ag-specific IgG2a, correlating with significantly reduced numbers of germinal center B cells,
Follicular Helper T cells (T<sub>FH</sub>) and spleen plasma cells. Surprisingly, Fyn-deficient B cells did not show significantly decreased AID expression and antibody production following <em>in vitro</em> activation with IL-4 and anti-CD40. Taken together, these results thus demonstrate that Fyn-mediated signaling is necessary for optimal humoral responses in a T-dependent immunization protocol.
[A] Lymph node

- B cell
  - BCR
  - IgM
  
  B cell ligation → B cell activation → move toward T cell zone

- B cell
  - CD40-CD40L interaction
  - Cytokines

B-T Border

- T cell primed by DC
  - BCL-6
  - SAP
  - ICOS

  Follicular Helper T cell (CXCR5)
  
  move toward B cell zone

Germinal Center
- Class switch recombination
- IgM -> IgG, IgE, IgA
- Somatic hypermutation (affinity maturation)
- Point mutation in Ig genes selecting for high affinity clones

Plasma cell
  
  Antigen

Memory cell

[B] Immunization protocol

0
- Pre-bleed
- Immunize: 200μl/injection
  - 10 μg of NP-KLH (10μl)
  - 4 mg of Alum (100μl)
  - in PBS (90μl)

7
- Bleed
- ELISA:
  - NP-specific IgM
  - High NP-IgG1
  - Total NP-IgG1

14
- Cardiac puncture
- ELISA:
  - NP-specific IgM
  - High NP-IgG1
  - Total NP-IgG1

  - Spleen
    - Flow:
      - plasma cells
        - CD138+, B220 lo/-
      - GC B cells:
        - GL7hi, B220+, Fas hi, IgD?
      - TFH:
        - CD4+, CXCR5 hi, PDI hi, GL7

We will be testing:
- Antibody responses - antigen specific
- Affinity maturation
- GC formation (flow cytometry, IHC)
- TFH numbers (Flow)
- Plasma cell numbers
- Signaling and Cytokines from B cells and TFHs
Figure 20

Background and research strategy.

[A] In addition to the B cell receptor (BCR) expression, naive B cells express surface IgM. Upon BCR ligation, activated B cells express CD40 and migrate toward the T cell zone. Once in the T cell zone, they will interact with activated T cells previously primed by a dendritic cell (DC). Follicular Helper T cells (T\textsubscript{FH}) have increased Bcl-6, SAP, iCOS and the chemokine receptor CXCR5, markers required for their activation and migration toward the B cell zone. Once antigen-specific B cells and T cells are in contact, B cells proliferate, form germinal centers (GC), undergo class switch recombination and somatic hypermutation.

[B] Basal antibody titers will be assessed for WT and Fyn KO mice prior to their immunization, 7 and 14 days post-immunization (p.i.). Additionally, spleens will be extracted to assess T cell, B cell and plasma cell numbers.
CHAPTER 2 – RESULTS

Fyn deficient animals have low basal IgG1 and IgG2a

Basal circulating antibodies against a given antigen may reflect the readiness for the immune system in neutralizing that antigen (and indirectly the infectious agent bearing that antigen) during the infection. Therefore, in order to better understand Fyn’s role in humoral immunity, we analyzed antibody levels in the serum of naïve Fyn-deficient and wild type B6.129 mice. Compared to wild type, Fyn-deficient mice had normal IgM levels and IgG2b, but had significantly decreased basal IgG1 levels and IgG2a (Figure 21). IgE levels were below the levels of detection (results not shown). These findings suggest that, in addition to the well-documented functions of Fyn in innate immunity (6, 25, 30, 31, 109), this kinase has a role in adaptive immunity through the regulation of antibody production.

Fyn kinase deficiency affects antigen-specific IgG1 and IgG2a production

In order to directly assess the role of Fyn kinase in antigen-specific antibody production, Fyn-deficient mice and their wild type littermates were immunized i.p. with 4-Hydroxy-3-nitrophenylacetyle coupled to keyhole limpet hemocyanin (NP-KLH) in alum. Serum NP-specific antibody levels were assessed at days 7, 14, 21 and 28 post-immunization (Figure 22, Figure 23). In line with the previous observations of basal IgM, I did not observe any significant difference in NP-specific IgM levels between WT and Fyn KO mice (Figure 22a).
Interestingly, Fyn KO mice were able to produce similar levels of NP-IgG1 compared to their WT control, suggesting that the low IgG1 titers observed in naïve mice can be compensated over the course of the immunization protocol (Figure 22b). Furthermore, I observed that Fyn KO mice drastically failed to produce NP-IgG2a while a slightly delayed trend was noted on the production of IgG2b (Figures 22c, 22d). Surprisingly, I did not observe any difference in spleen weight between Fyn KO and WT 14 days after the immunization (Figure 24). These data support the idea that Fyn kinase plays a role in antibody production, affecting selective sub-classes of immunoglobulins.

