# UC San Diego

## UC San Diego Electronic Theses and Dissertations

### Title

Oxidation-specific epitopes are targets of innate natural antibodies : potential implications in health and disease

### Permalink

https://escholarship.org/uc/item/4zd854hz

### Author

Chou, Meng-Yun Sandy

### Publication Date

2009

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Oxidation-Specific Epitopes are Targets of Innate Natural Antibodies **:** Potential Implications in Health and Disease

A Dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Meng-Yun Sandy Chou

Committee in charge:

Professor Joseph L. Witztum, Chair
Professor Maripat Corr
Professor Linda K. Curtiss
Professor Christopher K.Glass
Professor Donald Guiney
Professor Victor Nizet

2009

Copyright

Meng-Yun Sandy Chou, 2009

All rights reserved.

The dissertation of Meng-Yun Sandy Chou is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009

# DEDICATION

*Dedicated to*

*my parents, my grandmother*

*,and my brother for their love and support*

# TABLE OF CONTENTS

Signature Page……..…..iii

Dedication Page……iv

Table of Contents…….....v

List of Figures…….....x

List of Tables…….……..…..xiv

Acknowledgement…….……..…...xv

Curriculum Vitae…….…….....xvii

Abstract of the Dissertation……..…….....xix

**Chapter 1**

**INTRODUCTION ..... 1**

Atherosclerosis and inflammation ..... 2

Role of LDL in atherosclerosis ..... 4

Innate and adaptive immunity in atherosclerosis ..... 5

Antigens of atherosclerosis – Generation of oxidation-specific epitopes ..... 10

Natural Antibodies and B-1 cells ..... 12

Oxidation-specific epitopes are ubiquitous ..... 16

Specific Background and Research Goals ..... 19

References ..... 22

**Chapter2**

**CHARACTERIZATION OF MURINE HUMORAL RESPONSES TO OXIDATION-SPECIFIC EPITOPES ..... 35**

Abstract ..... 36

Introduction ..... 37

Most plasma IgM are natural antibodies of B-1 origin. ..... 37

T-cell independent antigens and oxidation-specific antigens ..... 38

Rationale and study design ..... 40

Results ..... 42

IgM antibodies against oxidation-specific epitopes are present in normal mice..... 42
IgM antibodies against oxidation-specific epitopes are present in germ-free mice..... 45
IgG antibodies against oxidation-specific epitopes in normal mice..... 49
Discussion..... 52
Acknowledgement..... 56
References..... 57

**Chapter 3**

**TOLL-LIKE RECEPTOR STIMULATION OF B-1 CELL INDUCES IGM PRODUCTION TO OXIDATION-SPECIFIC EPITOPES AND IL-10 SECRETION ..... 61**

Abstract..... 62
Introduction..... 63
Oxidation-specific epitopes are PAMPs recognized by PRRs..... 63
Toll-like Receptors (TLRs)..... 64
Toll-like receptors and atherosclerosis..... 65
Rationale and study design..... 67
Results..... 69
B-1 cells secrete IgM NAbs against oxidation-specific epitopes *in vitro*..... 69
IgM binding to native LDL..... 74
B-1 cell responses to both TLR4 and TLR2 agonists were in large part MyD88 dependent ..... 74
B-1 cell stimulation in vitro also induces IL-10 secretion..... 79
IL-10 inhibits IgM secretion induced by TLR stimulation from B-1 cells..... 82
IL-10 inhibits B-1 cell proliferation but not plasma cell differentiation..... 84
Effect of IL-10 administration *in vivo*..... 86
Discussion..... 90
Acknowledgement..... 94
References..... 95

**Chapter 4**

**OXIDATION-SPECIFIC EPITOPES ARE DOMINANT TARGETS OF INNATE NATURAL ANTIBODIES ..... 100**

Abstract..... 101
Introduction..... 102

Oxidation-specific epitopes are evolutionarily important epitopes ..... 102
Repertoire of B-1 cells is selected to bind evolutionarily important epitopes ..... 103
Rationale and study design ..... 105
Results ..... 107
Characterization of Rag1$^{-/-}$ recipients adoptively transferred with B-1 cells ..... 107
B-1 cells secrete IgM NAbs against oxidation-specific epitopes in vivo ..... 113
Oxidation epitopes are a dominant target of natural IgM antibodies. ..... 118
Generation of B-1 cell derived monoclonal antibodies ..... 121
Discussion ..... 125
Acknowledgement ..... 128
References ..... 129

**Chapter 5**

**B-1 CELL DERIVED NATURAL ANTIBODIES RECOGNIZE BIOLOGICALLY RELEVANT OXIDATION-SPECIFIC EPITOPES ..... 132**

Abstract ..... 133
Introduction ..... 134
IgM and its role in atherosclerosis ..... 134
IgM and its role in apoptotic cell clearance ..... 135
IgM and its role in autoimmunity ..... 136
Molecular mimicry between OxLDL and pathogens ..... 137
Rationale and study design ..... 139
Results ..... 141
B-1 cell derived natural IgM Abs recognize oxidation-specific epitopes on apoptotic cells ..... 141
Natural antibodies facilitate apoptotic cell uptake by macrophages in vivo ..... 144
B-1 cell derived natural IgM Abs recognize oxidation-specific epitopes in atherosclerotic lesions ..... 147
B-1 cell derived natural IgM Abs recognize pathogens ..... 152
Discussion ..... 157
Acknowledgement ..... 161
References ..... 162

**Chapter 6**

**NATURAL IGM ANTIBODIES IN HUMAN CORD BLOOD RECOGNIZE OXIDATION-SPECIFIC EPITOPES ..... 167**

Abstract ..... 168

Introduction ..... 169

B-1 cells in humans ..... 169

Natural Antibody in humans ..... 170

Rationale and study design ..... 172

Results ..... 173

Natural IgM Abs in human cord blood recognize oxidation-specific epitopes ..... 173

Natural IgM Abs in human cord blood recognize oxidation-specific epitopes on apoptotic cells ..... 175

Human Fab Abs recognize oxidation-specific epitopes on apoptotic cells ..... 176

Human Fab Abs recognize oxidation-specific epitopes in atherosclerotic lesions ..... 181

Discussion ..... 184

Acknowledgement ..... 188

References ..... 189

**Chapter 7**

**CONCLUSION AND FUTURE DIRECTIONS ..... 192**

Conclusion ..... 193

Future Directions ..... 206

References ..... 210

**Chapter 8**

**MATERIAL AND METHODS ..... 215**

Animals ..... 216

B-1 cell isolation ..... 217

B-1 cell cultures ..... 217

Adoptive transfer of B-1 cells ..... 218

Flow Cytometry ..... 218

Measurement of Antibody Titers ..... 219

Immunocompetition Assays ..... 220

Enzyme-linked Immunospot Assay ..... 221

Apoptotic cells and Immunofluorescence Microscopy....221
Immunocytochemistry....222
Cloning and Genetic Analysis of Hybridoma NA-17....223
Human Samples....223
In Vivo Uptake of Apoptotic Cells by Peritoneal Macrophages....224
CFSE Proliferation Assay....225
Measurement of Cytokines....225
Macrophage Binding Assay....226
Statistical Analysis....226
References....227

# LIST OF FIGURES

**Fig. 1-1** Simplified schematic illustration of in vitro formation of OxLDL and oxidation-specific epitopes……12

**Fig. 2-1** IgM titers to oxidation-specific antigens in conventional, SPF, and TcR$\alpha^{-/-}$ mice……43

**Fig. 2-2** IgM titers to oxidation-specific antigens in conventional and Ldlr$^{-/-}$ mice……..44

**Fig. 2-3** MDA-LDL specific IgM secreting cells are dominant in the spleens of conventionally raised C57BL/6 mice……...46

**Fig. 2-4** Antibody dilution binding curves of plasma IgM from germ free (A.), conventionalized (B.), and conventional (C.) Swiss-Webster mice to indicated antigens……..47

**Fig. 2-5** Titers of IgM Abs to oxidation-specific epitopes are present in conventional and germ-free Swiss Webster mice……48

**Fig. 2-6** IgG titers to oxidation-specific antigens in conventional, SPF, and TcR$\alpha^{-/-}$ mice……50

**Fig. 2-7** IgG titers to oxidation-specific antigens in conventional and Ldlr$^{-/-}$ mice……..51

**Fig. 3-1** In vitro stimulation of B-1 cells induces increased natural IgM Ab titers to oxidation-specific antigens……70

**Fig. 3-2** In vitro stimulation of B-1 cells induces increased natural IgM Ab titers to oxidation-specific antigens……71

**Fig. 3-3** Fold increase of natural IgM Ab to oxidation-specific antigens after in vitro stimulation of B-1 cells……..72

**Fig. 3-4** Natural IgM Abs produced *in vitro* show specificity to MDA-LDL and CuOx-LDL……73

**Fig. 3-5** Natural IgM Abs binding to plated native LDL could not be competed by native LDL in solution…….....75

**Fig. 3-6** In vitro stimu-lation of B-1 cells from TLR4$^{-/-}$, Myd88$^{-/-}$, and CD14$^{-/-}$ mice……..76

**Fig. 3-7** Fold increase of natural IgM Ab after in vitro stimulation of wild-type and Myd88$^{-/-}$ B-1 cells……78

**Fig. 3-8** TLR2 deficiency blocked stimulation of Pam3CSK4 in B-1 cells…….80

**Fig. 3-9** In vitro stimulation of B-1 cells induces increased IL-10 secretion……..81

**Fig. 3-10** IL-10 inhibits IgM secretion from B-1 cells in vitro……...82

**Fig. 3-11** IL-10 does not inhibit plasma cell differentiation of B-1 cells…….85

**Fig. 3-12** IL-10 inhibits B-1 cell proliferation……..88

**Fig. 3-13** Effect of IL-10 administration in vivo……..89

**Fig. 4-1** Characterization of B-1 cells after FACS sorting……108

**Fig. 4-2** Adoptive transfer of B-1 cells into *Rag1*$^{-/-}$ mice replenishes peritoneal cavity B-1 cell population……109

**Fig. 4-3** B-1 cells can be detected in the spleen after adoptive transfer of B-1 cells into *Rag1*$^{-/-}$ mice……..111

**Fig. 4-4** IgM to oxidation-specific epitopes are present in the plasma of B-1 reconstituted *Rag1*$^{-/-}$ mice free of T cells……..114

**Fig. 4-5** T cells increased IgM levels to oxidation-specific epitopes in B-1 reconstituted *Rag1*$^{-/-}$ mice……...116

**Fig. 4-6** Natural IgM Abs produced *in vivo* show specificity to MDA-LDL and CuOxLDL……...117

**Fig. 4-7** Pre-absorption of plasma of *Rag1*$^{-/-}$ plus B-1 cells with oxidation-specific antigens shows that oxidation-specific epitopes are dominant targets for NAbs……...119

**Fig. 4-8** ELISpot assay of frequencies of MDA-LDL specific IgM secreting cells (ISCs)……120

**Fig. 4-9** NA-17 recognizes MDA-related epitopes……..122

**Fig. 4-10** B-1 cell derived natural monoclonal antibody NA-17…….....123

**Fig. 4-11** Complete sequence of Clone NA-17……124

**Fig. 5-1** Natural IgM Abs bind to apoptotic thymocytes……142

**Fig. 5-2** Natural IgM Abs bind to apoptotic thymocytes but not normal thymocytes……143

**Fig. 5-3** Natural IgM antibodies facilitate apoptotic cell uptake by macrophages in vivo……145

**Fig. 5-4** NA-17 facilitates apoptotic cell uptake by macrophages in vivo…....146

**Fig. 5-5** Natural IgM antibodies are present in atherosclerotic lesions……148

**Fig. 5-6** NA-17 recognizes oxidation-specific epitopes present in atherosclerotic lesions……150

**Fig. 5-7** NA-17 inhibits MDA-LDL binding to macrophages……..151

**Fig. 5-8** Natural IgM Abs recognizes various pathogens……..153

**Fig. 5-9** E06 recognizes PC present on *Staphylococcus aureus*……155

**Fig. 5-10** E014 recognizes MDA-epitopes on *Group A Streptococcus*……...156

**Fig. 6-1** Human umbilical cord blood contains natural IgM antibodies to oxidation-specific antigens……...174

**Fig. 6-2** Human maternal plasma IgM antibodies show specificity to KLH…….176

**Fig. 6-3** Umbilical cord IgM binds to apoptotic cells in part via binding to MDA-related adducts……177

**Fig. 6-4** MAA-BSA also inhibits umbilical cord IgM binding to apoptotic cells……178

**Fig. 6-5** Human Fab KA2 and LA25 bind to late apoptotic cells but not early apoptotic cells or normal cells……179

**Fig. 6-6** Deconvolution microscopy shows binding of human Fab HA-25 to apoptotic cells but not normal cells……180

**Fig. 6-7** Staining of atherosclerotic lesions from rabbit aorta with human Fab clones……182

**Fig. 6-8** HA-2 inhibits oxidation-specific antigen binding to macrophages…….183

**Fig. 7-1** Oxidation-specific epitopes are a class of pathogen-associated molecular patterns (PAMPs) recognized by multiple arcs of innate immunity…201

# LIST OF TABLES

**Table 1-1** Comparisons between Innate and Adaptive Immunity……8

**Table 1-2** Presence of oxidation-specific epitopes under various pathological processes……...17

**Table 3-1** Cytokine profile of wild-type and *Myd88*$^{-/-}$ B-1 cells *in vitro* after stimulation with TLR agonists……...82

**Table 4-1** Levels of each antibody isotype in the plasma of wild-type C57BL/6 and B-1 reconstituted *Rag1*$^{-/-}$ mice……112

# ACKNOWLEDGEMENT

I am most grateful to my advisor, Professor Joseph L. Witztum, for his mentorship, support, and inspiration throughout the course of my research. He was not just a mentor to me scientifically, but also helped me through numerous personal hardships and cared about me. I also wish to express my gratitude to Dr. Christoph J. Binder, who helped me initiate my thesis project and has been a valuable mentor to me throughout my graduate studies.

I also want to thank my committee members for their time and helpful discussions: Professor Maripat Corr, who helped me develop the B-1 cell reconstituted *Rag1*$^{-/-}$ mouse model, Professor Linda K. Curtiss, Professor Christopher K. Glass, Professor Donald Guiney, and Professor Victor Nizet, who provided the resources and guided me with his expertise in microbiology.

Chapters 2,3,4,5, and 6, in part, has been accepted for publication in "Oxidation-specific Epitopes are a Dominant Target for Innate Natural Antibodies", Journal of Clinical Investigation, 2009, by MY Chou, L. Fogelstrand, K. Hartvigsen, LF Hansen, D. Woelkers, PX Shaw, J. Choi, T. Perkmann, F Bäckhed, YI Miller, S. Hörkkö, M. Corr, JL Witztum and CJ Binder.

Most of the work would not have been possible without the help and advice of my coworkers and collaborators. I would like to thank my collaborator Dr. Fredrik Bäckhed who provided the germ-free plasma samples, and also Ohn Chow and Dr. Yung-Chi Chang from the Nizet lab for providing the various strains of bacteria.

Finally I would like to thank all the members of the Witztum lab, for their help in various aspects of my research. In particular Drs. Linda Fogelstrand, Karsten Hartvigsen, Lotte Hansen, Douglas Woelkers and Yury Miller for their experimental assistance and helpful discussions. I would also like to thank Felicidad Almazan, Suzy Butler, Jennifer Pattison, and Florence Casanada for their excellent technical assistance.

I would lastly like to give special thanks to my grandmother Yung-Ho Lee Chou and my parents, Wen-Chung Chou and Mei-Chen Chou, as well as my brother Meng-Chieh Chou for their un-conditional love and support. I would like to thank Felicidad Almazan, for treating me like her own daughter all these years. Lastly I want thank my boyfriend Chi-Nung Ni for his encouragement and thoughtfulness during the final stages of my work.

I would also love to thank all my friends who has helped me throughout these years and other colleagues who are too numerous to name in full.

*Meng-Yun Sandy Chou Jan 09*

# CURRICULUM VITAE

## EDUCATION

**2009** Ph.D., Biomedical Sciences, University of California – San Diego

**2002** B.S., Life Sciences *with Honors,* National Tsing Hua University, Taiwan

## HONORS AND AWARDS

Academic Achievement Award, National Tsing Hua University (2000)

Ministry of Education Scholarship for Graduate Students, National Tsing Hua University (2002)

Biomedical Sciences Graduate Program Best Poster Award, University of California, San Diego (2006)

Award for Outstanding Research, Disorders of Lipid Metabolism Symposium (2007)

## PUBLICATIONS

1. **Chou MY**, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Bäckhed F, Miller YI, Hörkkö S, Corr M, Witztum JL, and Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies *J Clin Invest* 2009 (In press)

2. **Chou MY**, Hartvigsen K, Hansen LF, Fogelstrand L, Shaw PX, Boullier A, Binder CJ, Witztum JL. Oxidation-specific epitopes are important targets of innate Immunity *J Intern Med*. 263:479-88, 2008.

3. Hartvigsen K, **Chou MY**, Hansen LF, Shaw PX, Tsimikas S, Binder CJ, Witztum JL. The role of innate immunity in murine atherosclerosis. *J Lipid Res*. 2008 Dec 22 (In Press)

4. Binder CJ, **Chou MY**, Fogelstrand L, Hartvigsen K, Shaw PX, Boullier A, Witztum JL. Natural Antibodies in murine atherosclerosis. *Curr Drug Targets*. 9:190-5, 2008

5. Chen CC, **Chou MY**, Huang CH, Majumder A, Wu HY. A cis-spreading nucleoprotein filament is responsible for the gene silencing activity found in the promoter relay mechanism. *J Biol Chem.* 280:5101-12. 2005

# ABSTRACT OF THE DISSERTATION

Oxidation-Specific Epitopes are Targets of Innate Natural Antibodies **:**
Potential Implications in Health and Disease

by

Meng-Yun Sandy Chou

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2009

Professor Joseph L. Witztum, Chair

Atherosclerosis is a chronic inflammatory disease, characterized by the accumulation of oxidized lipoproteins and apoptotic cells, both containing various “oxidation-specific” neoepitopes. Adaptive immune responses to oxidation-specific epitopes have been shown to play an important role in atherogenesis. However, accumulating evidence suggests that these epitopes are also

recognized by components of innate immunity, such as scavenger receptors on macrophages, as well as natural antibodies (NAbs). NAbs are mostly of the IgM isotype which are selected by evolution and represent the humoral arc of innate immunity. In my thesis, I provide multiple lines of evidence that oxidation-specific epitopes constitute a dominant, previously unrecognized target of NAbs in both mice and humans. For example, using reconstituted mice expressing solely IgM NAbs, I showed that ~ 30% of all NAbs bind to model oxidation epitopes, as well as to atherosclerotic lesions and apoptotic cells. Similar observations were made for human natural IgM Abs. Because oxidative processes are ubiquitous, these epitopes might exert selective pressure to expand NAbs, which in turn may play an important role in mediating homeostatic functions consequent to inflammation and cell death. This is demonstrated by the ability of NAbs to facilitate apoptotic cell clearance. These findings provide novel insights into the functions of NAbs in mediating host homeostasis and into their roles in health and diseases, which may lead to novel diagnostic and possibly therapeutic approaches to deal with consequences of oxidative stress such as atherogenesis.

# CHAPTER I

## INTRODUCTION

## Atherosclerosis and inflammation

Complications that rise from atherosclerosis are the most common cause of death in the United States. Atherosclerosis is a disease of the vascular wall that leads to myocardial infarction, heart failure, peripheral vascular disease and stroke (1). It develops over decades and is believed to progress from intimal thickening to ever more complex lesions involving accumulation of cells derived from the circulation, proliferation of inherent vascular wall cells, elaboration of extracellular matrix and lipid accumulation, both extracellular bound to matrix, and intracellular, within macrophage foam cells.

The evolution from a normal artery to the complex lesions and ruptured plaques that result in clinical events involves a complex array of events. Atherosclerosis begins as fatty streaks characterized by the deposition of lipid-laden foam cells. Macrophage cholesteryl ester formation is believed to be due in large part to enhanced and unregulated uptake of oxidized, aggregated and variously other modified LDL and possibly other lipoproteins, and disturbed cellular responses that are unable to export the accumulated cholesterol load. As the lesions progress, many of the lipid filled cells undergo apoptosis, which are not sufficiently cleared, leading to an abnormal accumulation of apoptotic cells in the lesion (2).

In time, these apoptotic cells undergo necrosis, yielding the acellular characteristic of the advanced atherosclerotic plaques. Smooth muscle cell proliferation and secretion of a thick collagen cap over the necrotic lipid-filled

cores may stabilize the lesion, but eventually vulnerable areas of the plaque erode or rupture, leading to thrombosis, ischemia and clinical events or even death.

Atherosclerosis is now widely recognized as a chronic inflammatory disease(3). The evolving atherosclerotic lesion has all the characteristics of a slowly smoldering inflammatory disease (4), including the presence of immune cells such as monocyte/macrophages, dendritic cells, T cells, Natural Killer (NK) T and NK cells, and mast cells. Neutrophils are curiously absent and B cells have only rarely been described, though they are found in the immediate adventitia surrounding lesions and in draining lymph nodes. In addition, non-cellular components of immunity are prominently found in established lesions, including immunoglobulins, in part bound to specific disease-related antigens as immune complexes, as well as complement and C-reactive protein (CRP). Specific aspects of the immune function have been manipulated and altered which resulted in significant modulation of the course of atherogenesis, demonstrating the important role of immune mechanisms in disease progression (5).

Although multiple risk factors have been identified for atherosclerosis such as hypercholesterolemia, hypertension, diabetes, gender and obesity (6) that contribute variably to lesion formation, the growth of the atherosclerotic lesion is both initiated and sustained by elevated levels of low density lipoproteins (LDL) and low and/or dysfunctional HDL. Recent clinical trial data clearly document that lowering plasma LDL levels is a potent way to reduce the risk of clinical events and these trials all suggest that the clinical adage, “the lower the LDL level the

better" is correct (7,8). Yet, it is also clear that among individuals with the same LDL levels or exposed to the same therapeutic interventions, the expression of clinical events varies widely, indicating that many other factors are involved in the response of the artery wall to a given LDL level. It is for this reason that so much effort is being made to understand the fundamental mechanisms involved in lesion formation.

## Role of LDL in atherosclerosis

Low density lipoprotein (LDL) particles are the major cholesterol carriers in circulation and their physiological function is to carry cholesterol to the cells. Each native LDL particle is about 22nm in diameter and contains a highly hydrophobic core consisting of triglycerides (TG) and cholesteryl esters (CE) (9). This core is surrounded by a monolayer of phospholipids and a single copy of apolipoprotein B-100 (apo B-100). The main phospholipid components of LDL are phosphatidylcholine (PC) and sphingomyelin (SM). The LDL particles also contain lysophosphatidylcholine (lyso-PC), phosphatidylethanolamine (PE), diacylglycerol (DAG), ceramide (CER) and some phosphatidylinositol (9). In addition, the particle contains unesterified cholesterol (UC), of which about one-third is located in the core and two-thirds in the surface. The LDL particles are in a dynamic state, their structure and physical properties being dependent on their lipid composition as well as on the conformation of apoB-100.

While LDL has an essential physiological role in transporting serum cholesterol to peripheral tissues, increased plasma LDL cholesterol levels are

associated with increased risk of cardiovascular disease, as noted above. LDL is subject to oxidative modifications when trapped in the artery wall, progressing from minimally oxidized LDL (mmLDL) to oxidized LDL (OxLDL) (1,10,11). OxLDL is then taken up by macrophages through scavenger receptors, leading to foam cell formation, a key event in the initiation of atherosclerosis (1,11-14). The oxidation of LDL generates oxidized lipids that are both pro-inflammatory and ultimately pro-atherogenic (15).