*Germinal center formation, Splenic Plasma cell differentiation and Follicular Helper T cell development is impaired in Fyn deficient mice*

In regards to the diminished antibody response observed in Fyn-deficient mice, I hypothesized that plasma cell development was impaired in these animals. To test this, mice were immunized with NP-KLH and plasma cell numbers was determined by flow cytometry 14 days after the immunization. Consistent with the defects in antibody production, Fyn-deficient mice showed decreased numbers of splenic plasma cells (CD138^+^B220^-^ cells) (Figure 25a, 25b). Additionally, being that a large portion of plasma cells generated in response to T-dependent antigens is derived from GCs, we examined GC formation in these animals. GC B cells, defined as B220^+^GL7^+^Fas^hi^ were enumerated by flow cytometry 14 days following NP-KLH immunization.
observed that Fyn KO animals had significantly low levels of GC B cells compared to their littermate controls (Figures 26a, 26b). Furthermore, recent literature shows that $T_{FH}$ cells (CXCR5+ PD-1+) provide B cell help via CD40L, IL-21 and IL-4 (97-101). I subsequently decided to examine whether the decrease in GC B cell numbers was due – or somewhat related – to a decrease in the $T_{FH}$ population. Consistent with GC B cells and spleen plasma cells, $T_{FH}$ numbers were significantly reduced in Fyn-deficient mice (Figures 26c, 26d). Subsequently, these results support the hypothesis that Fyn kinase is involved in humoral immunity, affecting $T_{FH}$, GC B cell and the plasma cell population.
a)

b)

c)

d)
Figure 21

Fyn KO mice have significantly low basal levels of IgG1 and IgG2a. Sera were collected from naive Fyn KO mice as well as their littermate controls (WT) (n = 8). Circulating antibody titers were measured for IgM (a), IgG1 (b), IgG2a (c) and IgG2b (d). No detectable levels of IgG3 or IgE were measured. Data shown are mean ± SE of 3 independent experiments performed in triplicate. *p<0.05; **p<0.001; ***p<0.0001 based on Student's t test on Fyn WT and Fyn KO.
**Figure 22**

**Fyn KO animals have impaired antibody titers upon T-dependent immunization.** WT and Fyn KO mice were immunized intraperitoneally (i.p.) with 10 µg of NP-KLH + 4 mg of Alum (n = 8-12). NP- specific antibody titers were assessed every week for up to 28 days: IgM (a), IgG1 (b), IgG2a (c) and IgG2b (d). No detectable levels of NP-specific IgG3 or IgE were measured. Data shown are mean ± SE of 3 independent experiments. *p<0.05; **p<0.001; ***p<0.0001 based on Student’s t test on Fyn WT and Fyn KO.
Time (Day)

0 7 14 21 28 35 42 47

Measure basal antibody titers
i.p. injection of NP-KLH (10μg) + Alum (4mg)

Measure NP-specific antibody titers

Measure NP-specific antibody titers
Harvest organs -> day 14 [GC B, GC TFH, PC]

Boost

\textbf{GC B:} B220^{+}, GL7^{hi}, Fas^{hi}
\textbf{TFH:} CD4^{+}, CXCR5^{hi}, Fas^{hi}
\textbf{PC:} GL7^{hi}, B220^{lo}
Figure 23

Immunization strategy.

WT and Fyn KO mice were immunized intraperitoneally (i.p.) with 10 µg of NP-KLH + 4 mg of Alum. The first group of mice was monitored for 14 days to mirror the primary immunization protocol, during which NP-specific antibody titers were assessed at days 7 and 14. The second group of mice was immunized and monitored for a longer period of time as indicated on the figure. In addition to the primary immunization, these mice received a second inoculation of NP-KLH at day 42 (Boost) and were monitored for 5 days following the boost injection. The boost injection corresponds to the second encounter of the animal with the antigen stimulating the immunological memory of the mouse.
Figure 24

Fyn KO mice have normal spleen size after immunization.