## Innate and adaptive immunity in atherosclerosis

Both innate and adaptive immunity play an important role in the pathogenesis of atherosclerosis (5,15-17). Traditionally, innate immunity has been recognized as providing the first line of host defense against exogenous infectious agents, but increasingly it is apparent that endogenous, "self-ligands" are also targets of innate immunity. Recognition of pathogens by the innate immune system relies on a limited number of highly conserved receptors - "pattern recognition receptors" (PRR) - such as scavenger receptors (SR) and Toll-like receptors (TLRs) that are present on the cell surface or within the cell (18). These receptors recognize highly conserved motifs on specific pathogens or self-ligands, which are termed "pathogen-associated molecular patterns" (PAMPS). Ligation of such PRRs can lead to intracellular signaling pathways that lead to cell activation, for example, ligation of TLRs often trigger the NF-κB

signaling pathway, which leads to the secretion of cytokines and an acute inflammatory response to help clear infection.

The multipurpose monocyte-macrophage is the classic innate immune cell, that not only serves to identify pathogens leading to activation of inflammatory responses, but also to scavenging. In addition, by processing and presentation to T cells, it serves to activate adaptive immunity as well. Innate dendritic cells are even more specialized professional antigen presenting cells (APCs). Other important components of innate immunity include marginal zone (MZ) B cells and NK cells (19). Natural antibodies (NAbs) secreted by B-1 cells are a critical component of the humoral response of innate immunity that provides optimal protection against bacterial infections (20,21), but as I will show later, bind to an important set of endogenous self-epitopes as well.

Because innate immune responses are germ-line encoded and preformed, innate responses are rapid but can only respond to a limited set of antigens. Adaptive immunity, on the other hand, initiates specific immune responses to a limitless set of antigens through T cell receptors (TCRs) and B cell receptors (BCRs), which gain high specificity by somatic rearrangement (22). These responses include stimulation of B-2 cells to produce antibodies (Abs) by $CD4^+$ T helper cells and direct attack of antigen-bearing cells by $CD8^+$ cytotoxic T lymphocytes (CTL). The adaptive immune responses cooperate with innate immune responses to eliminate foreign particles. It is critical for the immune system to be able to distinguish self from non-self because Abs that develop

toward self antigens can give rise to autoimmune diseases or allograft rejection. **Table 1** summarizes some key features of innate and adaptive immunity.

Innate immune mechanisms are involved in the recruitment of monocytes and their differentiation into macrophages during early events of atherosclerosis (23,24). Endothelial cells are mainly activated by hypercholesterolemia, OxLDL, and hypertension (25-27), resulting in the upregulation of adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). This will lead to the migration of monocytes into subendothelial spaces (28-30). Cytokines such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and chemokines such as monocyte chemotactic protein-1 (MCP-1) are also involved in this process (31,32).

Within the artery wall, the recruited monocytes are then differentiated into macrophages. Progressive accumulation of macrophages and their uptake of OxLDL will eventually lead to the formation of atherosclerotic lesions. Mice that lack macrophage colony stimulating factor (M-CSF), MCP-1, or CCR2, the receptor for MCP-1, and thus lack differentiated macrophages in the lesions, show only minimal atherosclerosis(33-35).

Increasingly, it is being recognized that the PRRs of innate immunity also play an important role in atherosclerosis. Scavenger receptors such as CD36 and SR-A on macrophages were shown to bind and internalize OxLDL, leading to foam cell formation(36,37). Antigens generated from internalized OxLDL can also be presented through MHC class II restricted antigen presentation, linking innate responses to adaptive responses(38).

**Table 1-1 Comparisons between Innate and Adaptive Immunity**

| | Innate | Adaptive |
|---|---|---|
| **Response time** | **Fast(hours)** | **Slow(days)** |
| **Antigen** | **Conserved molecular PAMPs** | **Unique epitopes on pathogens** |
| **Cellular Components** | **Macrophages, B-1 cells, marginal zone B cells, NK T cells and mast cells** | **T cells and B-2 cells** |
| **Generation of specificity** | **Germ-line encoded** | **Somatic rearrangement** |
| **Effector Mechanisms** | **NAbs, cytokines, chemokines, cell-mediated toxicity, complement activation** | **Abs, cytokines, chemokines, CTL, complement activation** |

TLRs, on the other hand, can elicit inflammatory response directly. Activation of TLRs by exogenous ligands such as microbial components might directly associate with atherosclerosis because several microorganisms have been shown to be associated with atherosclerosis (39). Endogenous ligands, such as heat shock protein 60 (HSP60) and minimally modified LDL (mmLDL)

can bind to TLR4-CD14 complexes and elicit inflammatory response (40-42). TLR2 and TLR4 deficient atherosclerotic mouse model both showed decreased atherosclerosis, demonstrating a clear role for TLRs in atherogenesis (43).

B-1 cell derived NAbs, chiefly IgM and IgA, are another important branch of innate immunity. In an uninfected mouse, plasma IgMs are considered in large part to be B-1 cell derived NAbs (44). IgM responses to OxLDL have been found in both animal models of atherosclerosis and humans (45,46). It is not yet clear how NAbs in general will modulate atherogenesis. However, as I will demonstrate in my thesis, accumulating evidence suggests that oxidation-specific epitopes of OxLDL are an important target for NAbs and thus NAbs might play an important role in atherosclerosis. This topic will be discussed in more detail in the section **Natural Antibodies and B-1 cells**.

The endogenous “neo-antigens” of atherosclerosis such as OxLDL and exogenous antigens from microorganisms are targets for the adaptive immune system as well as the innate immune system. These antigens are presented to T cells by antigen presenting cells (macrophages, dendritic cells, B cells). Activated T cells then secrete a variety of cytokines that affect the progression of atherogenesis (5). T helper cells that secrete pro-inflammatory cytokines (Th1 cells) such as TNF$\alpha$ and interleukin-1 (IL-1) play similar roles in promoting atherosclerosis by promoting inflammatory response (47,48). Only low amounts of anti-inflammatory cytokines secreted by Th2 cells, such as IL-4, IL-5 and IL-10, can be detected in atherosclerotic lesions (49,50). IL-10 can potently suppress the secretion of pro-inflammatory cytokines (51). Overexpression of IL-10 in LDL

receptor (LDLR) deficient mice, a murine model for atherosclerosis, inhibited atherosclerosis (52). On the other hand, IL-10 deficiency increased the early phase of atherosclerotic lesion development and thrombotic activity in apoE$^{-/-}$ mice, confirming a protective role for IL-10 in atherosclerosis (53). Therefore, anti-inflammatory cytokines might be athero-protective.

T cells can also help activate B cells to produce Abs. Some T cell-independent antigens can activate B cells without the help of T cells. OxLDL is probably an example of such an antigen. Immunization of hypercholesterolemic rabbits and mice with models of OxLDL lead to high Ab titers to OxLDL and a reduction in atherosclerosis, suggesting immune responses to OxLDL might be important in modulating atherosclerosis (54-58). Circulating IgG and IgM Abs against OxLDL have been shown to be present in the plasma of animals and humans and form immune complexes with OxLDL in atherosclerotic lesions (59,60). These Abs closely correlate to the progression and regression of atherosclerosis in murine models and measures of lipid peroxidation (61-63).

## Antigens of atherosclerosis – Generation of oxidation-specific epitopes

Our lab has developed extensive evidence that OxLDL exists *in vivo* and plays a central role in the pathogenesis of atherosclerosis (59,62,64-66). When LDL enters the intima of the artery wall, it undergoes progressive stages of oxidation, which results in immunogenic modifications to the phospholipids, cholesterol, and protein moieties of LDL (65). The mechanisms responsible for

the *in vivo* conversion of native LDL into OxLDL remain to be defined, but is likely associated with lipoxygenases, myeloperoxidase-related mechanisms and the generation of reactive oxygen species (63,67,68). *In vitro*, however, this can be accomplished by incubation of LDL with copper ions or reactive aldehydes to generate $CuSO_4$-oxidizied LDL (Cu-OxLDL) and malondialdehye modified LDL (MDA-LDL), two models of OxLDL that are most commonly used to study the effects of OxLDL. For example, the polyunsaturated fatty acids (PUFA) found in phospholipids, or cholesteryl esters undergo peroxidation and generate highly reactive aldehydes, such as MDA and 4-hydroxynonenal (4-HNE), which in turn form covalent adducts with lysines of associated proteins or with amino groups of lipids such as phosphatidylethanolamine (PE) (**Fig.1-1, pathway 1.**) (69). MDA can undergo further reactions to generate more complex adducts such as malondialdehyde-acetaldehyde (MAA)-lysine, a lysine adduct with 2 MDAs and acetaldehyde (70).

In addition, oxidation of the PUFA chains typically found in the *sn2* position of intact phospholipids can generate oxidized phospholipids (OxPL). For example, oxidation of the sn2 PUFA can generate an intact phospholipid containing a reactive 5-carbon aldehyde, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC). The reactive carbonyl of POVPC can form adducts with the ε-amine of lysines of apoB, leading to OxPL-apoB adducts or OxPL-lipid adducts (**Fig.1-1, pathway 2.**) (27,71).

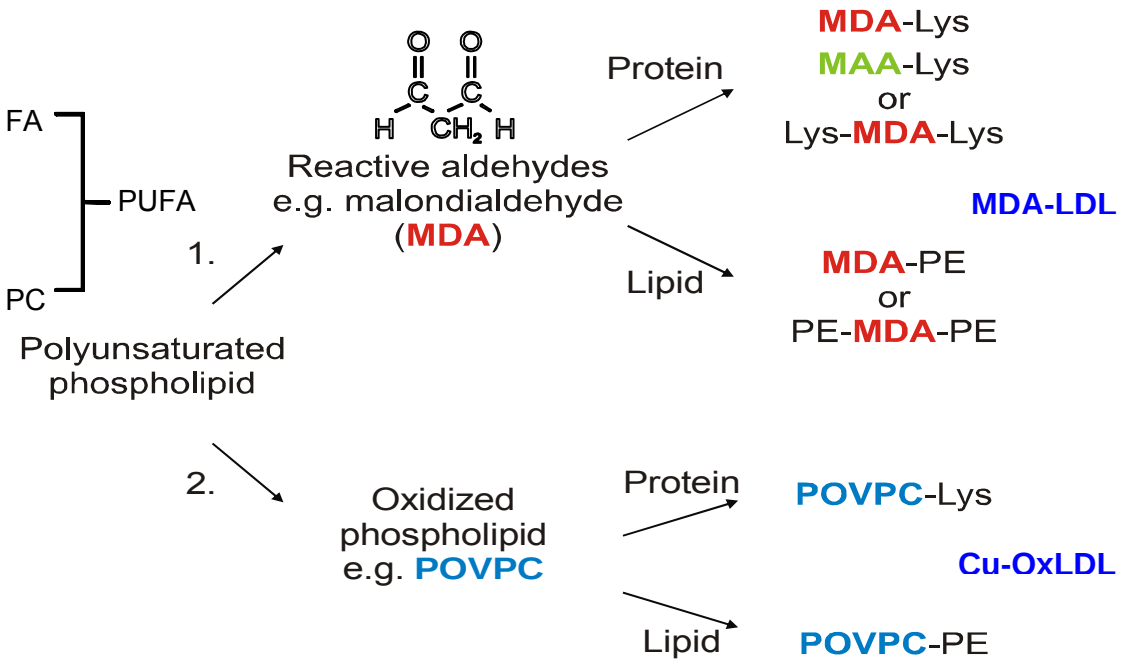



**Fig. 1-1 Simplified schematic illustration of *in vitro* formation of OxLDL and oxidation-specific epitopes.**

These are a just a few of the extensive modifications that lead to the generation of neo-antigens, which we termed “oxidation-specific epitopes”. As I demonstrate in my thesis, these oxidation-specific epitopes constitute a previously unrecognized, but major category of altered-self that are targets of NAbs, one important branch of innate immunity (72).

## Natural Antibodies and B-1 cells

NAbs are germ-line encoded Abs that spontaneously arise without exogenous antigen stimulation, and represent a primitive layer of the innate immune system(73,74). NAbs are predominantly of the IgM isotype, supported by

the fact that serum IgM levels are similar between mice raised under germ-free and antigenic-free conditions versus conventionally raised mice (44,75,76). Thus, in uninfected mice, most plasma IgM Abs are considered NAbs.

NAbs act as the first line of defense against bacterial and viral infections (20,77). Most pathogens elicit a humoral response starting with an early-rise of antigen-specific immunoglobulin (Ig)Ms derived largely from innate B-1 cells, followed by affinity maturation and isotype switching to give rise to antigen-specific IgG, IgA and IgE Abs that are mainly secreted by conventional B-2 cells (77). B-1 cells are a specialized set of B-lymphocytes that has been considered to be a part of innate immunity (78). They are the major source of NAbs (78,79).

B-1 cells differ from conventional B-2 cells in several ways, including their development, anatomical localization, and surface marker expression (80-83). B-1 cell development occurs primarily during fetal and neonatal life, whereas B-2 cell production is most active after birth (84). B-1 cells are found mainly in the pleural and peritoneal cavities in adult mice. They are absent from peripheral lymph nodes, and make up about 5% or less of splenic B cells (85). B-1 cells possess a unique surface phenotype, allowing their identification and isolation. In the peritoneal cavity, B-1 cells are $CD19^{+}$, $CD23^{-}$, $IgM^{hi}$, Mac-$1^{+}$, and $CD5^{+}$ (for B-1a cells), or $CD5^{-}$ (for B-1b cells). B-1a cells are thought to be the primary source of spontaneously secreted NAbs while B-1b cells are induced to produce NAb following exposure to antigen—though this distinction is not absolute. In the spleen, B-1 cells maintain the surface markers of peritoneal cavity B-1 cells except for the loss of Mac-1 and gain of CD43 expression (86,87). The levels of

CD5 expression is 5-10 fold lower in splenic B-1 cells than T cells, thus CD5 would not be a good marker for splenic B-1 cells (88). In adult mice, mature B-1 cells can replenish through self-renewal in the peritoneal and pleural cavities (89,90).

The maturation and secretion of NAbs by B-1 cells is thought to be largely thymus-independent and does not require conventional cognate T cell help (85). NAbs are germline-encoded due in part to the lack of terminal deoxynucleotidyl transferase (TdT) activity during early ontogeny when NAbs are formed. TdT inserts nontemplated nucleotides into the V-D-J splice sites and is responsible in part for creating the diversity of Abs produced by B-2 cells. NAbs thus exhibit a more restricted repertoire than that produced by B-2 cells (91).

NAbs have been shown to participate in immediate host defense against pathogens by recognizing PAMPs that are present on multiple pathogens (20). However, NAbs can also bind to a variety of apparent “self-antigens” such as various carbohydrates, for example $\alpha$1,3-dextran (92), glycolipids and intracellular molecules such as single-stranded DNA (93) or cell surface molecules on T-cells such as Thy1 (94). In addition, as first demonstrated by our laboratory, NAbs also bind to a variety of oxidation-specific epitopes formed for example on OxLDL, but also present on apoptotic cells, such as the phosphocholine moiety present on OxPL or on the surface of apoptotic cells.

Many of these neo-self epitopes are also present on or closely related to molecular structures that are present on infectious pathogens, which might explain the so called “poly-reactivity” that has been described for NAbs. One

example relevant to atherosclerosis is T15/EO6, a natural IgM Ab of B-1 origin, which recognizes the phosphocholine (PC) headgroup on both OxPL and capsular polysaccharides of *Streptococcus pneumoniae* (95). This Ab provides optimal protection against *S. pneumoniae* infections in mice (96). Immunization of cholesterol-fed *LDLR*$^{-/-}$ mice with heat-killed *S. pneumoniae* induced a near monoclonal generation of T15/EO6 that bound OxLDL and reduced atherosclerosis (97), suggesting a potential atheroprotective role for NAbs.

NAbs may also play a role in the recognition and removal of senescent cells, cell debris, and other neo-self antigens. Binding of NAbs to epitopes on membranes of damaged red blood cells activates the alternative complement pathway, which may then aid the clearance of senescent red blood cells (98). Other NAbs might bind to lysophospholipids formed on apoptotic cells, affecting apoptotic cell removal through similar complement dependent mechanisms (99). Mice that are deficient in secreted IgM (sIgM$^{-/-}$), when bred onto the lupus prone MRL-lpr strain, showed an accelerated incidence of autoimmune disease (100). Moreover, with increased age, sIgM$^{-/-}$ mice had an increased tendency to produce anti-double stranded DNA IgG autoantibodies (101). Therefore, NAbs have been postulated to contribute to the elimination of autoantigens exposed during stress, tissue damage, or even conventional cell turnover and thus protect from autoimmunity. For this reason, NAbs might be autoreactive by design (102,103). However, as we will suggest, the epitopes to which these NAbs bind may actually be subtle modifications of true self that in fact identify the structure as being damaged, and thus the target of immune clearance.

**Oxidation-specific epitopes are ubiquitous**

NAb can recognize oxidation-specific epitopes on OxLDL and may play a protective role in atherosclerosis (95,97). In the case of E06, it binds to OxLDL and blocks scavenger mediated uptake of OxLDL by macrophages. However, the presence of oxidation-specific epitopes is not restricted to OxLDL in atherosclerosis. Oxidation-specific epitopes can be found in many other disease conditions in which cells undergo oxidative stress, including apoptosis (104). Cells that undergo programmed cell death develop mitochondrial disruption, leading to generalized enhancement of oxidative events. In turn, a wide variety of lipids are oxidized, including cardiolipin, phosphatidylserine, and phosphatidylcholine (104-106). Such epitopes are greatly enriched in microparticles, the apoptotic blebs in circulation that bud off from apoptotic cells (107). Our lab has shown that syngeneic apoptotic cells, not promptly cleared, are highly immunogenic and proinflammtory. Mice that are immunized with syngeneic apoptotic cells also develop high autoAb titers to a variety of oxidation-specific epitopes (105). Furthermore, all of our oxidation-specific monoclonal Abs can bind to apoptotic cells or apoptotic bodies (106,108), further supporting that similar oxidation-induced surface structures are present on apoptotic cells as well. Since apoptosis is a universal biological event from the earliest stage of development, we hypothesize that this provides a strong evolutionary pressure for the selection of innate NAbs to efficiently clear such dying cells. Indeed,

Ogden et al. has shown that in the presence of complement, T15/E06 idiotypic Ab has the capacity to facilitate apoptotic cells clearance *in vivo* (109).

Aside from apoptotic cells, oxidation specific epitopes can also be found under a wide variety of pathological conditions, such as oxidatively damaged red blood cells (110). MDA-lysine epitopes were detected in the cytoplasm of neurons and astrocytes in patients with Alzheimer's disease as well as normal aging subjects, but were rarely detected in normal young subjects (111). Oxidative stress also plays a key role in inducing acute lung injury caused by infectious agents. OxPL formation was observed in lungs of human and animals infected with SARS, anthrax, or H5N1 avian influenza virus (112). **Table 1-2** is a summary of the presence of different oxidation-specific epitopes under various pathological processes.

**Table 1-2 Presence of oxidation-specific epitopes under various pathological processes**

| Condition | Tissue Affected | Animal Model | Oxidation-Specific Epitope Generated | Reference |
|---|---|---|---|---|
| Alcohol Abuse | Red blood cells/Liver | Rat, micropig human | Acetaldehyde and MDA-modified epitopes on proteins | Niemela et al. (113) |
| Excess iron deposition | Brain | Mouse | MDA-lysine epitopes | Castelnau et al. (114) |
| Genetic hemochromatosis | Liver | Human | MDA-lysine epitopes | Houglum et al. (115) |

**Table 1-2 Continued**

| Condition | Tissue Affected | Animal Model | Oxidation-Specific Epitope Generated | Reference |
|---|---|---|---|---|
| Passive Heymann nephritis | Kidney | Rat | MDA-lysine epitopes | Exner et al. (116) |
| | | | 4-hydroxynonenal (HNE)-lysine epitopes | Neale et al. (117) |
| Diabetic nephropathy | Kidney | Human | Advanced glycation end products | Horie et al. (118) |
| | | | MDA-lysine epitopes | |
| Atherosclerosis | Aorta | Rabbit | Advanced glycation end products | Palinski et al. (119) |
| | | Rabbit and human | MDA-lysine epitopes | Yla-Herttuala et al.(64) |
| | | | 4-HNE-lysine epitopes | Rosenfeld et al.(120) |
| | | Rabbit | Copper-oxidation-generated epitopes | Palinski et al. (121) |
| | | | Oxidized cardiolipin | Tuominen et al (106) |
| Apoptosis | Cells | Mouse | MDA-lysine epitopes | Chang et al. (108) |
| | | | OxPL (PC) | Chang et al. (105) |
| | | | Oxidized Cardiolipin | Tuominen et al. (106) |
| | | | Oxidized phosphatidylserine | Kagan et al. (104) |
| Alzheimer's | Brain | Human | MDA-lysine epitopes | Dei et al. (111) |
| | | | Advanced glycation end products | |
| Rheumatoid Arthritis | joint | Mouse | MDA-lysine epitopes | Binder et al. |
| | | | OxPL | (Unpublished) |
| Acute Lung Injury | Lung | Mouse human | OxPL (PC) | Imai et al.(112) |

## Specific Background and Research Goals

Recent evidence has shown that NAbs not only bind to PAMPs present on exogenous antigens, but can also bind to altered structures on endogenous antigens. The example of T15/E06, which binds to PC, demonstrates a molecular mimicry between such neo-self antigens and certain microbial antigens(60). Aside from E06, the PC moiety is recognized by at least 3 other innate PRRs, including CD36, SRB-1, and CRP(122-124). Because the innate immune system is conserved by natural selection, this implies that these innate PRRs that recognize PC as a PAMP must have been selected for their beneficial effects. In addition, it is now recognized that during fetal and neonatal period when B-1 cells develop, antigen recognition promotes B-1 cell selection and survival, leading to "positive selection" (94,125). Thus, the repertoire of B-1 cell derived NAbs is selected to bind to evolutionarily important epitopes. Since the presence of oxidation-specific epitopes is common on apoptotic cells, as discussed previously, **we hypothesize that the prevalence of oxidation-specific epitopes that occur during normal fetal development, might have provided the positive selection for an enriched population of B-1 cells binding to oxidation specific epitopes, consistent with the oxygen-rich environment that we live in. Because these epitopes are greatly expressed in inflammatory conditions, and molecular mimics are frequently present on pathogens, there would be even further developmental pressure to expand these B-1 clones, leading to the hypothesis that oxidation-specific epitopes are a**

**major target of innate NAbs.** Evidence supporting this hypothesis is presented in Chapters 2, 3, and 4. In Chapter 2, I examine plasma IgMs from various mouse models including the germ-free mice to demonstrate the presence of IgM Abs against oxidation-specific epitopes. In Chapter 3, B-1 cells were cultured *in vitro* and tested for the secretion of oxidation-specific IgM to demonstrate the origin of these Abs. Related *in vitro* studies are also presented in Chapter 3. In Chapter 4, a B-1 mouse model is created to study the B-1 cell-derived NAbs *in vivo*.