WT and Fyn KO mice were immunized intraperitoneally (i.p.) with 10 μg of NP-KLH + 4 mg of Alum and their spleens were extracted 14 days after the inoculation of the antigen. (a) Representative images of spleens from WT and Fyn KO animals. (b) Graph representing spleen weight. Data shown are mean ± SE of 3 spleens.
Figure 25

Fyn deficient mice have low splenic plasma cells upon immunization. WT and Fyn KO mice were immunized intra-peritoneally (i.p.) with 10 µg of NP-KLH + 4 mg of Alum (n = 8-12) and spleen plasma cells percentage was assessed 14 days post immunization (a, b). Data shown are mean ± SE of 3 independent experiments. *p<0.05; **p<0.001; ***p<0.0001 based on Student’s t test on Fyn WT and Fyn KO.
a) B220+ WT vs Fyn KO

b) GC B cells (%B220+)

Fas

5.12
1.21

C) CD4+ WT vs Fyn KO

CXCR5

PD-1

17.6
11.3

TFH (% of CD4+)

WT
Fyn KO

***

**
**Figure 26**

**Fyn deficient mice have low GC B cells and low T\textsubscript{FH} upon immunization.** WT and Fyn KO mice were immunized intra-peritoneally (i.p.) with 10 µg of NP-KLH + 4 mg of Alum (n = 8-12). Later, GC B cells (a, b) and T\textsubscript{FH} (c, d) percentage were assessed 14 days post immunization. Data shown are mean ± SE of 3 independent experiments. *p<0.05; **p<0.001; ***p<0.0001 based on Student’s t test on Fyn WT and Fyn KO.
**Fyn kinase is required for B cell antibody production**

T cell-secreted cytokines are required for B cell survival and isotype switching (110, 111). For example in mice, IL-4 is necessary to drive isotype switching to IgG1 and IgE, while IFNγ induces IgG2a production (112). In secondary lymphoid organs, T cells are the main producer of both IL-4 and IFNγ, which are required for B cell survival and isotype switching. Graham et al. demonstrated that IL-4 secreted by T_{FH} is pivotal for B cell survival (113). Additionally, Cannons et al. showed that Fyn-deficient T cells have low levels of IL-4 subsequent to α-CD3/α-CD28 stimulation (105). Furthermore, previous studies demonstrated that Fyn KO NK cells produce reduced amounts of IFN-γ (114). Hence, the obvious question was to know whether the phenotype observed in Fyn KO mice (i.e. reduced GC B cells and plasma cells) was due to low T cell-secreted IL-4 (due to the absence of Fyn) or if the defect extends to the B cell population. In fact, given that cytokines play an important role in the humoral response, it is possible that our observations are not only due to low T cell-derived IL-4 in Fyn KO T cells but instead to the combination of low T cell-derived IL-4 added to an impaired IL-4 receptor signaling in B cells. In order to determine whether Fyn-deficiency leads to a B-cell intrinsic defect downstream of the IL-4 receptor, Fyn KO B cells were isolated and stimulated in vitro with anti-CD40 and IL-4. Subsequent AID expression as well as IgG1, IgE and IgG2a production was measured by ELISA. Intriguingly, we did not observe any significant reduction in AID expression in Fyn KO B cells (data not shown). Furthermore, Fyn KO B cells produced significantly lower amounts of IgG1 and
IgE when stimulated with IL-4 (Figures 27a, 27b). These results demonstrate that, at least in vitro, Fyn-deficient B cells have impaired activation. Interestingly, we did not observe any significant difference in B cell proliferation when WT and Fyn KO B cells were cultured in anti-CD40 and IL-4 (Figure 27c). Taken together and consistent with previous reports, our results suggest that the impaired antibody production observed in Fyn KO involves both the B and the T cell compartments.