The traditional view of the function of NAbs is to provide protection against bacterial and viral infections. However, our studies with the NAb E06 provides new insights into the role of NAbs in health and disease. E06, which protects against *S. pneumoniae* infections in mice (96), also provides atheroprotective properties(97). NAbs have also been suggested to play a role in the clearance of senescent cells and other self-antigens, and thus, may provide protection against autoimmunity as well (83). IgM have been shown to facilitate phagocytic clearance of apoptotic cells through complement activation, thus helping to eliminate autoantigens that are formed during apoptosis (126). Therefore, aside from the critical role in mediating host defense against pathogens, NAbs might be naturally selected also due to their importance in maintaining homeostasis. **We hypothesize that NAbs against oxidation-specific epitopes are conserved because they provide advantageous properties not only for defense against pathogens but also in host responses to oxidative and inflammatory events.** This hypothesis is tested in Chapter 5, where I describe

studies of potential implications of natural IgM Abs in health and disease, such as the clearance of apoptotic cells, the ability of NAbs to recognize epitopes in the lesions and to inhibit OxLDL binding to macrophages, and the ability of NAbs to recognize pathogens.

Specific Abs against oxidation-specific epitopes have been found in humans (127), but it is unknown if these are NAbs. The presence of NAbs to oxidation-specific epitopes in humans is tested in Chapter 6 by analyzing IgM in human umbilical cord plasma. Umbilical cord plasma IgMs are considered to be the human equivalent of NAbs, because IgM molecules are too big to cross the placenta (128).

## References


1. **Glass, C. K. and J. L. Witztum**. 2001. Atherosclerosis. the road ahead. Cell **104**:503-516.

2. **Kockx, M. M.** 1998. Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects. Arterioscler. Thromb. Vasc. Biol. **18**:1519-1522.

3. **Hansson, G. K.** 2005. Inflammation, atherosclerosis, and coronary artery disease. N. Engl. J. Med. **352**:1685-1695.

4. **Ross, R.** 1999. Atherosclerosis--an inflammatory disease. N. Engl. J. Med. **340**:115-126.

5. **Hansson, G. K., P. Libby, U. Schonbeck, and Z. Q. Yan**. 2002. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ. Res. **91**:281-291.

6. **Rosamond, W., K. Flegal, K. Furie, A. Go, K. Greenlund, N. Haase, S. M. Hailpern, M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lloyd-Jones, M. McDermott, J. Meigs, C. Moy, G. Nichol, C. O'Donnell, V. Roger, P. Sorlie, J. Steinberger, T. Thom, M. Wilson, and Y. Hong**. 2008. Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation **117**:e25-146.

7. **LaRosa, J. C., S. M. Grundy, D. D. Waters, C. Shear, P. Barter, J. C. Fruchart, A. M. Gotto, H. Greten, J. J. Kastelein, J. Shepherd, and N. K. Wenger**. 2005. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. N. Engl. J. Med. **352**:1425-1435.

8. **Cannon, C. P., E. Braunwald, C. H. McCabe, D. J. Rader, J. L. Rouleau, R. Belder, S. V. Joyal, K. A. Hill, M. A. Pfeffer, and A. M. Skene**. 2004. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. N. Engl. J. Med. **350**:1495-1504.

9. **Hevonoja, T., M. O. Pentikainen, M. T. Hyvonen, P. T. Kovanen, and M. Ala-Korpela**. 2000. Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim. Biophys. Acta **1488**:189-210.

10. **Witztum, J. L.** 1994. The oxidation hypothesis of atherosclerosis. Lancet **344**:793-795.

11. **Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum**. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. **320**:915-924.

12. **Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg**. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. U. S. A **81**:3883-3887.

13. **Fogelman, A. M., I. Shechter, J. Seager, M. Hokom, J. S. Child, and P. A. Edwards**. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. Proc. Natl. Acad. Sci. U. S. A **77**:2214-2218.

14. **Yamada, Y., T. Doi, T. Hamakubo, and T. Kodama**. 1998. Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. Cell Mol. Life Sci. **54**:628-640.

15. **Binder, C. J., M. K. Chang, P. X. Shaw, Y. I. Miller, K. Hartvigsen, A. Dewan, and J. L. Witztum**. 2002. Innate and acquired immunity in atherogenesis. Nat. Med. **8**:1218-1226.

16. **Getz, G. S.** 2005. Thematic review series: the immune system and atherogenesis. Bridging the innate and adaptive immune systems. J. Lipid Res. **46**:619-622.

17. **Hansson, G. K. and P. Libby**. 2006. The immune response in atherosclerosis: a double-edged sword. Nat. Rev. Immunol. **6**:508-519.

18. **Janeway, C. A., Jr. and R. Medzhitov**. 2002. Innate immune recognition. Annu. Rev. Immunol. **20**:197-216.

19. **Delves, P. J. and I. M. Roitt**. 2000. The immune system. First of two parts. N. Engl. J. Med. **343**:37-49.

20. **Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel**. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science **286**:2156-2159.

21. **Boes, M., A. P. Prodeus, T. Schmidt, M. C. Carroll, and J. Chen**. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. Exp. Med. **188**:2381-2386.

22. **Delves, P. J. and I. M. Roitt**. 2000. The immune system. Second of two parts. N. Engl. J. Med. **343**:108-117.

23. **Gerrity, R. G.** 1981. The role of the monocyte in atherogenesis: II. Migration of foam cells from atherosclerotic lesions. Am. J. Pathol. **103**:191-200.

24. **Gerrity, R. G.** 1981. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am. J. Pathol. **103**:181-190.

25. **Kume, N., M. I. Cybulsky, and M. A. Gimbrone, Jr.** 1992. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J. Clin. Invest **90**:1138-1144.

26. **Leitinger, N., A. D. Watson, K. F. Faull, A. M. Fogelman, and J. A. Berliner**. 1997. Monocyte binding to endothelial cells induced by oxidized phospholipids present in minimally oxidized low density lipoprotein is inhibited by a platelet activating factor receptor antagonist. Adv. Exp. Med. Biol. **433**:379-382.

27. **Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner**. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J. Biol. Chem. **272**:13597-13607.

28. **Cybulsky, M. I. and M. A. Gimbrone, Jr.** 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science **251**:788-791.

29. **Walpola, P. L., A. I. Gotlieb, M. I. Cybulsky, and B. L. Langille**. 1995. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. Arterioscler. Thromb. Vasc. Biol. **15**:2-10.

30. **Nakashima, Y., E. W. Raines, A. S. Plump, J. L. Breslow, and R. Ross**. 1998. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. Arterioscler. Thromb. Vasc. Biol. **18**:842-851.

31. **de, M. R., M. Hoeth, R. Hofer-Warbinek, and J. A. Schmid**. 2000. The transcription factor NF-kappa B and the regulation of vascular cell function. Arterioscler. Thromb. Vasc. Biol. **20**:E83-E88.

32. **Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, T. Sarkioja, T. Yoshimura, E. J. Leonard, J. L. Witztum, and D. Steinberg**. 1991.

Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc. Natl. Acad. Sci. U. S. A **88**:5252-5256.

33. **Smith, J. D., E. Trogan, M. Ginsberg, C. Grigaux, J. Tian, and M. Miyata**. 1995. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. Proc. Natl. Acad. Sci. U. S. A **92**:8264-8268.

34. **Gosling, J., S. Slaymaker, L. Gu, S. Tseng, C. H. Zlot, S. G. Young, B. J. Rollins, and I. F. Charo**. 1999. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J. Clin. Invest **103**:773-778.

35. **Boring, L., J. Gosling, M. Cleary, and I. F. Charo**. 1998. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature **394**:894-897.

36. **Febbraio, M., D. P. Hajjar, and R. L. Silverstein**. 2001. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J. Clin. Invest **108**:785-791.

37. **Suzuki, H., Y. Kurihara, M. Takeya, N. Kamada, M. Kataoka, K. Jishage, O. Ueda, H. Sakaguchi, T. Higashi, T. Suzuki, Y. Takashima, Y. Kawabe, O. Cynshi, Y. Wada, M. Honda, H. Kurihara, H. Aburatani, T. Doi, A. Matsumoto, S. Azuma, T. Noda, Y. Toyoda, H. Itakura, Y. Yazaki, T. Kodama, and .** 1997. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature **386**:292-296.

38. **Nicoletti, A., G. Caligiuri, I. Tornberg, T. Kodama, S. Stemme, and G. K. Hansson**. 1999. The macrophage scavenger receptor type A directs modified proteins to antigen presentation. Eur. J. Immunol. **29**:512-521.

39. **Morre, S. A., W. Stooker, W. K. Lagrand, A. J. van den Brule, and H. W. Niessen**. 2000. Microorganisms in the aetiology of atherosclerosis. J. Clin. Pathol. **53**:647-654.

40. **Miller, Y. I., S. Viriyakosol, C. J. Binder, J. R. Feramisco, T. N. Kirkland, and J. L. Witztum**. 2003. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J. Biol. Chem. **278**:1561-1568.

41. **Xu, X. H., P. K. Shah, E. Faure, O. Equils, L. Thomas, M. C. Fishbein, D. Luthringer, X. P. Xu, T. B. Rajavashisth, J. Yano, S. Kaul, and M. Arditi**. 2001. Toll-like receptor-4 is expressed by macrophages in murine

and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. Circulation **104**:3103-3108.

42. **Kol, A., A. H. Lichtman, R. W. Finberg, P. Libby, and E. A. Kurt-Jones**. 2000. Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. J. Immunol. **164**:13-17.

43. **Curtiss, L. K. and P. S. Tobias**. 2007. The toll of Toll-like receptors, especially toll-like receptor 2, on murine atherosclerosis. Curr. Drug Targets. **8**:1230-1238.

44. **Haury, M., A. Sundblad, A. Grandien, C. Barreau, A. Coutinho, and A. Nobrega**. 1997. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur. J. Immunol. **27**:1557-1563.

45. **Palinski, W., R. K. Tangirala, E. Miller, S. G. Young, and J. L. Witztum**. 1995. Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis. Arterioscler. Thromb. Vasc. Biol. **15**:1569-1576.

46. **Salonen, J. T., S. Yla-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, K. Nyyssonen, W. Palinski, and J. L. Witztum**. 1992. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet **339**:883-887.

47. **Moyer, C. F., D. Sajuthi, H. Tulli, and J. K. Williams**. 1991. Synthesis of IL-1 alpha and IL-1 beta by arterial cells in atherosclerosis. Am. J. Pathol. **138**:951-960.

48. **Barath, P., M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester**. 1990. Detection and localization of tumor necrosis factor in human atheroma. Am. J. Cardiol. **65**:297-302.

49. **de Boer, O. J., A. C. van der Wal, C. E. Verhagen, and A. E. Becker**. 1999. Cytokine secretion profiles of cloned T cells from human aortic atherosclerotic plaques. J. Pathol. **188**:174-179.

50. **Frostegard, J., A. K. Ulfgren, P. Nyberg, U. Hedin, J. Swedenborg, U. Andersson, and G. K. Hansson**. 1999. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis **145**:33-43.

51. **D'Andrea, A., M. ste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri**. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte

interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J. Exp. Med. **178**:1041-1048.

52. **Pinderski, L. J., M. P. Fischbein, G. Subbanagounder, M. C. Fishbein, N. Kubo, H. Cheroutre, L. K. Curtiss, J. A. Berliner, and W. A. Boisvert**. 2002. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. Circ. Res. **90**:1064-1071.

53. **Caligiuri, G., M. Rudling, V. Ollivier, M. P. Jacob, J. B. Michel, G. K. Hansson, and A. Nicoletti**. 2003. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. Mol. Med. **9**:10-17.

54. **Palinski, W., E. Miller, and J. L. Witztum**. 1995. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. Proc. Natl. Acad. Sci. U. S. A **92**:821-825.

55. **Freigang, S., S. Horkko, E. Miller, J. L. Witztum, and W. Palinski**. 1998. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. Arterioscler. Thromb. Vasc. Biol. **18**:1972-1982.

56. **Zhou, X., G. Caligiuri, A. Hamsten, A. K. Lefvert, and G. K. Hansson**. 2001. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. Arterioscler. Thromb. Vasc. Biol. **21**:108-114.

57. **Ameli, S., A. Hultgardh-Nilsson, J. Regnstrom, F. Calara, J. Yano, B. Cercek, P. K. Shah, and J. Nilsson**. 1996. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler. Thromb. Vasc. Biol. **16**:1074-1079.

58. **George, J., A. Afek, B. Gilburd, H. Levkovitz, A. Shaish, I. Goldberg, Y. Kopolovic, G. Wick, Y. Shoenfeld, and D. Harats**. 1998. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. Atherosclerosis **138**:147-152.

59. **Yla-Herttuala, S., W. Palinski, S. W. Butler, S. Picard, D. Steinberg, and J. L. Witztum**. 1994. Rabbit and human atherosclerotic lesions

contain IgG that recognizes epitopes of oxidized LDL. Arterioscler. Thromb. **14**:32-40.

60. **Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum**. 2000. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest **105**:1731-1740.

61. **Horkko, S., C. J. Binder, P. X. Shaw, M. K. Chang, G. Silverman, W. Palinski, and J. L. Witztum**. 2000. Immunological responses to oxidized LDL. Free Radic. Biol. Med. **28**:1771-1779.

62. **Tsimikas, S., W. Palinski, and J. L. Witztum**. 2001. Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized LDL in LDL receptor-deficient mice. Arterioscler. Thromb. Vasc. Biol. **21**:95-100.

63. **Cyrus, T., D. Pratico, L. Zhao, J. L. Witztum, D. J. Rader, J. Rokach, G. A. FitzGerald, and C. D. Funk**. 2001. Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein e-deficient mice. Circulation **103**:2277-2282.

64. **Yla-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg**. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest **84**:1086-1095.

65. **Palinski, W., M. E. Rosenfeld, S. Yla-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum**. 1989. Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. U. S. A **86**:1372-1376.

66. **Palinski, W., V. A. Ord, A. S. Plump, J. L. Breslow, D. Steinberg, and J. L. Witztum**. 1994. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. Arterioscler. Thromb. **14**:605-616.

67. **Gaut, J. P. and J. W. Heinecke**. 2001. Mechanisms for oxidizing low-density lipoprotein. Insights from patterns of oxidation products in the artery wall and from mouse models of atherosclerosis. Trends Cardiovasc. Med. **11**:103-112.

68. **Nicholls, S. J. and S. L. Hazen**. 2005. Myeloperoxidase and cardiovascular disease. Arterioscler. Thromb. Vasc. Biol. **25**:1102-1111.

69. **Esterbauer, H., R. J. Schaur, and H. Zollner**. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. **11**:81-128.

70. **Xu, D., G. M. Thiele, M. L. Kearley, M. D. Haugen, L. W. Klassen, M. F. Sorrell, and D. J. Tuma**. 1997. Epitope characterization of malondialdehyde-acetaldehyde adducts using an enzyme-linked immunosorbent assay. Chem. Res. Toxicol. **10**:978-986.

71. **Horkko, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum**. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J. Clin. Invest **103**:117-128.

72. **Chou, M. Y., K. Hartvigsen, L. F. Hansen, L. Fogelstrand, P. X. Shaw, A. Boullier, C. J. Binder, and J. L. Witztum**. 2008. Oxidation-specific epitopes are important targets of innate immunity. J. Intern. Med. **263**:479-488.

73. **Manz, R. A., A. E. Hauser, F. Hiepe, and A. Radbruch**. 2005. Maintenance of serum antibody levels. Annu. Rev. Immunol. **23**:367-386.

74. **Casali, P. and E. W. Schettino**. 1996. Structure and function of natural antibodies. Curr. Top. Microbiol. Immunol. **210**:167-179.

75. **Thurnheer, M. C., A. W. Zuercher, J. J. Cebra, and N. A. Bos**. 2003. B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. J. Immunol. **170**:4564-4571.

76. **Bos, N. A., C. G. Meeuwsen, H. Hooijkaas, R. Benner, B. S. Wostmann, and J. R. Pleasants**. 1987. Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. Cell Immunol. **105**:235-245.

77. **Boes, M.** 2000. Role of natural and immune IgM antibodies in immune responses. Mol. Immunol. **37**:1141-1149.

78. **Herzenberg, L. A. and A. B. Kantor**. 1993. B-cell lineages exist in the mouse. Immunol. Today **14**:79-83.

79. **Lalor, P. A. and G. Morahan**. 1990. The peritoneal Ly-1 (CD5) B cell repertoire is unique among murine B cell repertoires. Eur. J. Immunol. **20**:485-492.

80. **Hardy, R. R.** 2006. B-1 B cells: development, selection, natural autoantibody and leukemia. Curr. Opin. Immunol. **18**:547-555.

81. **Hardy, R. R.** 2006. B-1 B cell development. J. Immunol. **177**:2749-2754.

82. **Hayakawa, K. and R. R. Hardy**. 2000. Development and function of B-1 cells. Curr. Opin. Immunol. **12**:346-353.

83. **Montecino-Rodriguez, E. and K. Dorshkind**. 2006. New perspectives in B-1 B cell development and function. Trends Immunol. **27**:428-433.

84. **Kantor, A. B. and L. A. Herzenberg**. 1993. Origin of murine B cell lineages. Annu. Rev. Immunol. **11**:501-538.

85. **Berland, R. and H. H. Wortis**. 2002. Origins and functions of B-1 cells with notes on the role of CD5. Annu. Rev. Immunol. **20**:253-300.

86. **Kawahara, T., H. Ohdan, G. Zhao, Y. G. Yang, and M. Sykes**. 2003. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. J. Immunol. **171**:5406-5414.

87. **Wells, S. M., A. B. Kantor, and A. M. Stall**. 1994. CD43 (S7) expression identifies peripheral B cell subsets. J. Immunol. **153**:5503-5515.

88. **Stall, A. M., S. M. Wells, and K. P. Lam**. 1996. B-1 cells: unique origins and functions. Semin. Immunol. **8**:45-59.

89. **Kantor, A. B., A. M. Stall, S. Adams, K. Watanabe, and L. A. Herzenberg**. 1995. De novo development and self-replenishment of B cells. Int. Immunol. **7**:55-68.

90. **Solvason, N., X. Chen, F. Shu, and J. F. Kearney**. 1992. The fetal omentum in mice and humans. A site enriched for precursors of CD5 B cells early in development. Ann. N. Y. Acad. Sci. **651**:10-20.

91. **Gu, H., I. Forster, and K. Rajewsky**. 1990. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J. **9**:2133-2140.

92. **Baumgarth, N., J. W. Tung, and L. A. Herzenberg**. 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol. **26**:347-362.

93. **Casali, P., S. E. Burastero, M. Nakamura, G. Inghirami, and A. L. Notkins**. 1987. Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. Science **236**:77-81.

94. **Hayakawa, K., M. Asano, S. A. Shinton, M. Gui, L. J. Wen, J. Dashoff, and R. R. Hardy**. 2003. Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. J. Exp. Med. **197**:87-99.

95. **Shaw, P. X., C. S. Goodyear, M. K. Chang, J. L. Witztum, and G. J. Silverman**. 2003. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells. J. Immunol. **170**:6151-6157.

96. **Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin**. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. **156**:1177-1185.

97. **Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and G. J. Silverman**. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat. Med. **9**:736-743.

98. **Lutz, H. U., F. Bussolino, R. Flepp, S. Fasler, P. Stammler, M. D. Kazatchkine, and P. Arese**. 1987. Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. Proc. Natl. Acad. Sci. U. S. A **84**:7368-7372.

99. **Kim, S. J., D. Gershov, X. Ma, N. Brot, and K. B. Elkon**. 2002. I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. J. Exp. Med. **196**:655-665.

100. **Boes, M., T. Schmidt, K. Linkemann, B. C. Beaudette, A. Marshak-Rothstein, and J. Chen**. 2000. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. Proc. Natl. Acad. Sci. U. S. A **97**:1184-1189.

101. **Ehrenstein, M. R., H. T. Cook, and M. S. Neuberger**. 2000. Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. J. Exp. Med. **191**:1253-1258.

102. **Bendelac, A., M. Bonneville, and J. F. Kearney**. 2001. Autoreactivity by design: innate B and T lymphocytes. Nat. Rev. Immunol. **1**:177-186.

103. **Manson, J. J., C. Mauri, and M. R. Ehrenstein**. 2005. Natural serum IgM maintains immunological homeostasis and prevents autoimmunity. Springer Semin. Immunopathol. **26**:425-432.

104. **Kagan, V. E., G. G. Borisenko, Y. Y. Tyurina, V. A. Tyurin, J. Jiang, A. I. Potapovich, V. Kini, A. A. Amoscato, and Y. Fujii**. 2004. Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. Free Radic. Biol. Med. **37**:1963-1985.

105. **Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum**. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J. Exp. Med. **200**:1359-1370.

106. **Tuominen, A., Y. I. Miller, L. F. Hansen, Y. A. Kesaniemi, J. L. Witztum, and S. Horkko**. 2006. A natural antibody to oxidized cardiolipin binds to oxidized low-density lipoprotein, apoptotic cells, and atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. **26**:2096-2102.

107. **Huber, J., A. Vales, G. Mitulovic, M. Blumer, R. Schmid, J. L. Witztum, B. R. Binder, and N. Leitinger**. 2002. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Arterioscler. Thromb. Vasc. Biol. **22**:101-107.

108. **Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum**. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc. Natl. Acad. Sci. U. S. A **96**:6353-6358.

109. **Ogden, C. A., R. Kowalewski, Y. Peng, V. Montenegro, and K. B. Elkon**. 2005. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. Autoimmunity **38**:259-264.

110. **Sambrano, G. R. and D. Steinberg**. 1995. Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. Proc. Natl. Acad. Sci. U. S. A **92**:1396-1400.

111. **Dei, R., A. Takeda, H. Niwa, M. Li, Y. Nakagomi, M. Watanabe, T. Inagaki, Y. Washimi, Y. Yasuda, K. Horie, T. Miyata, and G. Sobue**. 2002. Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease. Acta Neuropathol. (Berl) **104**:113-122.

112. **Imai, Y., K. Kuba, G. G. Neely, R. Yaghubian-Malhami, T. Perkmann, L. G. van, M. Ermolaeva, R. Veldhuizen, Y. H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J. S. Peiris, A. S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C. J. Binder, and J. M. Penninger**. 2008. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell **133**:235-249.

113. **Niemela, O., S. Parkkila, S. Yla-Herttuala, C. Halsted, J. L. Witztum, A. Lanca, and Y. Israel**. 1994. Covalent protein adducts in the liver as a result of ethanol metabolism and lipid peroxidation. Lab Invest **70**:537-546.

114. **Castelnau, P. A., R. S. Garrett, W. Palinski, J. L. Witztum, I. L. Campbell, and H. C. Powell**. 1998. Abnormal iron deposition associated with lipid peroxidation in transgenic mice expressing interleukin-6 in the brain. J. Neuropathol. Exp. Neurol. **57**:268-282.

115. **Houglum, K., G. A. Ramm, D. H. Crawford, J. L. Witztum, L. W. Powell, and M. Chojkier**. 1997. Excess iron induces hepatic oxidative stress and transforming growth factor beta1 in genetic hemochromatosis. Hepatology **26**:605-610.

116. **Exner, M., M. Susani, J. L. Witztum, A. Hovorka, L. K. Curtiss, S. Spitzauer, and D. Kerjaschki**. 1996. Lipoproteins accumulate in immune deposits and are modified by lipid peroxidation in passive Heymann nephritis. Am. J. Pathol. **149**:1313-1320.

117. **Neale, T. J., P. P. Ojha, M. Exner, H. Poczewski, B. Ruger, J. L. Witztum, P. Davis, and D. Kerjaschki**. 1994. Proteinuria in passive Heymann nephritis is associated with lipid peroxidation and formation of adducts on type IV collagen. J. Clin. Invest **94**:1577-1584.