**Fyn KO B cells have impaired IL-4R signaling**

As mentioned earlier, B cell IL-4 receptor plays an important role in B cell survival and isotype switching to IgG1 and IgE. The observed low titers of IgG1 in Fyn KO mice prompted us to investigate IL-4 receptor signaling in Fyn-deficient B cells. We hypothesized that IL-4 receptor signaling is somewhat affected in B cells, in addition to the previously reported low T cell-derived IL-4 in the absence of Fyn (105). To test this hypothesis, we stimulated WT and Fyn KO naïve B cells with 30 ng/ml of IL-4 for a total of 60 minutes distributed in five time points. While STAT6 phosphorylation seemed unaffected, we observed that STAT3 and STAT5 phosphorylation were significantly impaired upon IL-4 receptor stimulation (Figure 28). These intriguing findings suggest that Fyn kinase might have a role in the JAK-STAT pathway. We hypothesize that Fyn kinase has a proximal position in the signaling cascade and might be part of the JAK-STAT complex (Figure 29). In fact, our laboratory recently discovered a previously unappreciated role of Fyn kinase in FcεRI-induced STAT5 phosphorylation in mast cells (64), where Fyn KO mast cells had significantly impaired STAT5
activation.
Fyn deficiency affects B cell antibody production but not B cell proliferation. WT and Fyn KO naive B cells were isolated from mice (n = 4; 6-8 weeks of age) and cultured in anti-CD40 (10µg/ml) and IL-4 (10ng/ml) for 7 days. IgG1 (a) and IgE (b) titers were measured at the end of the 7th day of culture. In order to assess the role of Fyn kinase in proliferation, naive B cells were purified from WT and Fyn KO mice and cultured with either anti-CD40 + IL-4 and the proliferation was assessed by Tritium (3H) incorporation assay (c). Data shown are mean ± SE of 2-4 independent experiments. *p<0.05; **p<0.001; ***p<0.0001 based on Student's t test on Fyn WT and Fyn KO.
Figure 28

Fyn KO B cells have impaired STAT3 and STAT5 activation upon IL-4 stimulation. WT and Fyn KO naive B cells were isolated from mice and stimulated with IL-4 (30ng/ml) at 37°C for indicated times. Cells were lysed and phosphorylated forms of STAT3 (pY705 and pS727), STAT5 (pY694) and STAT6 (pY641) were assessed by western blot. Non-phosphorylated proteins were used as loading controls. Representative image of 3 independent experiments.
B cell

IL-4 Ra Y  IL-4  JAK1  JAK3  STAT3

IL-13 Ra Y  IL-4  JAK1  JAK2  TYK2

STAT3

IL-4 responsive genes

I-c  MHC-II  IL-4 Ra  CD23
Proposed role of Fyn in IL-4R-mediated STAT3 and STAT5 activation

Cytokine receptor signaling involves JAK and STAT molecules. For example, the ligation of IL-4 to its receptor will induce intracellular phosphorylation of JAK1 and JAK3, which will then phosphorylate STAT6 molecules. Phospho-STAT6 will dimerize and translocate to the nucleus and activate IL-4 responsive genes such as CD23 and the MHC-II.
CHAPTER 3 – DISCUSSION

The innate immune system is the first line of defense against pathogenic infection. In the context of human disease, the adaptive immune system is the second and mostly the last crucial arm necessary to clear the infection. Intracellular pathogens are principally eradicated through cell-mediated cytotoxicity mechanisms while extracellular pathogens are mainly cleared by the joint action of neutralizing antibodies and phagocytosis. Subsequently, elevated titers of circulating antigen-specific antibodies will promote rapid and efficient protection against forthcoming pathogenic infection.

The immune system produces 5 immunoglobulin isotypes ranging from IgM, IgD, IgG, IgE to IgA. IgG isotypes are the most abundant circulating antibodies. They are divided in different sub-classes (IgG1, IgG2a, IgG2b and IgG3) and these sub-classes bind to specific IgG receptor family members (FcγRI, FcγRIII and FcγRIV are the activating receptors while FcγRIIB is the inhibitory). In fact, recent studies conducted in mice show that antigen-specific IgG1 preferentially binds to the inhibitory IgG receptor FcγRIIB with the highest affinity (115). The ligation of IgG1 to FcγRIIB will lead to increased phosphatase mobilization through ITIM activation, resulting in an increase of the activation threshold. Furthermore, the same study provided the evidence that IgG2a binds with moderate and high affinity to the activating IgG receptors FcγRIII and FcγRIV respectively. Therefore, IgG2a and IgG2b will be two sub-classes capable of eliciting cell activation upon receptor ligation. In terms of physiological
relevance in mice, high titers of antigen-specific IgG1 will likely result into a “failure” of the immune system to activate the defense mechanism during infection while low titers might predict high susceptibility to infection. Here we show that Fyn KO mice do not only have significantly low levels of basal IgG1 and IgG2a (Figure 21) but in addition, have low antigen-specific IgG2a and delayed IgG1 kinetics when compared to their littermate controls (Figure 22). Intriguingly enough is the fact that Fyn kinase deficiency specifically affects some subclasses and not others, suggesting selective regulatory pathways in antibody production.