118. **Horie, K., T. Miyata, K. Maeda, S. Miyata, S. Sugiyama, H. Sakai, S. C. van Ypersole de, V. M. Monnier, J. L. Witztum, and K. Kurokawa**. 1997. Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. J. Clin. Invest **100**:2995-3004.

119. **Palinski, W., T. Koschinsky, S. W. Butler, E. Miller, H. Vlassara, A. Cerami, and J. L. Witztum**. 1995. Immunological evidence for the presence of advanced glycosylation end products in atherosclerotic lesions of euglycemic rabbits. Arterioscler. Thromb. Vasc. Biol. **15**:571-582.

120. **Rosenfeld, M. E., W. Palinski, S. Yla-Herttuala, S. Butler, and J. L. Witztum**. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. Arteriosclerosis **10**:336-349.

121. **Palinski, W., S. Yla-Herttuala, M. E. Rosenfeld, S. W. Butler, S. A. Socher, S. Parthasarathy, L. K. Curtiss, and J. L. Witztum**. 1990. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis **10**:325-335.

122. **Boullier, A., P. Friedman, R. Harkewicz, K. Hartvigsen, S. R. Green, F. Almazan, E. A. Dennis, D. Steinberg, J. L. Witztum, and O. Quehenberger**. 2005. Phosphocholine as a pattern recognition ligand for CD36. J. Lipid Res. **46**:969-976.

123. **Chang, M. K., C. J. Binder, M. Torzewski, and J. L. Witztum**. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. Proc. Natl. Acad. Sci. U. S. A **99**:13043-13048.

124. **Gillotte-Taylor, K., A. Boullier, J. L. Witztum, D. Steinberg, and O. Quehenberger**. 2001. Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. J. Lipid Res. **42**:1474-1482.

125. **Hayakawa, K., M. Asano, S. A. Shinton, M. Gui, D. Allman, C. L. Stewart, J. Silver, and R. R. Hardy**. 1999. Positive selection of natural autoreactive B cells. Science **285**:113-116.

126. **Quartier, P., P. K. Potter, M. R. Ehrenstein, M. J. Walport, and M. Botto**. 2005. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. Eur. J. Immunol. **35**:252-260.

127. **Witztum, J. L. and D. Steinberg**. 2001. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? Trends Cardiovasc. Med. **11**:93-102.

128. **Merbl, Y., M. Zucker-Toledano, F. J. Quintana, and I. R. Cohen**. 2007. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. J. Clin. Invest **117**:712-718.

# CHAPTER 2

## CHARACTERIZATION OF MURINE HUMORAL RESPONSES TO OXIDATION-SPECIFIC EPITOPES

**Abstract**

Natural antibodies (NAbs) are predominantly IgMs produced by B-1 cells. They are germ-line encoded and spontaneously arise without any exogenous antigen exposure. Thus, the repertoire of B-1 cell derived NAbs are selected to bind to evolutionary important epitopes. Oxidation-specific epitopes generated in vivo under oxidative stress, which are common to many pathological conditions, might constitute one such class of epitopes that can be recognized by NAbs. As an initial step to demonstrate that oxidation-specific epitopes might be a major target for NAbs, I showed that IgM Abs to oxidation-specific epitopes were present in conventionally raised mice as well as in mice raised under specific pathogen free (SPF) conditions. The IgM responses to malondialdehyde (MDA)-modifications were the most robust, many fold higher than to the prototypic B-1 cell antigen α1,3-Dextran. Moreover, IgM responses to oxidation-specific epitopes were also found in germ-free mice, grown under strict gnotobiotic conditions, which entirely lacked exogenous pathogen exposure. The above evidence strongly supported my hypothesis that these oxidation-specific IgMs were most likely B-1 cell derived NAbs.

## Introduction

### Most plasma IgM are natural antibodies of B-1 origin.

NAbs are part of the humoral arc of innate immunity. They are the product of natural selection, exhibiting a genetically determined and stable repertoire that is largely independent of external antigenic stimuli (1-3). NAbs have been defined in various ways, for example, as the IgM present in normal plasma (4), but more stringently, as germ-line encoded Abs that arise without any exogenous immune exposure or bacterial colonization of the gut (5). They are produced at tightly regulated and stable levels in healthy individuals, and are found in all vertebrate species (6,7). In normal, uninfected mice, most plasma IgM are NAbs that are derived from innate B-1 cells. Therefore, by characterizing the IgM population in various mouse models, we can gain insight into how the NAb population might respond to oxidation-specific epitopes.

Although the mechanisms leading to selection and expansion of B-1 cells are unclear, it is now appreciated that antigen selection during fetal and neonatal period leads to positive selection (6,8,9). Since this appears to occur equally well in mice raised in germ-free environments (1,10,11), this selection is of necessity made by endogenous “self-antigens”. Thus, the repertoire of B-1 cells is selected to bind to evolutionarily important epitopes. This is in contrast to developing, self-reactive B-2 cells, in which such antigen encounter early in life leads to negative selection via apoptosis (clonal deletion), or anergy. Oxidation-specific epitopes have been shown to be present not only in the context of atherosclerosis but also

on apoptotic cells and under inflammatory conditions in general. Therefore, I hypothesize that oxidation-specific epitopes might constitute one such class of evolutionarily important epitopes that B-1 cell derived NAbs are selected to recognize.

**T-cell independent antigens and oxidation-specific antigens**

Antigens recognized by B-1 cells include phosphocholine (12), dextran (13) and common bacterial carbohydrate antigens (14). These are all T-cell independent type 2 (TI-2) antigens, which are antigens that stimulate antibody production in the absence of MHC class II-restricted T cell help. TI antigens fall into two major categories: T-cell independent type 1 (TI-1) and TI-2 antigens. TI-2 antigens are usually polysaccharide antigens that are large molecular weight and have repeating antigenic epitopes. $\alpha$1,3-dextran is an example of a prototypic TI-2 antigen recognized by B-1 cells that will be used throughout the study.

TI-2 antigens have the ability to activate the complement cascade and have poor *in vivo* degradability. TI-1 antigens, such as lipopolysaccharide (LPS), have the characteristic of being mitogenic for B cells (15). A third class, T cell-dependent antigens (TD), consists of soluble proteins or peptides. These antigens can associate with MHC molecules at the surface of an antigen presenting cell (APC), allowing the APC to interact with T cells to generate an immune response.

Although B-1 cells were thought to play a major role in the response to TI-2 antigens, they do not respond to some TI-2 antigens such as trinitrophenyl (TNP), or nitrophenyl (NP) presented on Ficoll (16). B-1 cells can also respond to many TD antigens. Therefore, while the B-1 cells show selectivity to the antigenic determinants to which they respond, it's not strictly along the lines of TI vs TD.

To study immune responses to oxidation-specific epitopes, several oxidation-related antigens were used as model antigens. The formation and possible epitopes on MDA-LDL and Cu-OxLDL were described in Chapter 1. 4-HNE modified mouse serum albumin (4-HNE-MSA) and PC modified bovine serum albumin (PC-BSA) were also included as model antigens of oxidation. 4-HNE modified LDL occur in the atheroma of hyperlipidemic rabbits and autoantibodies against HNE-modified proteins exist in plasma of rabbits and humans (17,18). Aside from such endogenously formed aldehyde lipid peroxidation products, such as MDA, HNE, or others to modify proteins and cause them to become immunogenic, as demonstrated in the case of atherosclerosis (19-21), it is also possible that they can react with DNA bases and induce mutations as well (22). Many genotoxic substances, including carcinogens, are metabolically activated through free radical reactions and can induce lipid peroxidation (23,24). The lipid peroxidation products formed in this process could inhibit certain DNA repair systems (25) and thus indirectly increase the rate of spontaneous mutation. Therefore, these oxidation modifications of proteins generated *in vivo* are of pathophysiological relevance. One hypothesis for the natural selection of NAbs against oxidation-specific epitopes is to help

clear these otherwise immunogenic and cytotoxic lipid peroxidation products. In addition, many of these oxidized lipids are highly proinflammatory, which would provide yet another powerful selecting pressure.

**Rationale and study design**

As an initial step to test the hypothesis that oxidation-specific epitopes are a major target of innate NAbs, it seemed reasonable to first characterize murine humoral IgM responses to oxidation-related antigens in mice. IgM immune responses to oxidation-related antigens have previously been demonstrated in wild-type C57BL/6 mice and shown to be increased in atherosclerotic apo E deficient mice (26).

In order to demonstrate that these IgM responses to oxidation-related antigens are indeed of NAb origin, IgM responses in specific pathogen free (SPF) mice were assessed to determine whether the development of these IgM responses depend on the exposure to certain pathogens. Moreover, germ-free Swiss Webster mice that were maintained under strict gnotobiotic conditions were also tested as these mice have had no exogenous antigen exposure. Titers to oxidation-specific mice should be observed in both the SPF and germ-free mice if such IgMs to oxidation-specific antigens were true NAbs.

T-cell receptor $\alpha$ deficient (TCR $\alpha^{-/-}$) mice were also tested to assess the impact of T-cell help on the development of oxidation-specific IgM titers. LDL receptor deficient ($Ldlr^{-/-}$) mice have higher cholesterol levels compared to wild-

type mice even when fed a regular chow diet. $Ldlr^{-/-}$ mice were also tested to assess how mild hypercholesterolemia would affect the IgM population to oxidation-specific epitopes.

Aside from oxidation-related antigens, IgM titers to the prototypic B-1 cell antigen $\alpha$1,3-dextran were also tested as a reference to compare to an antigen known to stimulate natural IgM production.

The main focus of this chapter is to characterize humoral IgM responses to oxidation-specific epitopes in various mouse models. The characterization of IgM responses to oxidation-specific epitopes in SPF and germ-free mouse models, and in hypercholesterolemic mice, is an essential initial study to support my hypothesis. Characterization of IgM responses to oxidation-specific epitopes in these relevant murine models will provide useful information on how natural IgM responses are affected by these perturbations. IgG responses were also tested in these mouse models.

## Results

### IgM antibodies against oxidation-specific epitopes are present in normal mice.

To characterize the murine humoral IgM responses to defined oxidation-specific epitopes, I assessed specific IgM titers in plasma of naïve, non-atherosclerotic C57BL/6 mice. As previously observed (26), prominent IgM titers to oxidation-epitopes, such as OxPL, MDA, 4-HNE, and PC, can be detected even in normal, conventionally housed mice, whereas IgM titers to "native LDL" are minimal or undetectable (**Fig. 2-1**) (see Chapter 3 on apparent low-titered binding to native LDL). Among these, the IgM responses to MDA-modifications were consistently found to be the most robust, and the titers were many fold higher than those to the prototypic B-1 cell antigen α1,3-Dextran. In addition, when comparing the plasma IgM titers to those in age-matched mice bred under SPF conditions, a similar response pattern was observed (**Fig.2-1, upper panel**), suggesting that the basal titers of these IgM Abs are largely independent of non-commensal exposure to microbial pathogens.

Moreover, I found that oxidation-specific IgM levels were also present in T-cell deficient mice (TCR $\alpha^{-/-}$) - although slightly lower - indicating that in large part these responses do not require T-cells (**Fig.2-1, lower panel**). The $Ldlr^{-/-}$ mice, when put on a high-fat, high-cholesterol atherogenic diet, show increased IgM titers to epitopes of OxLDL with increased atherosclerosis (27). In my studies,

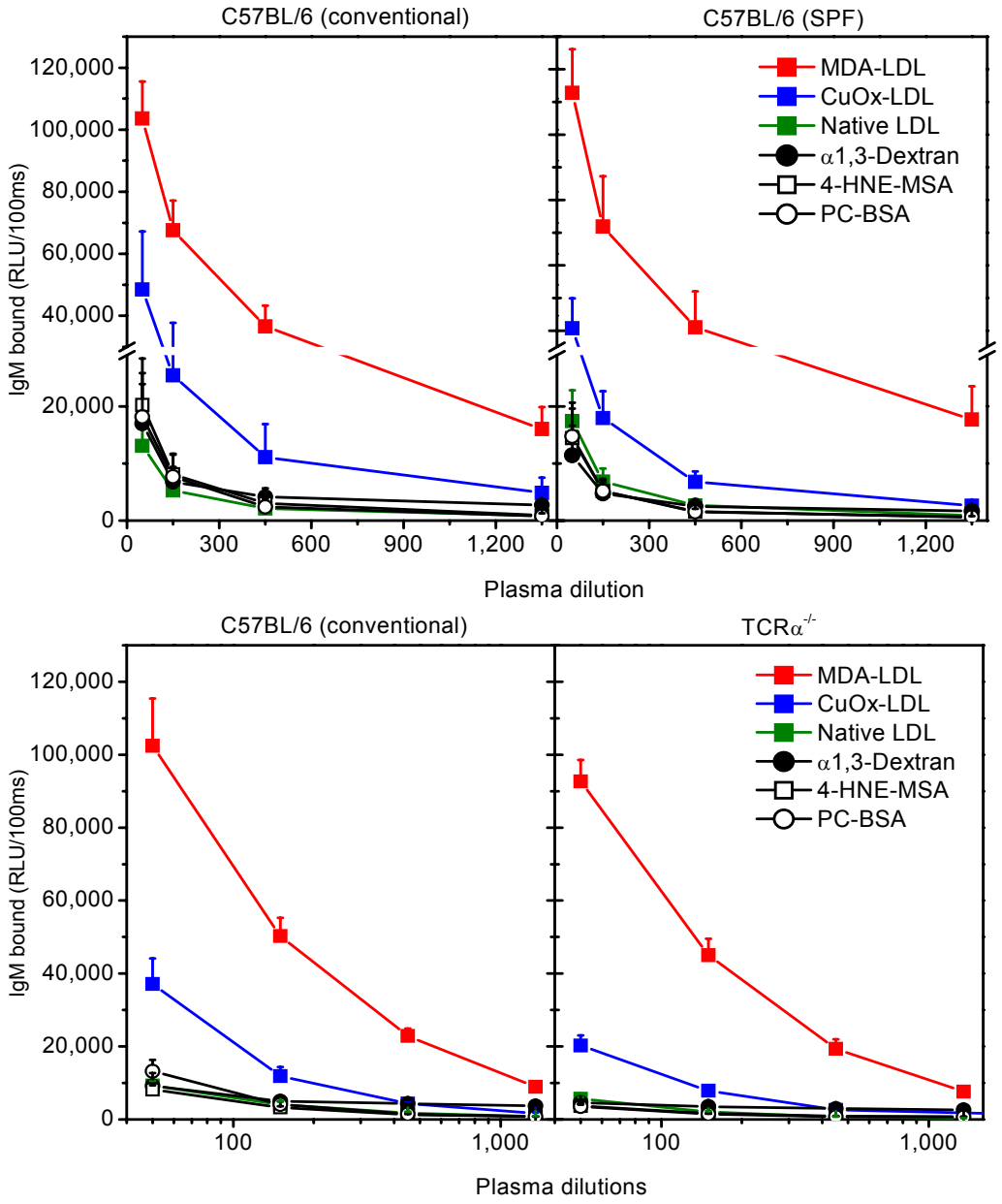



**Fig.2-1 IgM titers to oxidation-specific antigens in conventional, SPF, and TCRα$^{-/-}$ mice.** C57BL/6 SPF and TcRα$^{-/-}$ mice both have similar IgM titers to oxidation-specific antigens compared to age-matched wild type C57BL/6 mice. IgM binding to indicated antigens were tested by ELISA. Plasma from 11 or 6 weeks old female conventionally raised C57BL/6 mice (n=4 for each age group), were compared to female age-matched SPF (n=4, 11 weeks old) and TcRα$^{-/-}$ mice (n=4, 6 weeks old). Values are means and SEM.

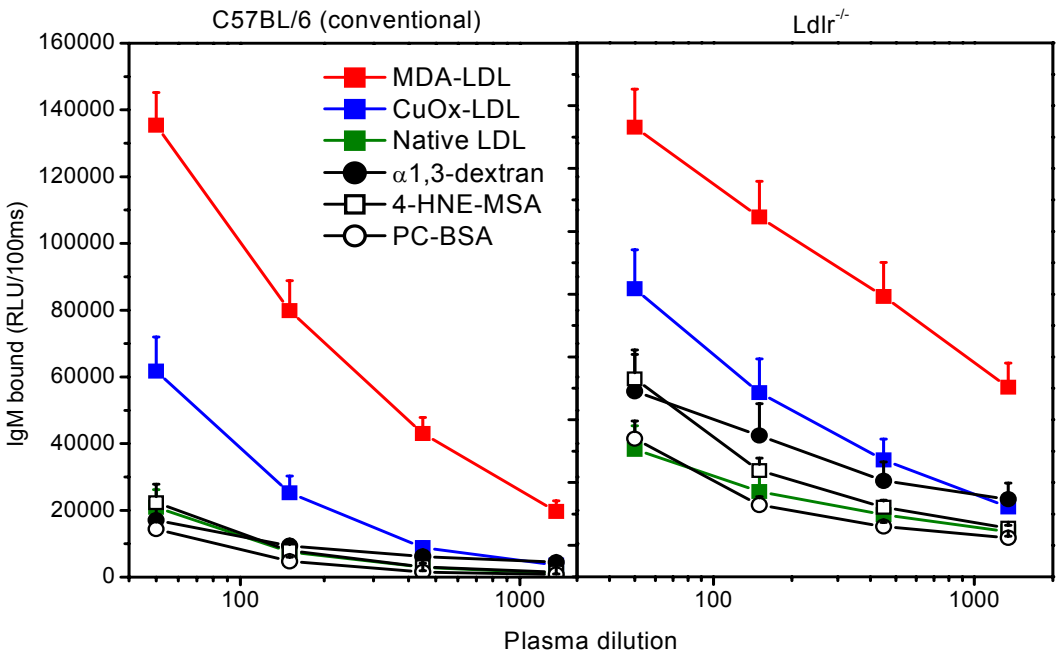



**Fig.2-2 IgM titers to oxidation-specific antigens in conventional and Ldlr$^{-/-}$ mice.** Ldlr$^{-/-}$ mice develop higher titers of IgM to indicated oxidation-specific antigen over time compared to conventional C57BL/6 mice. Plasma from 7 month old female conventionally raised (n=4) and Ldlr$^{-/-}$ (n=4), both in C57BL/6 background, were tested by ELISA. Values are means and SEM.

I examined plasma from Ldlr$^{-/-}$ on a regular chow diet. The Ldlr$^{-/-}$ mice on a regular show diet showed slightly higher IgM titers to oxidation-specific antigens over a 7 month period compared to age-matched conventionally housed C57BL/6 mice (**Fig.2-2**). This observation supports previous report that IgM responses to oxidation-specific epitopes are expanded with increased atherosclerosis (26).

In mice, IgMs are in large part derived from antibody secreting cells in the spleen (28). The frequencies of IgM secreting cells (ISC) against candidate

oxidation-specific epitopes in the spleens of conventionally housed C57BL/6 mice were tested with enzyme-linked immunosorbent spot (ELISpot) assay. This assay is much less sensitive to small differences, but nevertheless, IgM Secreting Cells (ISCs) with specificity for MDA-LDL were much higher, reflective of the IgM titers observed in plasma. ISCs to other oxidation-specific epitopes were marginally higher than to the PBS control, but were not statistically different (**Fig. 2-3**).

**IgM antibodies against oxidation-specific epitopes are present in germ-free mice**

Previous data suggest that IgM titers to an array of oxidation-specific epitopes may in fact represent IgM generated even in the absence of response-eliciting antigen-exposure. In confirmation of this, robust IgM titers to oxidation-specific epitopes were observed in the serum of “germ-free mice”, which were raised in strict gnotobiotic conditions, and were shown by DNA analysis to be completely free of gut bacteria (**Fig.2-4, A.**). In particular, IgM titers to MDA-LDL and MAA-BSA were the most prominent among all IgM titers measured. MAA (malondialdehyde-acetaldehyde-adducts) is a specific and prominent chemical moiety generated when lysines are modified with MDA; in this case conjugated to BSA. CuOx-LDL can also be detected in the germ-free mice, while 4-HNE-LDL and PC-BSA levels were low in the germ-free mice.

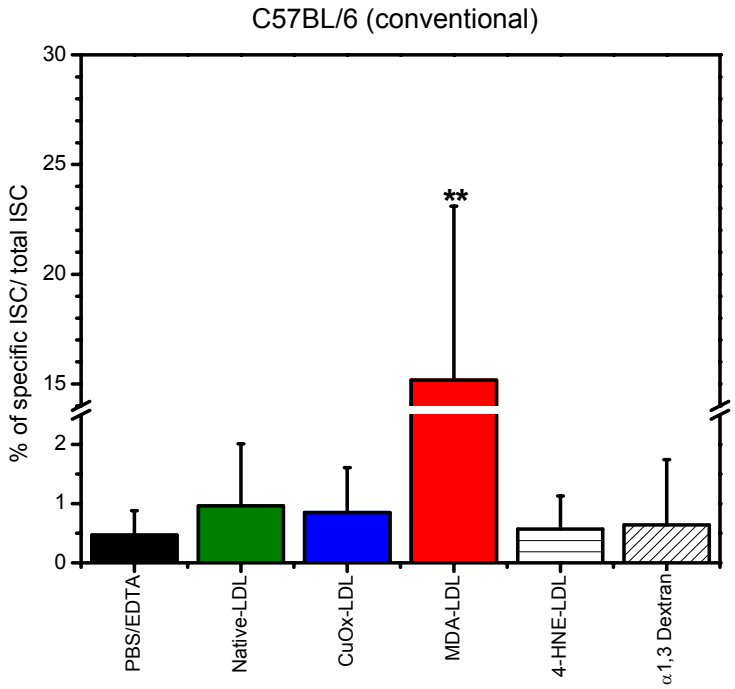



**Fig.2-3 MDA-LDL specific IgM secreting cells are dominant in the spleens of conventionally raised C57BL/6 mice.** Splenocytes from conventionally raised 12 week old female C57BL/6 mice (n=4) were tested by ELISpot assay for frequencies of IgM-secreting cells (ISCs) as described in Methods. Values represent the number of ISCs to the indicated antigen as a percent of total ISCs (mean and SD). The experiment shown is one representative of 3 individual experiments. ** $p<0.01$, compared to all other antigens. (One-way ANOVA with Tukey-Kramer Multiple Comparison Test)

Furthermore, compared to the germ-free state, reconstitution of germ-free mice with gut bacteria ("conventionalized mice") for only two weeks led to increases in IgMs to oxidation-specific antigens such as CuOxLDL, 4-HNE-LDL, PC-BSA as well as B-1 prototypic antigen $\alpha$1,3-Dextran. The responses to MDA-LDL and MAA-BSA however, remained similar between germ-free and

conventionalized mice (**Fig.2-4, B.** and **Fig.2-5**). Thus, commensal bacteria might be able to induce the expansion of IgM responses to certain oxidation-specific epitopes.

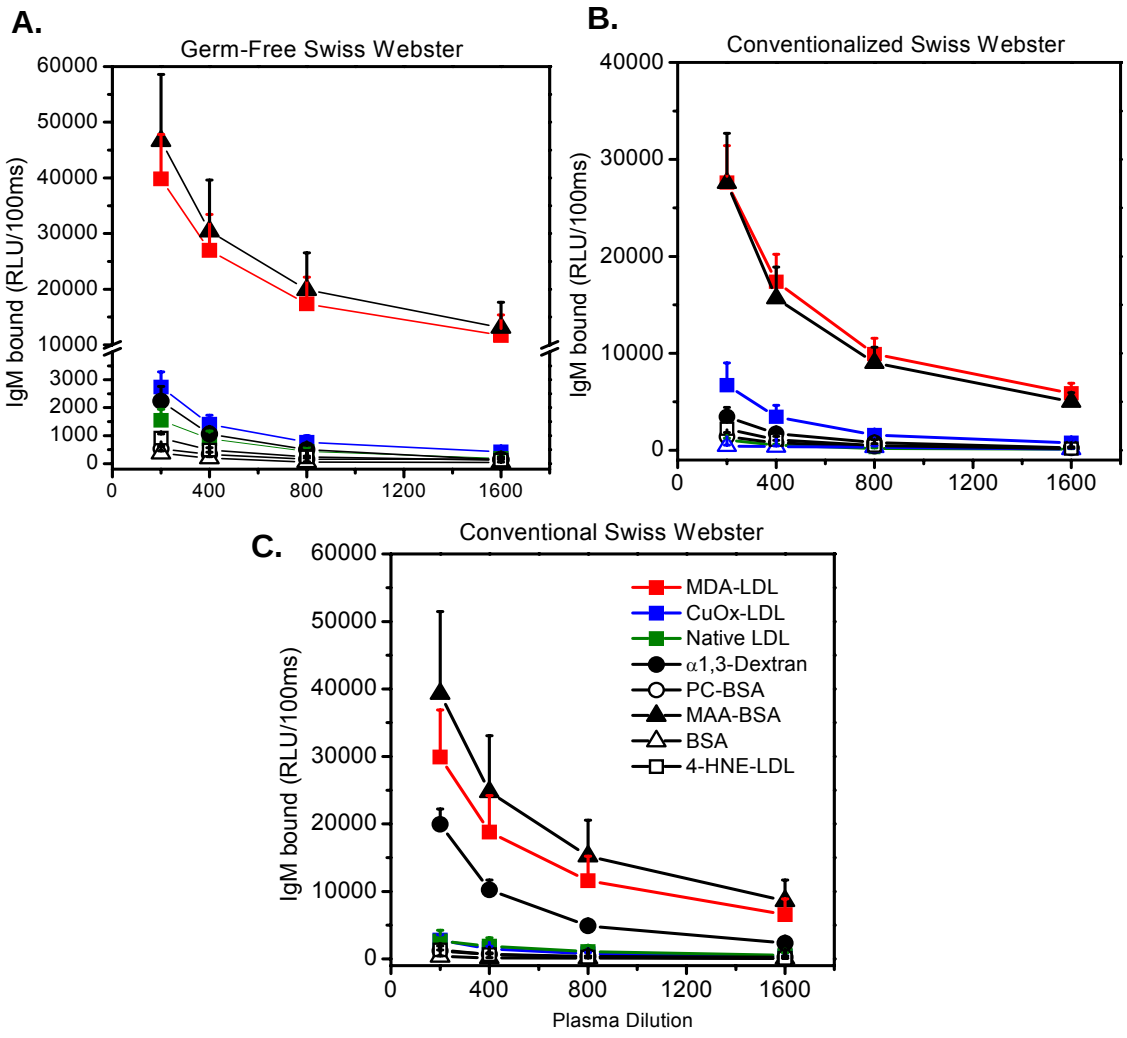



**Fig2-4 Antibody dilution binding curves of plasma IgM from germ free (A.), conventionalized (B.), and conventional (C.) Swiss-Webster mice to indicated antigens.** Plasma samples were from 14-16 week old female and male mice (n=7 for conventional, n=11 for conventionalized, n=9 for germ-free). Values are means and SEM.

Moreover, similar specific IgM responses were measured in age-matched conventionally raised mice of the same genetic background (**Fig.2-4, C.** and **Fig.2-5**). Together the above data showed that IgMs specific for oxidation-specific epitopes are present in germ-free mice, supporting their NAb origin (**Fig.2-5**).

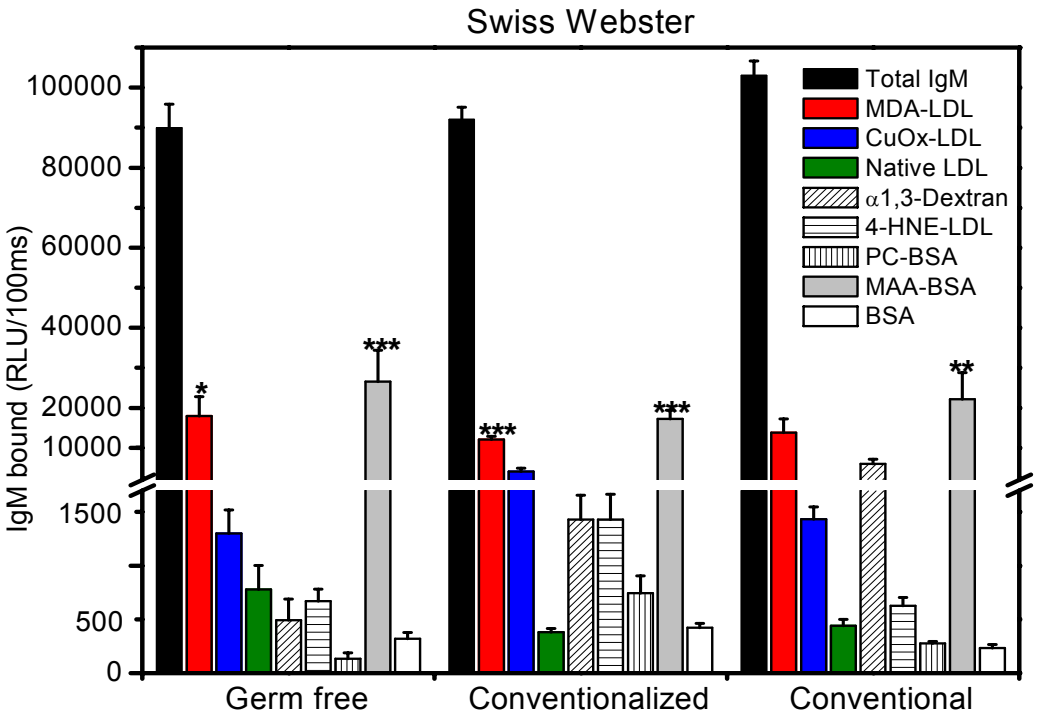



**Fig.2-5 Titers of IgM Abs to oxidation-specific epitopes are present in conventional and germ-free Swiss Webster mice.** Plasma from 14-16 week old female and male conventionally raised (n=7), conventionalized (germ-free colonized with bacterial flora) (n=11), and germ-free (n=9) mice were diluted 1:400 and tested for binding to the indicated antigens. Values are means and SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.002$ compared to α1,3-Dextran. (One-way ANOVA with Tukey-Kramer Multiple Comparison Test).

**IgG antibodies against oxidation-specific epitopes in normal mice**

IgG responses to oxidation-specific epitopes were also measured in these murine models. In conventionally housed C57BL/6 mice, IgG response to MDA-LDL and α1,3-dextran were found to be the most dominant (**Fig.2-6**). IgG responses to OxPL, 4-HNE, and PC can also be detected but to a much lower extent. IgG titers to native LDL were not detected. A similar response pattern was observed in age-matched C57BL/6 mice bred under SPF conditions (**Fig2-6, upper panel**). In TCR $\alpha^{-/-}$ mice, IgG responses were diminished as expected due to the lack of T cells, except for the weak IgG response to T-cell independent antigen α1,3-dextran (**Fig.2-6, lower panel**).

IgG responses to oxidation-specific antigens in $Ldlr^{-/-}$ mice on a regular chow diet followed a similar pattern as age-matched conventionally housed C57BL/6 mice except for α1,3-Dextran (**Fig.2-7**). IgG titers to α1,3-Dextran were higher in $Ldlr^{-/-}$ mice compared to age-matched conventionally housed wild-type mice. On the other hand, IgG responses to oxidation-specific epitopes were not elevated in $Ldlr^{-/-}$ mice compared to conventionally housed wild-type mice.

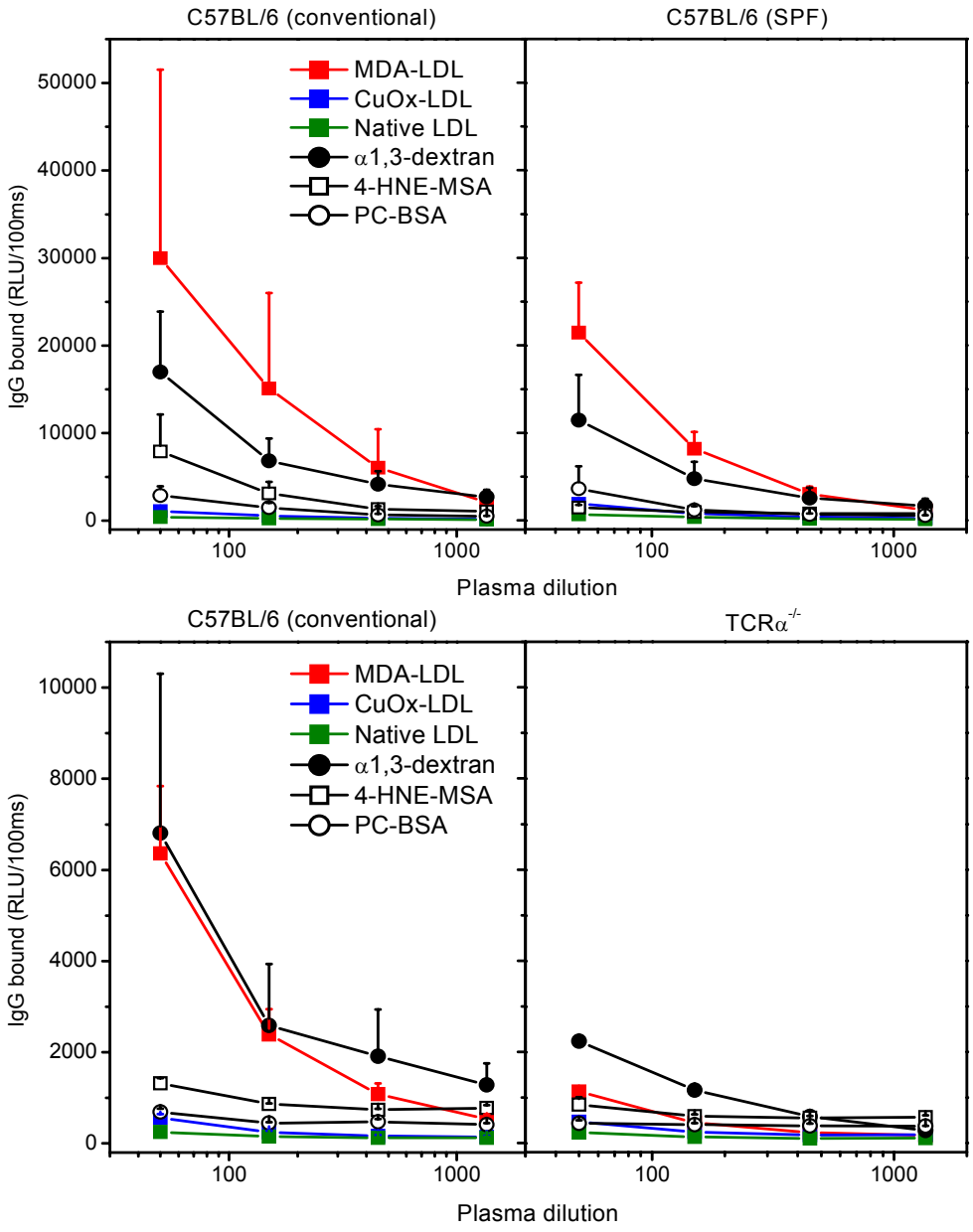



**Fig.2-6 IgG titers to oxidation-specific antigens in conventional, SPF, and TcRα$^{-/-}$ mice.** Conventional and SPF C57BL/6 mice both have IgG titers to oxidation-specific antigens while TcRα$^{-/-}$ mice did not. IgG binding to indicated antigens were tested by ELISA. Plasma from 11 or 6 weeks old female conventionally raised C57BL/6 mice (n=4 for each age group), were compared to female age-matched SPF (n=4, 11 weeks old) and TcRα$^{-/-}$ C57BL/6 mice (n=4, 6 weeks old). Values are means and SEM.

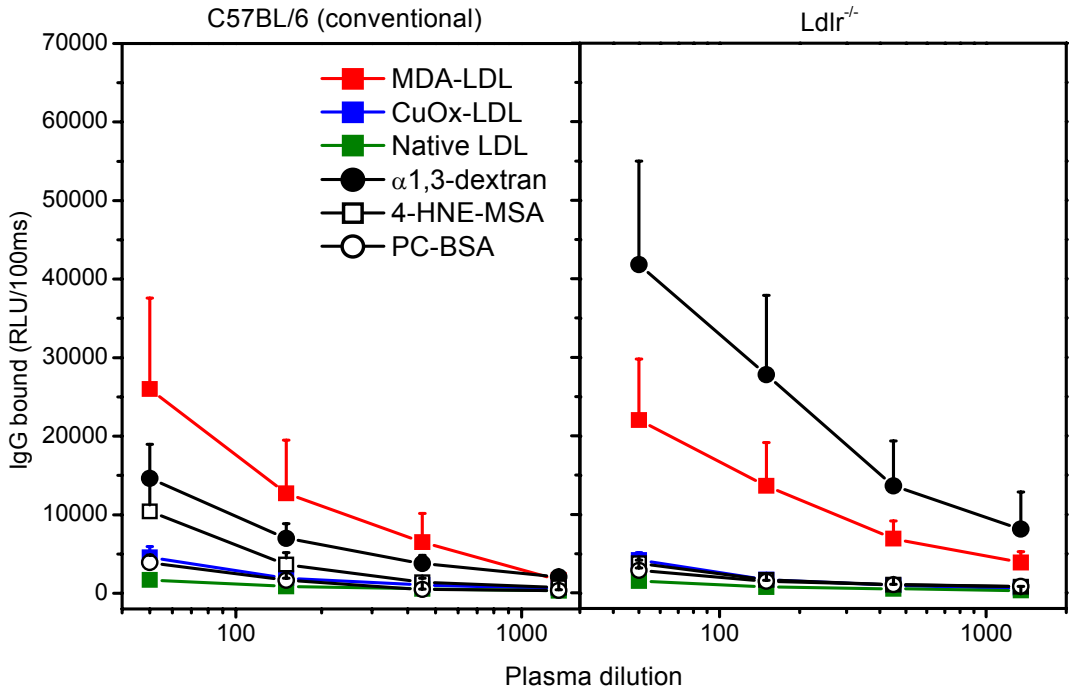



**Fig.2-7 IgG titers to oxidation-specific antigens in conventional and Ldlr$^{-/-}$ mice.** Ldlr$^{-/-}$ mice had similar titers of IgG to indicated oxidation-specific antigen compared to conventional C57BL/6 mice except for α1,3-dextran. Plasma from 7 month old female conventionally raised (n=4) and Ldlr$^{-/-}$ (n=4) mice, both in C57BL/6 background, were tested by ELISA. Values are means and SEM.

## Discussion

Consistent with the view that the IgM pool of uninfected mice in large part represent NAbs (6), my results show that mice maintained in SPF conditions showed a similar response to oxidation-specific epitopes compared to conventionally raised mice in the same genetic background. This suggests that the basal titers of these IgMs are largely independent of exposure to non-commensal bacteria.

Moreover, the IgM titers to oxidation-specific epitopes were higher compared to titers against a recognized TI-2 antigen, $\alpha$1,3-dextran, with IgM titers to MDA-LDL being the most robust in all mouse models tested. This supports my hypothesis that oxidation-specific epitopes might be a major target for NAbs.

Consistent with the concept that IgM in uninfected mice are in large part NAbs secreted from the spleen, there is a high frequency of ISCs to oxidation-specific epitopes, chiefly MDA, in the spleens of wildtype mice. This presents another piece of evidence demonstrating that oxidation-specific epitopes might be a dominant target for B-1 cell derived natural IgM Abs.

Furthermore, fulfilling classic criteria of NAbs, IgMs to oxidation-related antigens were present in relatively high titer even in germ-free mice maintained under strict gnotobiotic conditions, demonstrating again the NAb origin of these oxidation-specific IgMs. In germ-free mice, there were only minimal IgM titers

against α1,3-dextran. The introduction of normal gut flora into the germ-free mice (conventionalized mice in **Fig.2-4** and **Fig.2-5**) increased the titers of certain oxidation-specific IgM, including those directed to OxLDL and 4-HNE LDL, but not to MDA-LDL. Thus, differences exist between the different oxidation-specific IgM and some, but not all can be stimulated by commensal bacteria.

In mice, NAbs are mainly secreted by B-1 cells, which for the most part secrete these Abs in the absence of antigenic stimulation, though they do respond to other non-cognate stimuli. Indeed, many B-1 cells are thought to typically be unresponsive to expansion by specific antigenic stimuli. However, some antigens, such as PC and α1,3-dextran, have been shown to stimulate antigen-specific NAb production by B-1 cells (6). Thus, the increase in the conventionalized mice of IgM to PC-BSA and OxLDL, in which PC of OxPL is a dominant epitope (29), may be reflective of such exogenous antigenic stimulation.

Additional evidence that an encounter later in life with antigens could further serve to expand a given B-1 cell clone (30,31) is shown with the example of $Ldlr^{-/-}$ mice. In this case the $Ldlr^{-/-}$ mice, which had a higher level of plasma cholesterol and higher degree of atherosclerosis compared to wild-type mice, also showed higher IgM titers towards oxidation-related antigens.

As noted in the background, NAb production by B-1 cells is thymus independent. Consistent with this, I also found that $TcR\alpha^{-/-}$ mice showed similar IgM responses to oxidation-specific epitopes compared to age-matched

conventionally raised mice, indicating that in large part these IgM responses do not require cognate T-cell help.

Circulating IgG autoantibodies to OxLDL have previously been described in mouse models of atherosclerosis such as $Ldlr^{-/-}$ and $ApoE^{-/-}$ mice that were put on a high fat diet (27,32). However, IgG responses to oxidation-specific epitopes in naïve, non-atherosclerotic mice have not been carefully studied. In the conventionally raised wild-type C57BL/6 mice, IgG titers to oxidation-specific antigens are present, IgG response to MDA-LDL being the most robust. However, IgG titers to MDA-LDL were not significantly higher compared to the B-1 cell prototypic antigen $\alpha$1,3-Dextran. IgG titers to other oxidation-related antigens were even lower than the IgG titer to $\alpha$1,3-Dextran. A similar pattern was observed in SPF mice. These results suggested that even though oxidation-specific epitopes might be a dominant target for IgM NAbs, it is probably necessary to have a strong antigen exposure to induce high titers of adaptive IgG Abs to these oxidation epitopes.

In $TcR\alpha^{-/-}$ mice, IgG responses to all oxidation-specific epitopes were diminished except for the TI-2 antigen $\alpha$1,3-dextran. This suggests that the IgG responses towards oxidation-specific epitopes are completely T-cell dependent adaptive response. IgG titers to $\alpha$1,3-dextran have previously been reported to be primarily $IgG_3$ responses (33). Because the assay for total IgG used in these particular studies did not distinguish between the different subclasses of IgG Abs,

this could be $IgG_3$ responses against $\alpha$1,3-dextran. $IgG_3$ is another major isotype of NAbs secreted by B-1 cells.

On the other hand, IgG titers to oxidation-related antigens were not found to be expanded in the chow fed $Ldlr^{-/-}$ mice like the IgM responses, suggesting an adaptive immune response was not elicited toward oxidation-specific antigens in $Ldlr^{-/-}$ mice on a regular chow diet. We do know from multiple experiments however, that with high-fat, high-cholesterol diets and the development of atherosclerosis, that IgG titers to these epitopes are greatly increased (27,32,34).

In conclusion, strong IgM responses to oxidation-related antigens can be found in conventionally raised and SPF C57BL/6 mice as well as Swiss Webster mice. There is also a high frequency of ISCs specific for MDA-LDL in the spleens of wildtype C57BL/6 mice. Since IgMs are thought to represent NAbs in uninfected mice, the above evidence supports the hypothesis that oxidation-specific epitopes might be a dominant target for NAbs. The presence of IgM titers against oxidation-specific epitopes in germ-free mice further supports the hypothesis. Although these studies describe in a qualitative sense the prevelence of high titers of IgM to oxidation-specific epitopes, they do not directly indicate the extent to which these represent dominant targets. The next step would be to confirm the hypothesis in pure B-1 cell derived NAb populations, in both *in vitro* and *in vivo* studies, in which the relative extent of IgM to oxidation-specific epitopes are compared to total IgM generated. Such studies will be described in Chapters 3 and 4.

**Acknowledgement**

Chapters 2, in part, has been accepted for publication in “Oxidation-specific Epitopes are a Dominant Target for Innate Natural Antibodies”, Journal of Clinical Investigation, 2009, by MY Chou, L. Fogelstrand, K. Hartvigsen, LF Hansen, D. Woelkers, PX Shaw, J. Choi, T. Perkmann, F Bäckhed, YI Miller, S. Hörkkö, M. Corr, JL Witztum and CJ Binder. The dissertation author was the primary investigator and author of this paper.

## References


1. **Haury, M., A. Sundblad, A. Grandien, C. Barreau, A. Coutinho, and A. Nobrega**. 1997. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur. J. Immunol. **27**:1557-1563.

2. **Vasconcellos, R., A. Nobrega, M. Haury, A. C. Viale, and A. Coutinho**. 1998. Genetic control of natural antibody repertoires: I. IgH, MHC and TCR beta loci. Eur. J. Immunol. **28**:1104-1115.

3. **Mouthon, L., A. Nobrega, N. Nicolas, S. V. Kaveri, C. Barreau, A. Coutinho, and M. D. Kazatchkine**. 1995. Invariance and restriction toward a limited set of self-antigens characterize neonatal IgM antibody repertoires and prevail in autoreactive repertoires of healthy adults. Proc. Natl. Acad. Sci. U. S. A **92**:3839-3843.

4. **Jayasekera, J. P., E. A. Moseman, and M. C. Carroll**. 2007. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. J. Virol. **81**:3487-3494.

5. **Hooijkaas, H., R. Benner, J. R. Pleasants, and B. S. Wostmann**. 1984. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. Eur. J. Immunol. **14**:1127-1130.

6. **Baumgarth, N., J. W. Tung, and L. A. Herzenberg**. 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol. **26**:347-362.

7. **Avrameas, S.** 1991. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. Immunol. Today **12**:154-159.

8. **Hardy, R. R., C. J. Wei, and K. Hayakawa**. 2004. Selection during development of VH11+ B cells: a model for natural autoantibody-producing CD5+ B cells. Immunol. Rev. **197**:60-74.

9. **Qian, Y., K. L. Conway, X. Lu, H. M. Seitz, G. K. Matsushima, and S. H. Clarke**. 2006. Autoreactive MZ and B-1 B-cell activation by Faslpr is coincident with an increased frequency of apoptotic lymphocytes and a defect in macrophage clearance. Blood **108**:974-982.

10. **Bos, N. A., C. G. Meeuwsen, H. Hooijkaas, R. Benner, B. S. Wostmann, and J. R. Pleasants**. 1987. Early development of Ig-secreting cells in young

of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. Cell Immunol. **105**:235-245.

11. **Thurnheer, M. C., A. W. Zuercher, J. J. Cebra, and N. A. Bos**. 2003. B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. J. Immunol. **170**:4564-4571.

12. **Masmoudi, H., T. Mota-Santos, F. Huetz, A. Coutinho, and P. A. Cazenave**. 1990. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. Int. Immunol. **2**:515-520.