In the context of T-dependent immunization, antibody production is dependent on GC B cells and T\textsubscript{FH}. In fact, T\textsubscript{FH}-derived IL-4 is not only necessary to drive and maintain B cell survival but is also important to induce isotype switch to IgE and IgG1. While low levels of T\textsubscript{FH}-derived IL-4 result in decreased B (plasma) cell population, nothing is known about the selective pattern of Fyn deficiency to affect only IgG1 and IgG2a but not the other immunoglobulin subclasses. One possible explanation is that Fyn kinase either recruits or activates key factors required for the transcription and/or the translation of the γ1, γ2a DNA loci or their corresponding mRNA respectively.

Furthermore, in vivo, antibody production is an ensemble of complex mechanisms involving different cell types (such as DCs, T cells and B cells) and occurring in in different anatomical compartments (spleen and lymph node, just to mention these). Therefore, it is challenging to assess the relative contribution of each cell type (and biological compartment) as a part of a system. As
mentioned earlier, Fyn-deficient T cells secrete less IL-4 after stimulation. We ought to assess the contribution of Fyn in B cell IL-4Rα signaling by measuring cell proliferation and antibody production. We observed an in vitro correlate to our in vivo data, where in vitro stimulation of Fyn KO B cells with anti-CD40 + IL-4 resulted in significantly low levels IgG1 and IgE compared to the control B cells. Additionally, biochemistry studies show that Fyn KO B cells have decreased STAT3 and STAT5 activation upon IL-4 receptor ligation (Figure 28). We did not observe significant changes in B cell proliferation when the cells were cultured with IL-4, consistent with our in vivo observation of low titers of IgG2a.

Activation-induced cytidine deaminase (AID) is a key enzyme in isotype switching, class switch recombination and affinity maturation. These processes are required for the generation of a variety of adequate subclasses of antibodies. Our unpublished observations do not support the hypothesis that Fyn kinase deficiency affects AID expression levels. In fact, we noticed that Fyn KO B cells had comparable levels of AID enzyme after two days of stimulation with anti-CD40 + IL-4.

Overall, our findings suggest that Fyn kinase plays a key role in antibody production, selectively regulating the amount of IgG1 and IgG2a, not only in steady state conditions but also in the context of immunization, at least in a mouse model. However, specific mechanisms by which this new regulatory pathway operates need to be elucidated. Taken together, we report here a previously under-appreciated function of Fyn kinase in immunoglobulin subclass production, with the hope that these findings will at a certain point contribute to
the understanding of human IgG-related disorders, given that human IgG1 and IgG3 are very similar to mouse IgG2a and IgG2b respectively.
CONCLUDING REMARKS

Src family tyrosine kinases have pivotal regulatory roles in the immune response, affecting both the innate and the adaptive immune system. Our study, solely focused on the study of Fyn and Lyn elucidates previously unreported function of these two kinases in the regulation of IgG-associated mechanisms. What we found have considerable implications in the understanding of IgG-related diseases. For example, we show that Lyn could now be a potential target for the treatment of anaphylaxis. Furthermore, we have been able to demonstrate that mast cells can be activated by antigen-specific IgG molecules via Fcγ receptor ligation, implying that targeting the IgE and FcεRI pathway should not be the only way to alleviate symptoms associated with allergic asthma (where mast cells have the central role). Therefore, we suggest considering a therapy that would target both the IgG and the IgE pathways, while staying specific to mast cells.

In addition to reducing the amount of mediator release upon receptor stimulation, we also found that Fyn kinase deficiency downregulated IgG1 and IgG2a levels in naïve mice. After immunizing these mice, we noticed that Fyn KO mice had significantly low titers of antigen-specific IgG2a. As mentioned earlier, this IgG subclass is a very potent neutralizing antibody responsible for pathogen clearance through ADCC and phagocytosis. Consequently, higher titers of antigen-specific IgG2a represent a good baseline insuring rapid protection. Understanding the regulatory roles of Fyn kinase in the production of mouse
IgG2a is essential for further studies in human subjects. In fact, mouse IgG2a is highly similar to human IgG1 for their abilities to fix complement molecules and bind protein antigens. IgG1 is the most abundant circulating immunoglobulin subclass and is the most suitable for therapeutic use against pathogens and cancer cells.
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