13. **Forster, I. and K. Rajewsky**. 1987. Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic, newborn mice. Eur. J. Immunol. **17**:521-528.

14. **Pecquet, S. S., C. Ehrat, and P. B. Ernst**. 1992. Enhancement of mucosal antibody responses to Salmonella typhimurium and the microbial hapten phosphorylcholine in mice with X-linked immunodeficiency by B-cell precursors from the peritoneal cavity. Infect. Immun. **60**:503-509.

15. **Mond, J. J., A. Lees, and C. M. Snapper**. 1995. T cell-independent antigens type 2. Annu. Rev. Immunol. **13**:655-692.

16. **Hayakawa, K., R. R. Hardy, M. Honda, L. A. Herzenberg, A. D. Steinberg, and L. A. Herzenberg**. 1984. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. Proc. Natl. Acad. Sci. U. S. A **81**:2494-2498.

17. **Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum**. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. **320**:915-924.

18. **Palinski, W., S. Yla-Herttuala, M. E. Rosenfeld, S. W. Butler, S. A. Socher, S. Parthasarathy, L. K. Curtiss, and J. L. Witztum**. 1990. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis **10**:325-335.

19. **Ameli, S., A. Hultgardh-Nilsson, J. Regnstrom, F. Calara, J. Yano, B. Cercek, P. K. Shah, and J. Nilsson**. 1996. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler. Thromb. Vasc. Biol. **16**:1074-1079.

20. **Freigang, S., S. Horkko, E. Miller, J. L. Witztum, and W. Palinski**. 1998. Immunization of LDL receptor-deficient mice with homologous

malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. Arterioscler. Thromb. Vasc. Biol. **18**:1972-1982.

21. **Horkko, S., C. J. Binder, P. X. Shaw, M. K. Chang, G. Silverman, W. Palinski, and J. L. Witztum**. 2000. Immunological responses to oxidized LDL. Free Radic. Biol. Med. **28**:1771-1779.

22. **Esterbauer, H., R. J. Schaur, and H. Zollner**. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. **11**:81-128.

23. **Brambilla, G., A. Martelli, and U. M. Marinari**. 1989. Is lipid peroxidation associated with DNA damage? Mutat. Res. **214**:123-127.

24. **Vaca, C. E., J. Wilhelm, and M. Harms-Ringdahl**. 1988. Interaction of lipid peroxidation products with DNA. A review. Mutat. Res. **195**:137-149.

25. **Krokan, H., R. C. Grafstrom, K. Sundqvist, H. Esterbauer, and C. C. Harris**. 1985. Cytotoxicity, thiol depletion and inhibition of O6-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibroblasts. Carcinogenesis **6**:1755-1759.

26. **Palinski, W., S. Horkko, E. Miller, U. P. Steinbrecher, H. C. Powell, L. K. Curtiss, and J. L. Witztum**. 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J. Clin. Invest **98**:800-814.

27. **Palinski, W., R. K. Tangirala, E. Miller, S. G. Young, and J. L. Witztum**. 1995. Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis. Arterioscler. Thromb. Vasc. Biol. **15**:1569-1576.

28. **Kawahara, T., H. Ohdan, G. Zhao, Y. G. Yang, and M. Sykes**. 2003. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. J. Immunol. **171**:5406-5414.

29. **Horkko, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum**. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J. Clin. Invest **103**:117-128.

30. **Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum**. 2000. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest **105**:1731-1740.

31. **Kearney, J. F.** 2000. Immune recognition of OxLDL in atherosclerosis. J. Clin. Invest **105**:1683-1685.

32. **Palinski, W., V. A. Ord, A. S. Plump, J. L. Breslow, D. Steinberg, and J. L. Witztum**. 1994. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. Arterioscler. Thromb. **14**:605-616.

33. **Moreno, C. and J. Esdaile**. 1983. Immunoglobulin isotype in the murine response to polysaccharide antigens. Eur. J. Immunol. **13**:262-264.

34. **Tsimikas, S., W. Palinski, and J. L. Witztum**. 2001. Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized LDL in LDL receptor-deficient mice. Arterioscler. Thromb. Vasc. Biol. **21**:95-100.

# CHAPTER 3

## TOLL-LIKE RECEPTOR STIMULATION OF B-1 CELL INDUCES IGM PRODUCTION TO OXIDATION-SPECIFIC EPITOPES AND IL-10 SECRETION

**Abstract**

Emerging evidence suggests that oxidation-specific epitopes can serve as a class of pathogen-associated molecular patterns (PAMPs) that are recognized by innate pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and scavenger receptors. Thus, the activation of TLRs might regulate vital immune functions of B-1 cells, such as the secretion of NAbs and cytokines. Indeed, when B-1 cells were cultured and stimulated *in vitro* with TLR2 and TLR4 agonists, there was increased production of IgM and oxidation-specific IgMs. Interestingly, in response to TLR stimulation, oxidation-specific IgMs increased to a greater extent than total IgM or IgM to $\alpha$1,3-dextran. TLR activation also induced the secretion of IL-10, which in this case possibly serves as a negative regulator for B-1 cells, as the co-administration of IL-10 together with TLR4 agonist blocked TLR-induced B-1 cell activation through inhibiting cell proliferation. These data demonstrated that oxidation-specific IgMs are B-1 cell derived NAbs. They also demonstrated that TLR activation can regulate B-1 cell secretion of IgM through direct stimulation of B-1 cell proliferation or plasma cell differentiation, and/or indirectly, through stimulation of B-1 cell cytokine secretion, which in turn can regulate B-1 cell proliferation in an autocrine manner. These observations suggest an integrated innate immune response to inflammation and infection.

## Introduction

### Oxidation-specific epitopes are PAMPs recognized by PRRs

Innate immunity utilizes pattern recognition receptors (PRRs) that recognize conserved structures on pathogens - the “pathogen-associated molecular patterns” (PAMPs) - to sense the invasion of microbes. Our lab presented evidence that oxidation-specific epitopes can act as a class of PAMPs that can be recognized by PRRs such as scavenger receptors, as well as other components of innate immunity.

For example, the phosphocholine (PC) headgroup, which is present on oxidized phospholipids (OxPL) (1), apoptotic cells (2), and also on the cell-wall polysaccharide of *S. pneumoniae* (3) can be recognized by a natural Ab, E06/T15, as well as C-reactive protein (CRP), an acute-phase protein that participates in the innate immune response against microorganisms (4).

The binding of E06 to the PC headgroup on OxLDL can inhibit OxLDL uptake by macrophages through scavenger receptors such as CD36 and SR-BI (5,6). Our lab subsequently showed that E06 can bind to apoptotic cells and inhibit apoptotic cell uptake by macrophages as well (7). Furthermore, OxLDL and oxidized red blood cells can both compete for the uptake of apoptotic cells by macrophages (8). This evidence strongly suggests the PC moiety as a common ligand on OxLDL and apoptotic cells. Thus, the PC moiety of OxPL is a PAMP that is recognized by at least four innate PRRS: CD36, SR-BI, CRP and NAbs.

Toll-like receptors (TLRs) are an important family of innate PRRs. Activation of TLRs on innate effector cells induces local inflammatory events and thus resolves infection. Consistent with a more generalized role for TLRs, recent reports have established that they also participate in sterile inflammatory diseases such as atherosclerosis (9). OxLDL and its components have been identified as ligands of TLRs and their coreceptors (10,11). However, how the TLRs can regulate B-1 cell function is not clear. LPS, a TLR4 agonist, has been shown to induce proliferation and migration of B-1 cells to the spleen (12,13). A recent report by Genestier et al. has shown that all TLR agonists, with the exception of TLR3 and TLR5 agonists, stimulate B-1 cell proliferation and differentiation into mature plasma cells (14). However, whether TLR activation of B-1 cells can induce the production of oxidation-specific IgM remains unknown.

**Toll-like Receptors (TLRs)**

The above evidence suggests that the TLR family participates in the activation of B-1 cells. The TLR family represents an important class of highly conserved receptors that recognizes various PAMPs associated with different classes of pathogens and thus links innate immune responses to adaptive immunity (15). There are now 12 TLRs identified in mice, among them TLR4 and TLR2 are the most extensively studied in the context of atherosclerosis (16,17). TLR4 together with MD-2 form a receptor complex that recognizes lipopolysaccharide (LPS) with the help of LPS binding protein (LBP) and CD14 (18). TLR2 forms heterodimers with either TLR1 or TLR6. TLR2/6 heterodimer

detects bis-acylated lipopeptides, whereas TLR2/1 heterodimer recognizes tris-acylated lipopeptides. Interestingly, the scavenger receptor CD36, a receptor for OxLDL, has recently been identified as a co-receptor for TLR2 (17). Upon stimulation of TLRs, adaptor proteins associate with the TLR signaling domain named Toll/Interleukin-1 receptor (TIR) domain and activate downstream signaling cascades, which eventually turn on transcription factors NF-κB and AP-1, leading to production of proinflammatory cytokines.

Myeloid differentiation factor 88 (MyD88) is a critical adapter molecule for TLR signaling that carries the TIR domain. MyD88 can associate with all TLRs except TLR3 and is required for the induction of inflammatory cytokines. TLR3 utilizes a MyD88-independent signaling pathway that involves two other adaptor proteins TRIF-related adaptor molecule (Tram) and TIR-domain-containing adapter-inducing interferon-β (Trif) (15,19). TLR2 is solely dependent on the MyD88 signaling pathway while TLR4 can signal through both MyD88 dependent and independent pathways (19).

**Toll-like receptors and atherosclerosis**

Enhanced expression of TLR4 has been detected in atherosclerotic plaques of humans and $ApoE^{-/-}$ mice (20). A deficiency in TLR4 and MyD88 is associated with reduction in lesion size, lipid content, expression of proinflmmatory cytokines and macrophage infiltration (21), suggesting a role for TLR signaling in the pathogenesis of atherosclerosis. TLR2 deficiency in $Ldlr^{-/-}$

mice also led to a reduction of atherosclerosis, implying a proatherogenic role (22). Indeed, the administration of a synthetic TLR2/TLR1 agonist, $Pam3CysSerLys_4$ (Pam3CSK4), dramatically increased disease burden and could be blocked by TLR2 deficiency (22). This suggested the existence of endogenous ligands that can bind to TLRs and affect atherosclerosis. Minimally modified LDL (mmLDL), a form of mildly oxidized LDL that has been shown to bind TLR4 on macrophages and induce the production of certain chemokines and cytokines (23), constitutes a potential endogenous TLR4 ligand in atherogenesis. The endogenous ligand(s) for TLR2 is still unknown. However, OxPL was shown by our lab (11) and others (24) to be a ligand for CD36, a TLR2 co-receptor. Therefore, oxidized lipoproteins and lipids could also be potential endogenous ligands for TLR2. The effector cells that respond to TLR activation in atherosclerosis have not yet been well defined. With respect to B-2 cells, recent studies have shown that TLR activation is required as a third signal besides B cell receptor (BCR) crosslinking and T cell help to activate B-2 cells in mice and humans (25,26). Whether the same applies to B-1 cells is not known. A recent study by Ng et al., showed that TLR9 stimulation of B-1 cells rendered them responsive to B cell-activating factor (BAFF) costimulation, as evidenced by increased proliferation and secretion of IL-6 and IL-10 (27). Another study demonstrated that up-regulation of IgA and co-stimulatory molecules CD80 and CD86 on B-1 cells by treating cells with porin of *Shigella dysenteriae* is dependent on TLR2 and TLR6 (28). **Therefore**, **the vital immune functions of B-1 cells, such as the secretion of NAbs and cytokines, might be regulated**

**by the ligation of TLRs, reflecting an integrated innate immune response to inflammation and infection.**

## Rationale and study design

I will test the hypothesis that the ligation of TLRs will lead to B-1 cell activation such as cell proliferation, IgM secretion and cytokine secretion. My study will focus on TLR2 and TLR4 since these TLRs has been extensively studied in the context of atherosclerosis, and oxidatively modified LDL might act as endogenous ligands for these two TLRs. Therefore, it will be interesting to see if TLR2 and TLR4 activation of B-1 cells can induce the secretion of oxidation-specific IgMs.

Pure B-1 cells from C57BL/6 will be cultured *in vitro* and stimulated with TLR2 and TLR4 agonists as well as IL-5, a known stimulant for B-1 cells (29). Control cells will be left untreated. Cell culture supernatants will be harvested after 7 days and assessed for oxidation-specific IgM production. The presence of oxidation-specific IgM in pure B-1 cell cultures will again confirm the NAb origin of these IgM Abs.

Cell proliferation and cytokine production by B-1 cells will also be assessed, especially the production of IL-10, since B-1 cells are the main source of B-cell-derived IL-10 (30). Other cytokines such as IL-6 and pro-inflammatory

cytokines such as IL-1 and TNF-$\alpha$ will be tested as well. Based on these *in vitro* findings, the *in vivo* relevance of the most robust observations can then be investigated.

Next, in order to determine the role of adaptor molecules in the TLR signaling pathways on TLR-mediated activation of B-1 cells, B-1 cells from knockout mice strains deficient in TLR itself or major components of the TLR signaling pathways such as CD14 and MyD88 will be utilized and stimulated as described previously with TLR agonists. The secretion of IgM and cytokines will be tested to assess how the lack of these critical proteins will affect TLR-mediated activation of B-1 cells.

The main focus of this chapter is first to directly demonstrate that B-1 cells produce oxidation-specific IgM and thus support my hypothesis that part of the NAb population are directed against oxidation-specific epitopes. Secondly, I would like to demonstrate that B-1 cells can be activated by the ligation of TLR2 and/or TLR4 to secrete NAbs to oxidation-specific epitopes as well as cytokines, forming an integrated innate immune response to infection and inflammation.

## Results

### B-1 cells secrete IgM NAbs against oxidation-specific epitopes *in vitro*

In Chapter 2, I presented evidence that IgM Abs to oxidation-specific epitopes are present in germ-free mice, indicating the NAb origin of these oxidation-specific IgM Abs. To directly demonstrate that these oxidation-specific IgM are NAbs derived from innate B-1 cells, I isolated B-1 cells from naïve mice by FACS sorting. B-1 cells were sorted as the CD19+, CD23- population. The CD3 marker was also used to actively exclude T cells. Purified B-1 cells were then stimulated *in vitro* with different stimuli and culture supernatants were tested for specific IgM Abs. IL-5, a TLR-4 ligand ($KdO_2$-LipidA) (31), as well as a combination of TLR-2 ligands (FSL-1 and Pam3CSK4), all induced B-1 cells to secrete IgM against oxidation-specific epitopes (**Fig.3-1**). Interestingly, the basal secretion of oxidation-specific IgM (i.e. that occurring in the absence of stimuli) were strikingly more prominent than IgM against the prototypic B-1 cell antigen α1,3-dextran (**Fig.3-1**).

The cell numbers that were recovered after 7 days of stimulation were also counted. The average cell number recovered from TLR4 and TL2 stimulated conditions were higher (average $0.82x10^6$ cells/well, $0.74x10^6$ cells/well, respectively) compared to IL-5 ($0.43x10^6$ cells/well) and unstimulated conditions ($0.35x10^6$ cells/well). When **Fig.3-1** was re-plotted taking cell number into consideration, I found that IL-5 actually induced a higher level of IgM secretion on a per cell basis compared to TLR2 and TLR4 agonist stimulation (**Fig.3-2**). IgM

responses to MDA-LDL and CuOx-LDL were still induced, while response to PC-BSA was only simulated by IL-5 stimulation.

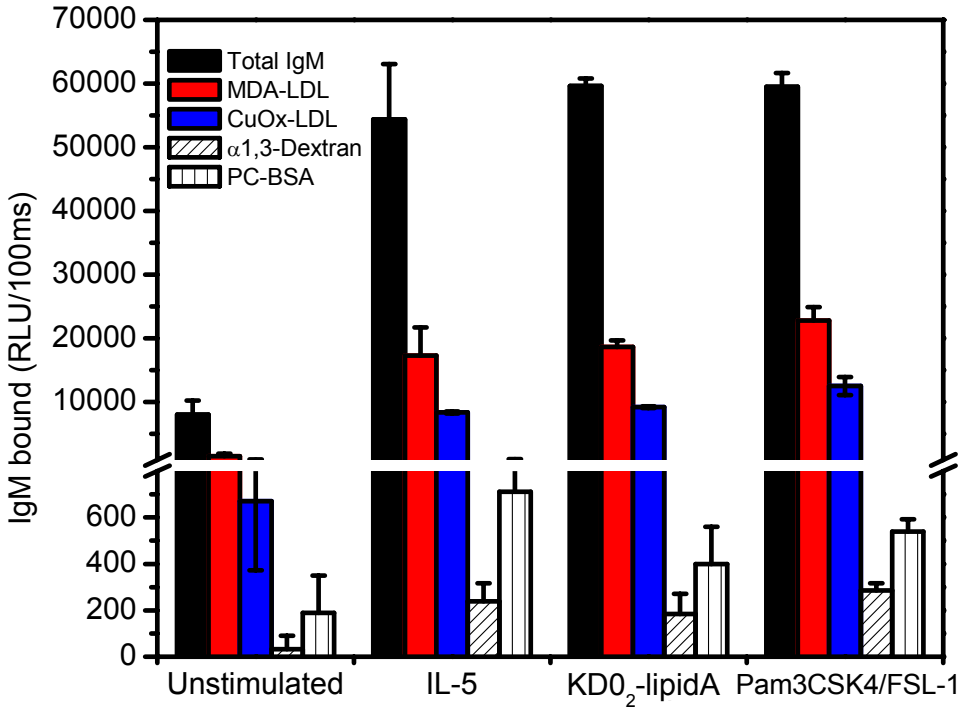



**Fig.3-1 *In vitro* stimulation of B-1 cells induces increased natural IgM Ab titers to oxidation-specific antigens.** Purified B-1 cells were cultured in 24 well plates in triplicates at a cell density of $1x10^6$ cells per well in 500μl culture media. Cells were stimulated with IL-5 (50ng/ml), $KdO_2$-lipidA (100ng/ml), or TLR2 agonists (a combination of Pam3CSK4 (300ng/ml) and FSL-1 (1μg/ml)) and incubated at 37°C for 7 days. Control B-1 cells were unstimulated. Cell culture supernatants were harvested after 7 days and IgM Ab titers analyzed by ELISA at 1:45 dilution. After stimulation, the production of IgM Abs to MDA-LDL and CuOx-LDL was induced. Values are means and SD. The experiment shown is one representative of 3 separate experiments.

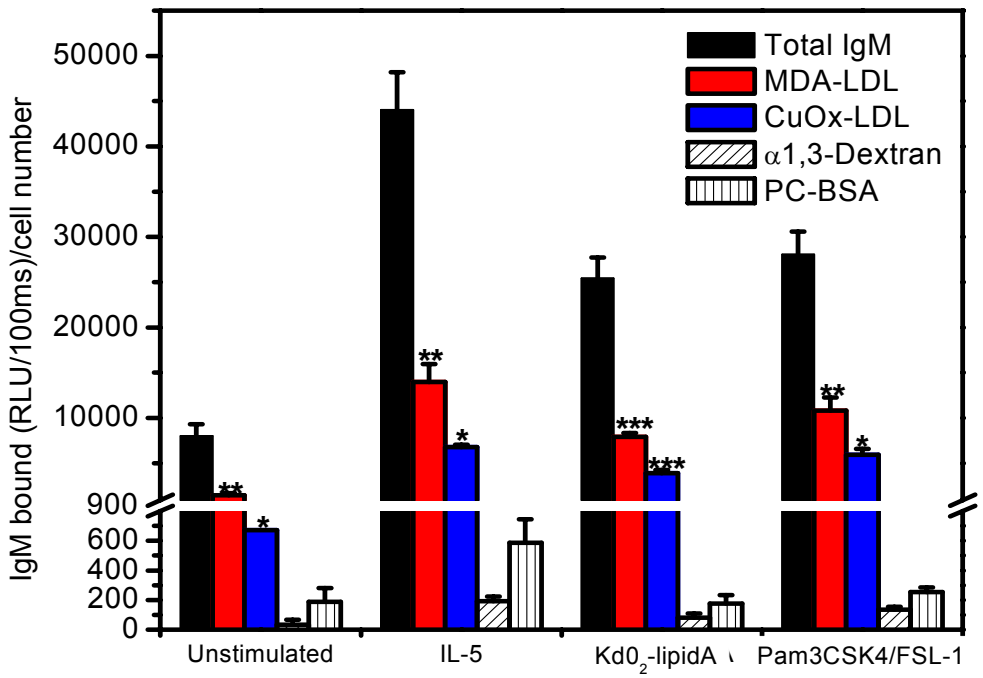



**Fig.3-2 *In vitro* stimulation of B-1 cells induces increased natural IgM Ab titers to oxidation-specific antigens.** Results are from the same *in vitro* stimulation experiment shown in **Fig.3-1**. Results are normalized to cell number recovered after 7 days under unstimulated condition. Values are means and SEM. The experiment shown is one representative of 3 separate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to α1,3-Dextran. (Repeated measures ANOVA with Tukey-Kramer Multiple Comparison Test)

In response to stimulation with $KdO_2$-lipidA and TLR2 agonists, the oxidation-specific IgM increased to a greater extent than IgM to α1,3-dextran (**Fig.3-3**). Moreover, in analogy to the robust anti-MDA-responses *in vivo*, MDA-specific IgM were the most dominant set of IgM Abs secreted by B-1 cells *in vitro* that we measured. Although I only tested a narrowly selected set of antigens, the

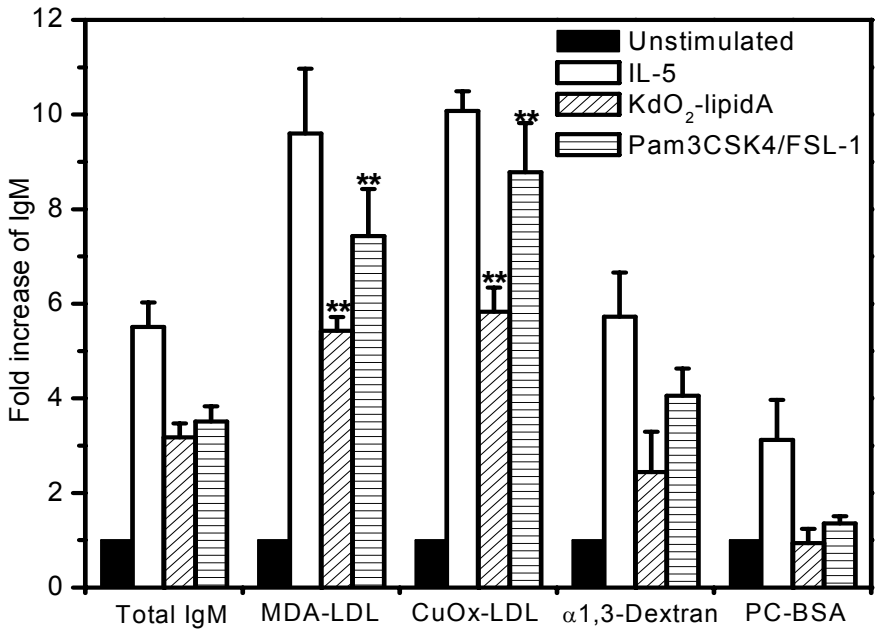



**Fig.3-3 Fold increase of natural IgM Ab to oxidation-specific antigens after *in vitro* stimulation of B-1 cells.** Results are from the same *in vitro* stimulation experiment shown in **Fig.3-1**. Data are plotted as fold increase compared to unstimulated condition. After stimulation, the production of IgM was increased more than 3-fold under all stimulated conditions compared to unstimulated B-1 cells. IgMs to MDA-LDL and CuOx-LDL were increased 5 to 10 fold, while IgMs to $\alpha$1,3-Dextran increased 2-6 fold. The increase of MDA-LDL and CuOx-LDL were significantly higher than $\alpha$1,3-Dextran when stimulated with $KdO_2$-lipidA and TLR2 agonists. ** $p<0.01$ compared to $\alpha$1,3-Dextran under the same condition (Repeated measures ANOVA with Tukey-Kramer Multiple Comparison test).

anti-MDA antibodies constituted up to approximately 30% of total IgM secreted (**Fig.3-1 and 3-2**). These Abs were highly specific for MDA-modifications, as only MDA-LDL, but neither OxLDL nor native LDL competed for the binding (**Fig. 3-4**).

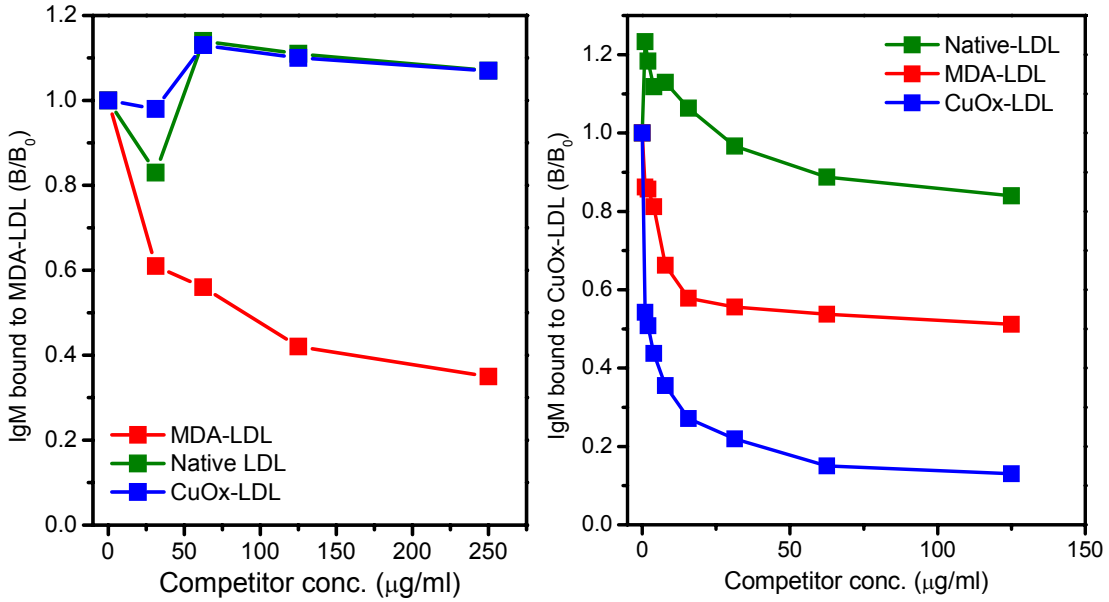



**Fig.3-4 Natural IgM Abs produced *in vitro* show specificity to MDA-LDL and CuOx-LDL**. For competition immunoassay, supernatant from purified B-1 cell cultures stimulated with LPS (100ng/ml) or IL-5 (50ng/ml) were diluted to 1:20 and incubated in the presence of indicated concentrations of competitors overnight. After incubation, IgM binding to MDA-LDL and CuOx-LDL was then tested by ELISA. Data are the mean of triplicate determinations, expressed as ratio of IgM binding to MDA-LDL or CuOx-LDL in the presence of competitor to the binding in the absence of competitor ($B/B_0$). Data shown are from one representative experiment.

IgM that bound to plated CuOx-LDL were competed by both CuOx-LDL itself and MDA-LDL, but not native LDL. The binding avidities of the IgM in the supernatants for MDA-LDL and OxLDL were also calculated using the Klotz methods (32). The dissociation constants (Kds) for MDA-LDL was $1.46 \times 10^{-7}$, and for CuOx-LDL was $4.03 \times 10^{-9}$, similar to values we previously determined for IgM in plasma of mice immunized against these epitopes (2).

**IgM binding to native LDL**

In these studies, I tested IgM binding to antigens coated on microtiter wells using standard solid-phase ELISA techniques (33). In some experiments, we saw low to modest levels of IgM binding to "native LDL," which appeared to vary with different LDL preparations. However, in all cases the binding to plated native LDL could not be competed by the same preparation of native LDL in solution (**Fig.3-5**), but could be competed to some degree by MDA-LDL and CuOx-LDL, suggesting that the LDL became modified in some way during the plating process. It should be emphasized that in competition immunoassays, I never observed native LDL in solution competing for binding to any of the modified LDL preparations (e.g. as shown in **Fig.3-4** and **Fig.4-6**).

**B-1 cell responses to both TLR4 and TLR2 agonists were in large part MyD88 dependent**

Since stimulation with the TLR4 agonist, $KdO_2$-lipidA, induces B-1 cells to secrete more natural IgM Abs and certain cytokines, I wanted to test if this response was dependent on the TLR4 signaling pathway. B-1 cells from $TLR4^{-/-}$, $MyD88^{-/-}$ and $CD14^{-/-}$ mice were isolated and stimulated as previously described *in vitro*. Surprisingly, $TLR4^{-/-}$ B-1 cells still responded modestly to $KdO_2$-lipidA stimulation (**Fig.3-6, A.**). B-1 cells from $MyD88^{-/-}$ mice responded to $KdO_2$-lipidA as well, even though to a lesser degree compared to both wild-type and $TLR4^{-/-}$ B-1 cells, suggesting that $KdO_2$-lipidA is partially dependent on

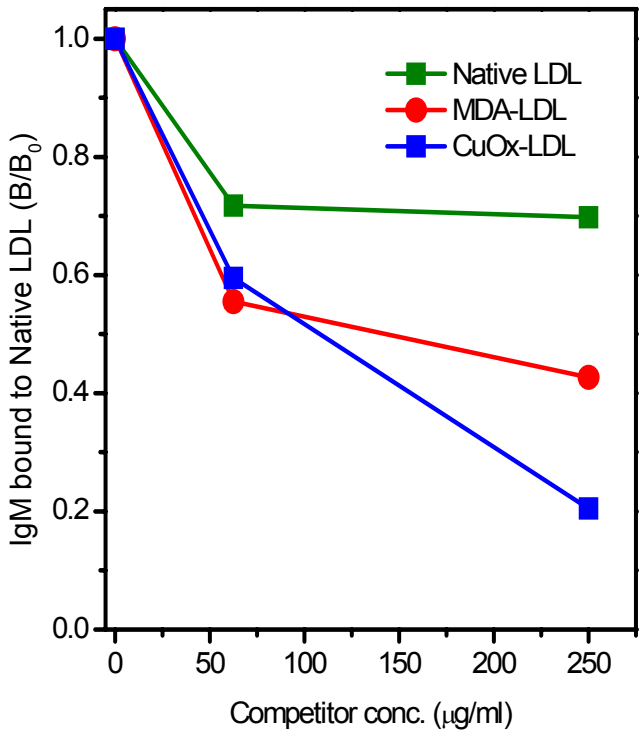



**Fig.3-5 Natural IgM Abs binding to plated native LDL could not be competed by native LDL in solution.** For competition immunoassay, supernatant from purified B-1 cell cultures stimulated with LPS (100ng/ml) were diluted to 1:20 and incubated in the presence of indicated concentrations of competitors overnight. After incubation, IgM binding to native LDL was then tested by ELISA. Data are the mean of triplicate determinations, expressed as ratio of IgM binding to native LDLin the presence of competitor to the binding in the absence of competitor ($B/B_0$). Data shown are from one representative experiment.

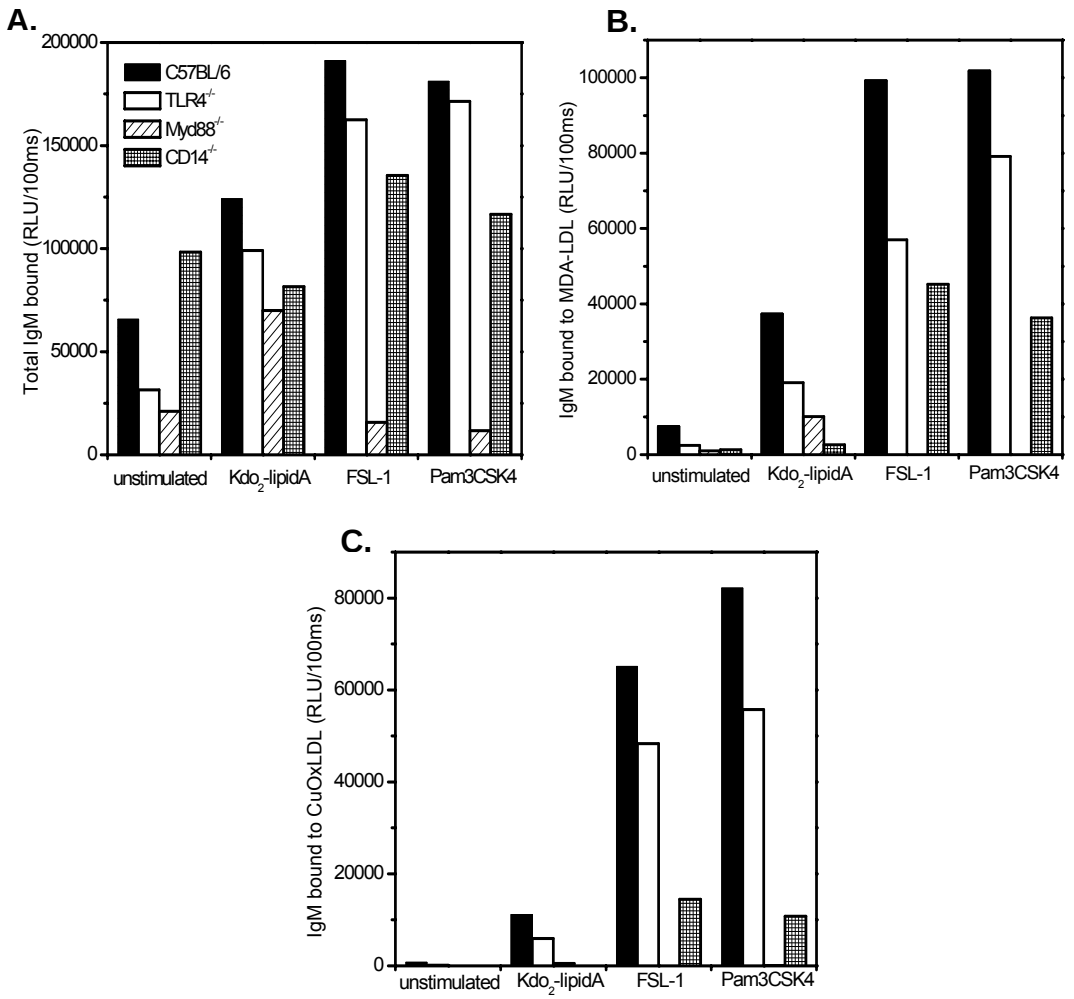



**Fig.3-6 *In vitro* stimu-lation of B-1 cells from TLR4$^{-/-}$, MyD88$^{-/-}$, and CD14$^{-/-}$ mice.** B-1 cells purified from wild-type, TLR4$^{-/-}$, MyD88$^{-/-}$ and CD14$^{-/-}$ mice were cultured and stimulated with $KdO_2$-lipidA (100ng/ml), Pam3CSK4 (300ng/ml), or FSL-1 (1μg/ml). Control cells were left unstimulated. Cell culture supernatants were harvested after 7 days and IgM Ab titers analyzed by ELISA at 1:5 dilution.

the MyD88 signaling pathway. CD14 does not seem to play a role in $KdO_2$-lipidA stimulation of B-1 cells, as $KdO_2$-lipidA stimulation did not induce increased IgM secretion compared to unstimulated $CD14^{-/-}$ B-1 cells. The same trend was observed in IgMs to MDA-LDL and CuOx-LDL when stimulated (**Fig.3-6, B. and C.**).

The lack of TLR4 or CD14 did not affect TLR2 agonist stimulation of B-1 cells, as expected (**Fig.3-6**). The lack of MyD88, however, completely abolished TLR2 agonist stimulation of B-1 cells. In $MyD88^{-/-}$ cells, only minimal levels of IgM could be detected after TLR2 agonist stimulation, and no IgM to MDA-LDL and CuOx-LDL were detected. This is expected since MyD88 is the sole signaling pathway for TLR2.

The same phenomenon was observed in a separate *in vitro* stimulation experiment utilizing $MyD88^{-/-}$ B-1 cells. When taking cell number into account, MyD88 deficiency only had a minor effect on $KdO_2$-lipidA induced IgM Abs, which decreased from 5 fold in wild-type B-1 cells to about 3.5 fold in $MyD88^{-/-}$ B-1 cells compared to unstimulated cells (**Fig.3-7, left panel**). However, $KdO_2$-lipidA induced MDA-LDL-specific IgM were diminished by MyD88 deficiency (**Fig.3-7, right panel**), suggesting that after TLR4 stimulation, oxidation-related IgMs might be induced through the MyD88 dependent signaling pathway.

Unlike TLR4 stimulation, TLR2 stimulation of B-1 cells seems to be solely dependent on MyD88-mediated signaling pathway, as MyD88 deficiency greatly

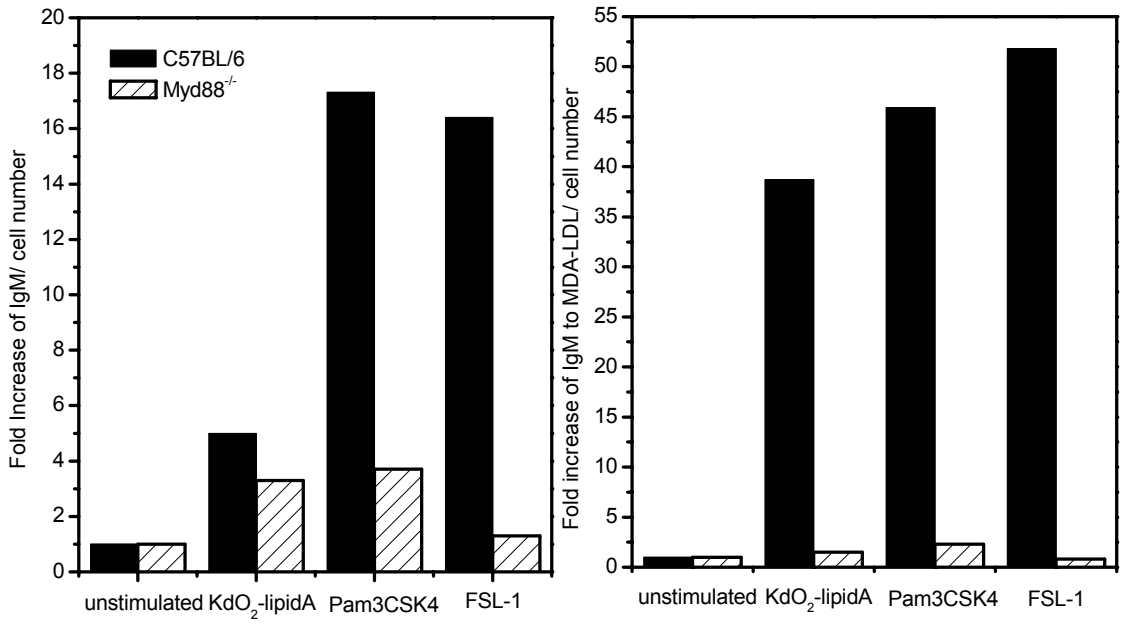



**Fig.3-7 Fold increase of natural IgM Ab after *in vitro* stimulation of wild-type and MyD88$^{-/-}$ B-1 cells.** Purified B-1 cells from wild-type and Myd88$^{-/-}$ mice were cultured and stimulated as previously described. Cell culture supernatant were harvested after 7 days and tested by ELISA. Data are plotted as fold increase compared to unstimulated condition and normalized to cell number recovered after 7 days under unstimulated condition. After stimulation, the production of IgM and MDA-LDL specific IgM was increased in wild-type B-1 cells. TLR agonist induced IgM increase is reduced or eliminated in MyD88$^{-/-}$ B-1 cells.

diminished TLR2 agonist induced IgM secretion from 16 fold in wild-type B-1 cells to less than 4 fold in MyD88$^{-/-}$ B-1 cells (**Fig.3-7, left panel**). The same reduction was observed in MDA-LDL specific IgM for MyD88$^{-/-}$ B-1 cells (**Fig.3-7, right panel**).

The above results suggested that while TLR4 stimulation of B-1 cells is only partially dependent on the MyD88 signaling pathway, TLR2 stimulation of B-

1 cells seems to be solely dependent on MyD88 mediated signaling pathway. However, TLR2 forms heterodimers with either TLR1 or TLR6. Pam3CSK4 is an agonist for the TLR2/TLR1 heterodimer while FSL-1 is an agonist for the TLR2/TLR6 heterodimer. Therefore, the stimulation effect could also be mediated through either TLR1 or TLR6. Utilizing $TLR2^{-/-}$ B-1 cells, I showed that TLR2 is critical for Pam3CSK4 mediated stimulation of B-1 cells. In the absence of TLR2, the induction of IgM Abs, antigen-specific IgM Abs as well as IL-10 secretion was all blocked (**Fig.3-8**). The increased production of IL-10 after stimulation will be discussed in more detail in the following section.

**B-1 cell stimulation in vitro also induces IL-10 secretion**

Since B-1 cells are the main source of B-cell-derived interleukin IL-10 (30), I wanted to test if IL-10 secretion is also induced in B-1 cells that are stimulated. Indeed, IL-10 can be detected in *in vitro* B-1 cell cultures even without stimulation. Stimulation with IL-5, $KdO_2$-lipidA or TLR2 agonists increased IL-10 secretion (**Fig.3-9**). The same phenomenon was observed in a separate experiment, where the cytokine profile of B-1 cells stimulated *in vitro* was examined with xMAP® technology developed by Luminex (Austin, TX, USA), which allows the detection of multiple cytokines within a single sample. When stimulated, IL-10 increased dramatically to a level that is at least 10 fold higher compared to other cytokines tested (**Table 3-1, wild-type B-1 cells**).

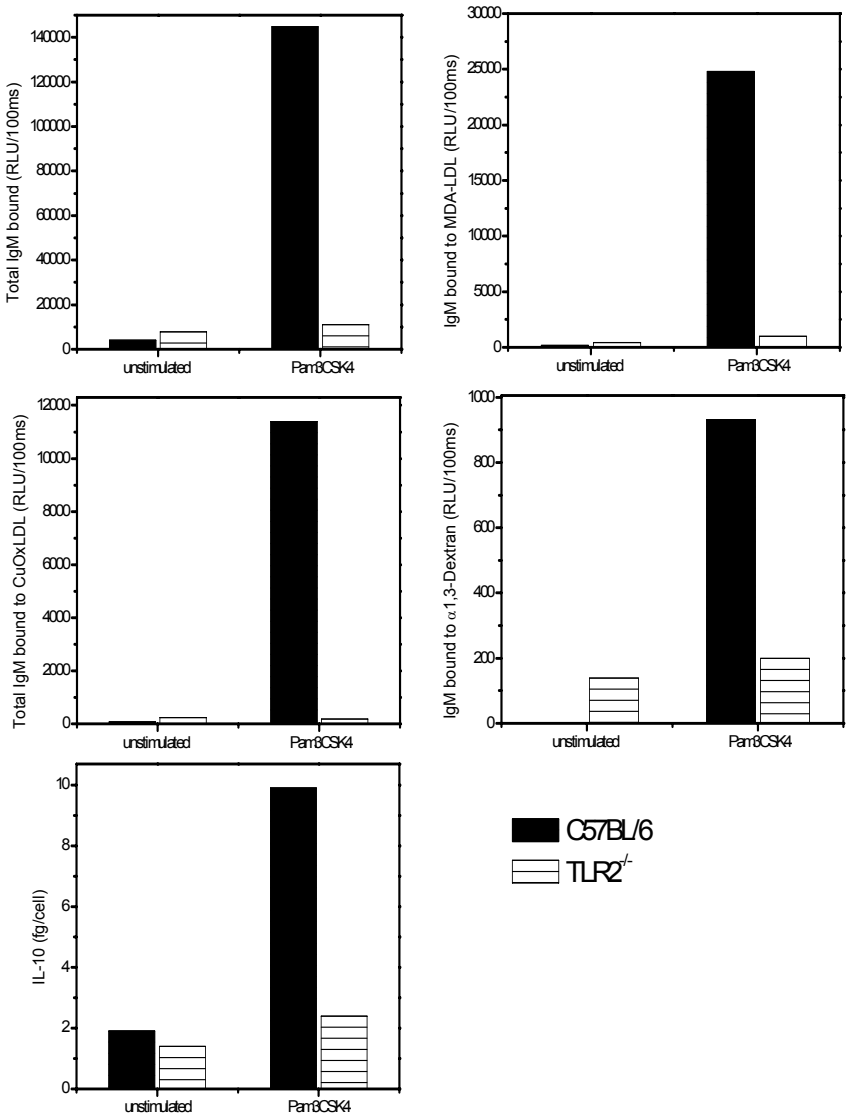



**Fig.3-8 TLR2 deficiency blocked stimulation of Pam3CSK4 in B-1 cells.** B-1 cells purified from wild-type and TLR2$^{-/-}$ mice were cultured and stimulated with Pam3CSK4 (300ng/ml), or left untreated. Cell culture supernatants were analyzed for IgM Ab levels and IL-10 secretion by ELISA at 1:5 dilution.

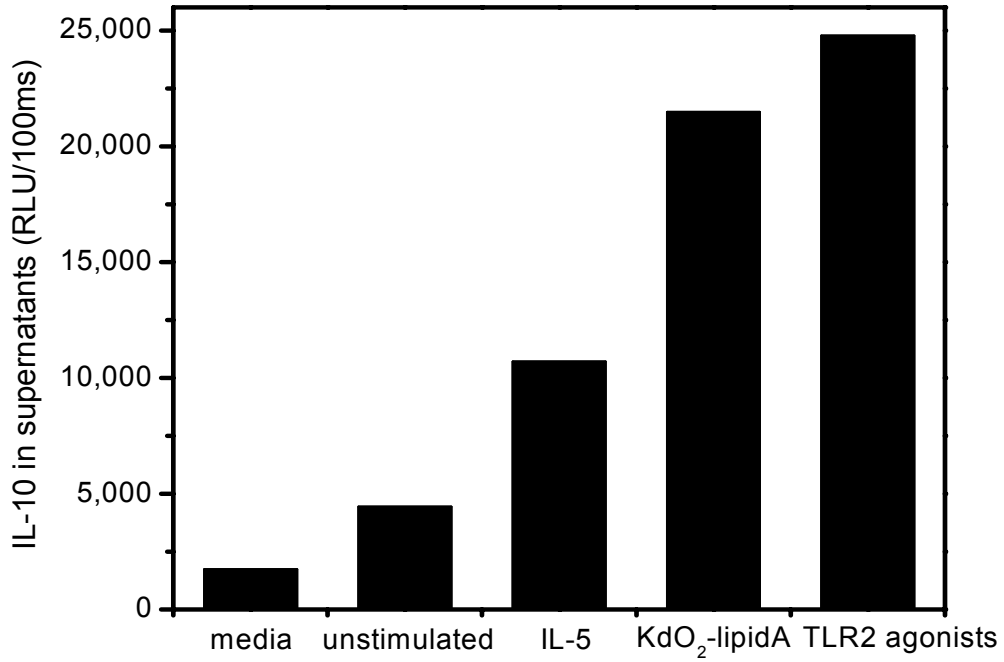



**Fig.3-9 *In vitro* stimulation of B-1 cells induces increased IL-10 secretion.** Purified B-1 cells were cultured and stimulated with IL-5 (50ng/ml), $KdO_2$-lipidA (100ng/ml), or TLR2 agonists (a combination of Pam3CSK4 (300ng/ml) and FSL-1 (1μg/ml)) and incubated at 37°C for 7 days. Control B-1 cells were unstimulated. Cell culture supernatants were harvested after 7 days and tested for IL-10 secretion by ELISA at 1:2 dilution. The experiment shown is one representative experiment.

Under unstimulated condition, IL-10 was also the most abundant cytokine secreted among those tested. Aside from IL-10, IL-6, IL-1β and IL-9 were also modestly induced after stimulation. Interestingly, TNF-α was induced only after TLR2 agonist stimulation but did not increase in response to TLR4 stimulation.

MyD88 deficiency also attenuated the induction of IL-10 secretion after TLR stimulation. As shown In **Table 3-1**, induction of IL-10 after $KdO_2$-lipidA

**Table 3-1 Cytokine profile of wild-type and MyD88$^{-/-}$ B-1 cells *in vitro* after stimulation with TLR agonists**

| | Wild-type B-1 cells | | | | MyD88$^{-/-}$ B-1 cells | | | |
|---|---|---|---|---|---|---|---|---|
| pg/ml | Media | Lipid A | Pam3 | FSL-1 | Media | Lipid A | Pam3 | FSL-1 |
| IL-10 | 9 | 1681 | 955 | 603 | 11 | 35 | 9 | 6 |
| IL-6 | 1 | 40 | 55 | 49 | 2 | 2 | 3 | 2 |
| IL-1$\alpha$ | -- | -- | 4 | 3 | -- | -- | -- | -- |
| IL-1$\beta$ | -- | 52 | 52 | 45 | -- | -- | -- | -- |
| IL-9 | -- | 26 | 13 | 44 | -- | -- | -- | -- |
| IL-12(p70) | -- | 3 | 4 | 1 | 1 | -- | -- | -- |
| IL-13 | -- | -- | 7 | 14 | -- | -- | -- | -- |
| TNF-a | -- | -- | 29 | 26 | 0.5 | -- | -- | -- |

stimulation was blunted in MyD88$^{-/-}$ B-1 cells, and the effect of TLR2 agonists on IL-10 induction was completely eliminated with MyD88 deficiency. All other cytokines were also undetectable in MyD88$^{-/-}$ B-1 cells after stimulation.

**IL-10 inhibits IgM secretion induced by TLR stimulation from B-1 cells**

Results above showed that B-1 cells can secrete IL-10 when stimulated. Therefore, the next important question is what effect does IL-10 have on the B-1 cells that secrete them? To investigate the effect of IL-10 on activated B-1 cells, B-1 cells were cultured and stimulated with $KdO_2$-lipidA in the presence and absence of added recombinant mouse IL-10 (rmIL-10). As shown in **Fig. 3-10**,

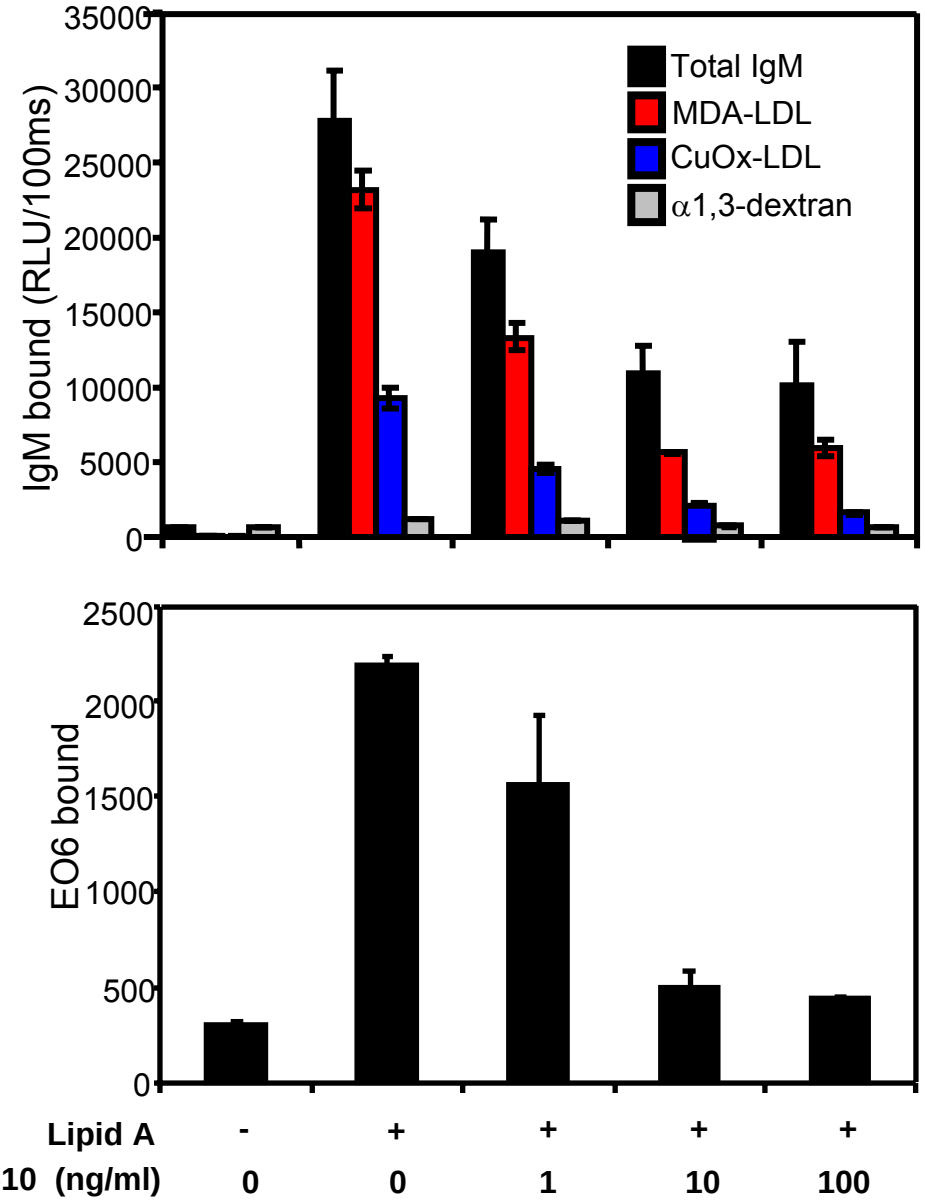



**Fig.3-10 IL-10 inhibits IgM secretion from B-1 cells *in vitro*.** B-1 cells were incubated with 100 ng/ml $KdO_2$-lipid A and rmIL-10 at indicated concentrations. Culture supernatants were analyzed for IgMs with ELISA at 1:5 dilution. EO6/T15 levels were also measured using an idiotypic-specific antibody AB1-2. Total IgM or antigen-specific IgM as well as EO6 secretion by B-1 cells were inhibited in the presence of IL-10.

$KdO_2$-lipidA dramatically induced the secretion of total IgM and antigen-specific IgMs, as well as E06, the prototypic B-1a cell-derived IgM NAb. However, when rmIL-10 was added together with $KdO_2$-lipidA at the beginning of the incubation, there was a dose-dependent inhibitory effect of rmIL-10. At 100 ng/ml, rmIL-10 caused a 60-70% decrease in all IgMs tested. In contrast, incubation of B-1 cells with rmIL-10 alone had no effect on IgM secretion.

**IL-10 inhibits B-1 cell proliferation but not plasma cell differentiation**

For these *in vitro* experiments, even though I started out with the same number of cells in each well, after 7 days of TLR agonist stimulation, there were always more live cells in stimulated wells compared to unstimulated as described previously. This suggests that TLR agonist stimulation also induces B-1 cell proliferation. Therefore, the effect of IL-10 inhibition on IgM secretion could be either through the inhibition of IgM secretion of B-1 cells itself, or the inhibition of B-1 cell proliferation induced by TLR stimulation.

To investigate if IL-10 affected the IgM secretion of B-1 cells, the differentiation of B-1 cells into IgM secreting plasma cells were tested. B-1 cells were incubated with $KdO_2$-LipidA in the presence and absence of IL-10 for 3 days, and the population of live cells analyzed for surface expression of the plasma cell marker CD138. Approximately 50% of B-1 cells expressed CD138 on their cell surface after stimulation with $KdO_2$-LipidA and can be defined as Ab secreting plasma cells, compared with less than 10% among the unstimulated B-1 cells. The presence of IL-10 did not alter the percentage of plasma cells compared with

$KdO_2$-LipidA alone (**Fig. 3-11**). This suggests that IL-10 does not inhibit B-1 cell differentiation into plasma cells.

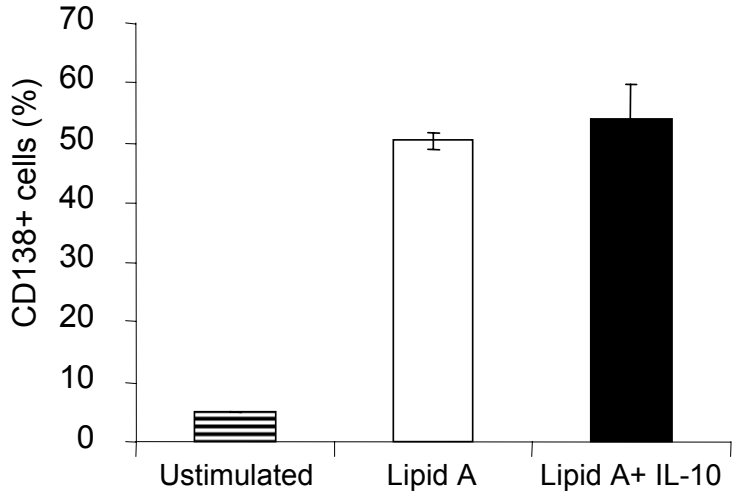



**Fig.3-11 IL-10 does not inhibit plasma cell differentiation of B-1 cells.** B-1 cells were cultured for 3 days with $KdO_2$-lipid A (100 ng/ml), $KdO_2$-Lipid A + IL-10 (100 ng/ml), or control medium. Cells were stained with biotinylated anti-CD138 antibody and PE-conjugated streptavidin and analyzed with FACS. CD138 positive cells were determined based on staining with isotypic control antibody. Only live cells were analyzed based on forward and side scatter. Results are combined data from 3 different experiments.

When the percentages of live and dead cells were analyzed in these B-1 cell cultures, the percentage of live cells was higher in B-1 cells stimulated with $KdO_2$-lipidA compared to $KdO_2$-lipidA plus rmIL-10. The absolute number of live cells was also higher in B-1 cells incubated with Lipid A-$KdO_2$ alone (data not shown). The lower number of cells in the presence of IL-10 after 3 days in culture could be due to either decreased proliferation or increased cell death. $^3$H-Thymidine incorporation assay of B-1 cells showed a major $KdO_2$-lipidA induced

proliferation response, which was decreased in the presence of rmIL-10 (**Fig. 3-12, A**), suggesting that IL-10 can cause decreased proliferation of B-1 cells. Proliferation was also analyzed by staining of B-1 cells with CFSE before culture followed by FACS analysis after 4 days. Again, there was a marked decrease in the number of proliferating cells in the presence of IL-10 compared to $KdO_2$-lipidA alone (**Fig. 3-12, B**). Apoptosis was assessed by staining for Annexin V or FITC-conjugated zVAD-FMK together with propidium iodide. These data failed to support an enhanced rate of apoptosis in the IL-10 treated B-1 cells under these conditions (data not shown). Thus, in cell culture, IL-10 appears to inhibit IgM secretion from TLR activated B-1 cells by inhibiting B-1 cell proliferation.

**Effect of IL-10 administration *in vivo***

The *in vitro* data demonstrated that IL-10 exerts inhibitory effects on proliferation and IgM secretion from B-1 cells. In order to confirm this *in vivo*, $KdO_2$-lipidA and/or rmIL-10 were injected intraperitoneally (i.p.) into C56BL/6 mice to activate B-1 cells. Three days after the first injection, rmIL-10 or PBS were administered as a second i.p. injection. Seven days after the first injection, plasma IgM titers were determined by ELISA and peritoneal cell populations characterized by FACS.

There was a robust increase in total as well as MDA-LDL- and CuOx-LDL-specific IgMs following $KdO_2$-lipidA injection (**Fig.3-13, A.**). There was a tendency towards lower IgM levels with the combination of $KdO_2$-lipidA and IL-10, although the data were not statistically significant. In the peritoneal cell population, the

numbers of B-1 cells were significantly lower in the mice injected with $KdO_2$-lipidA plus rmIL-10 (0.8 x $10^6$ B-1 cells) compared with $KdO_2$-lipidA alone (2 x $10^6$ cells) (**Fig.3-13, B. and C.**). These results *in vivo* are consistent with the *in vitro* effects of IL-10 on B-1 cell proliferation and IgM secretion.

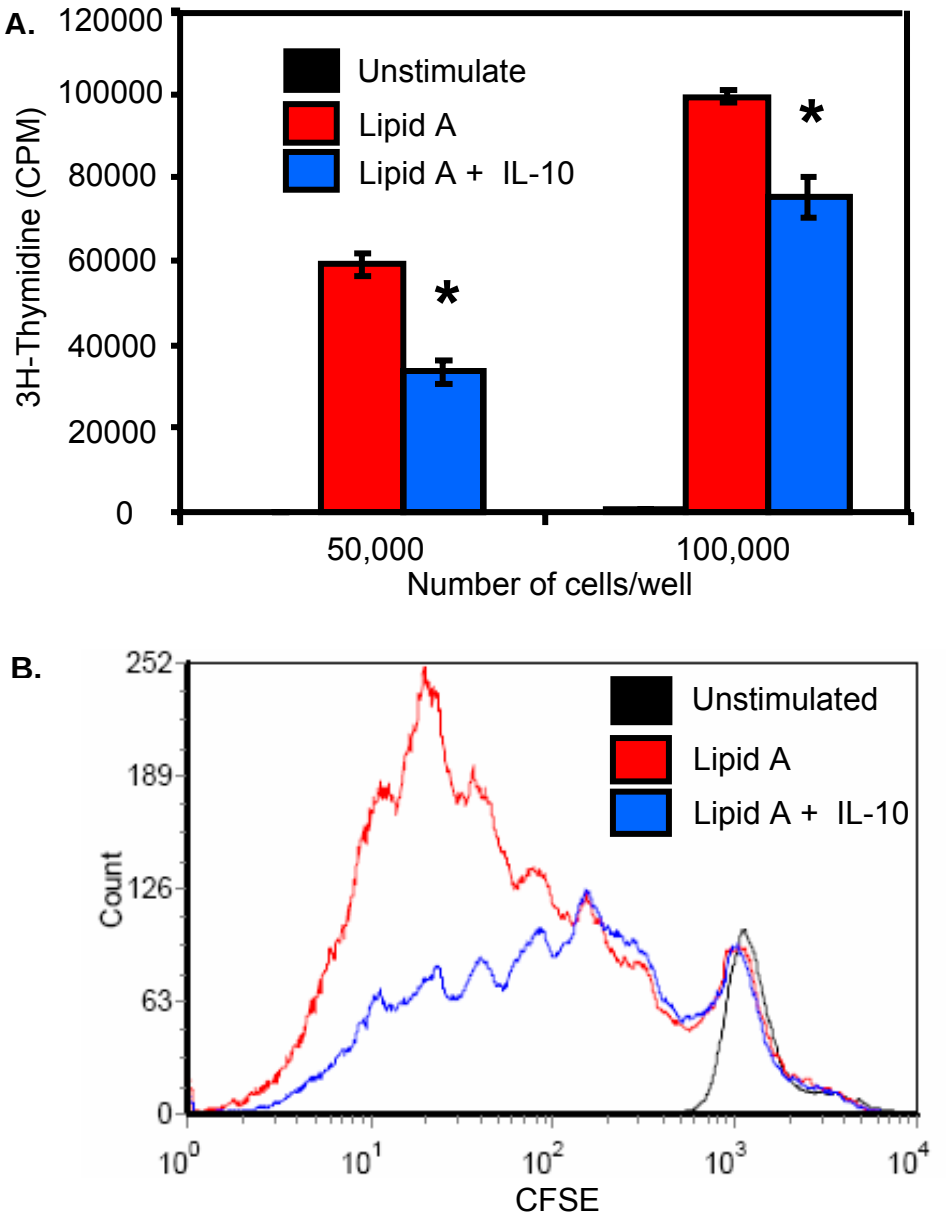



**Fig.3-12 IL-10 inhibits B-1 cell proliferation. A.** B-1 cells were incubated with $^3$H-Thymidine (1 uCi/well) during the last 16 hours of culture. Incorporated thymidine was determined by liquid scintillation counting. *$p<0.05$ vs. $KdO_2$-LipidA (Student's unpaired t test) **B**. Purified B-1 cells were stained with CFSE and cultured for 4 days with 1 μg/ml $KdO_2$-lipidA, $KdO_2$-Lipid A + IL-10 (100 ng/ml), or control medium. Histogram shows only live cells based on forward scatter and side scatter.

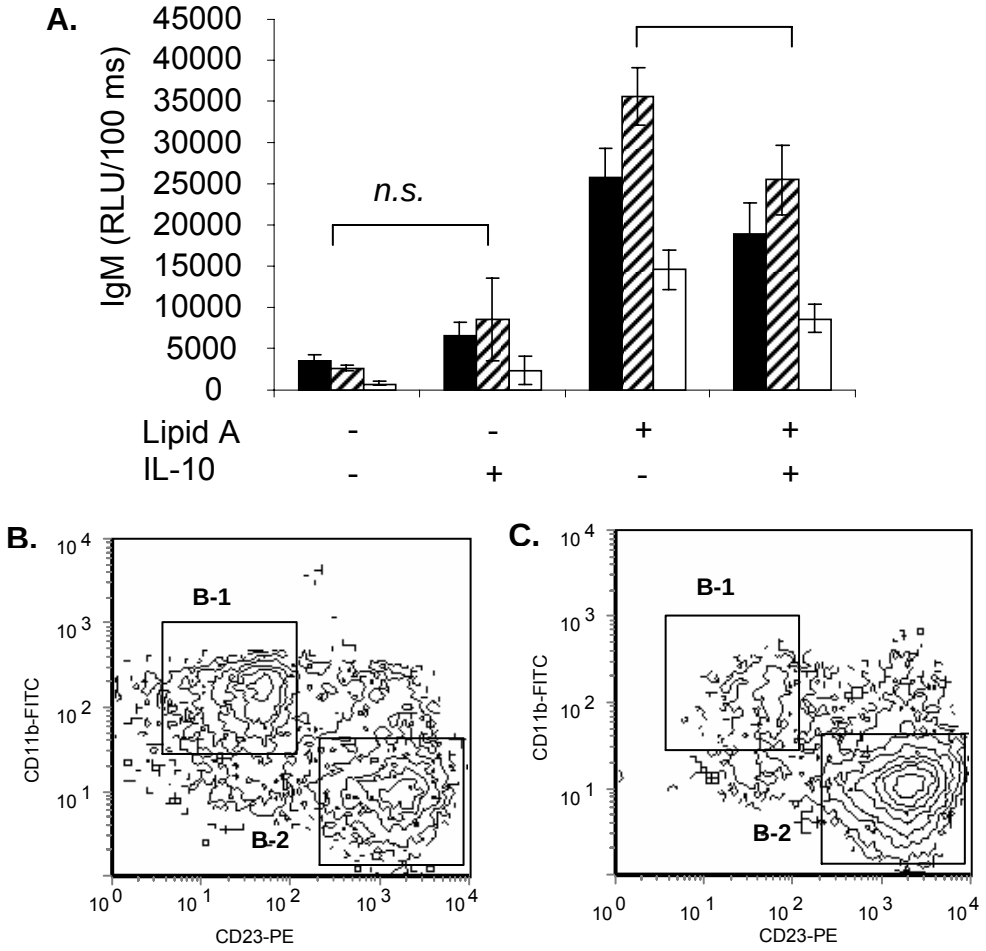



**Fig.3-13 Effect of IL-10 administration *in vivo*.** C57BL/6 12 weeks old male mice were injected i.p with $KdO_2$-lipidA 5 μg (n=5), $KdO_2$-lipidA 5 μg and rmIL-10 5 μg (n=5), rmIL-10 5 μg (n=3), or PBS (n=5). After 7 days, mice were sacrificed and analyzed for plasma IgM and peritoneal B cells. **A.** Total IgM (at 1:10,000 dilution, black bars), MDA-LDL IgM (1:1,000 dilution, striped bars) and Cu-OxLDL IgM (1:1,000 dilution, white bars). **B**. Peritoneal B-1 ($CD11b^+CD23^+$) and B-2 ($CD11b^+CD23^-$) cells in one representative mouse injected with $KdO_2$-lipidA. **C.** Peritoneal B-1 and B-2 cells in one representative mouse injected with $KdO_2$-lipidA and rmIL-10. The plots are peritoneal cells after gating on $CD19^+$ cells.

## Discussion

My studies showed that B-1 cells in culture, purified from naïve uninfected mice, gave rise to a whole array of IgM secreting cells with specificity for various oxidation epitopes, confirming the NAb origin of these oxidation-specific IgM. Indeed, such IgM were already detectable in supernatants of unstimulated B-1 cells in culture, and the production of these IgM in response to stimulation, especially that of TLRs, increased to a greater extent than total IgM or IgM to classic B-1 cell antigens, such as $\alpha$1,3-Dextran (34), with the exception of PC-BSA. In the case of PC-BSA, our ELISA method is probably not sensitive enough to detect the low amount of IgM against PC-BSA present in B-1 cell culture, since the readings were near background levels. A possible explanation for the low levels of IgM to PC-BSA in B-1 cell culture might be that PC-BSA only contains one specific epitope, whereas MDA-LDL and CuOxLDL both contain a mixture of epitopes on their protein surface and thus can be recognized by multiple clones of IgM.

Moreover, when taking the number of cells recovered after 7 days of stimulation into account, IL-5 seemed to induce B-1 cells to secrete more IgM per cell compared to TLR agonists stimulation, whereas TLR stimulation seemed to induce the proliferation of B-1 cells. Evidence supporting this claim comes from both the higher number of cells recovered after 7 days after TLR stimulation, and the thymidine incorporation assay and CFSE proliferation assay (**Fig.3-12**), which showed increased cell proliferation after $KdO_2$-lipidA stimulation. Therefore, B-1 stimulants might activate B-1 cells through different mechanisms.

In analogy to the robust anti-MDA-responses *in vivo* observed in different mouse models described in Chapter 2, MDA-specific IgM were the most dominant set of IgM Abs secreted by B-1 cells *in vitro* that were measured, and amazingly, constituted up to 30% of total IgM. These MDA-specific IgMs produced *in vitro* were specific for MDA-LDL, as shown by the competition immunoassay. Meanwhile, IgM Ab binding to CuOx-LDL could be competed about 50% by MDA-LDL. This is due to the fact that CuOx-LDL also shares MDA type adducts, in addition to OxPL and other epitopes. Native LDL, on the other hand, showed no competition for either MDA-LDL or CuOx-LDL.

I also demonstrated that TLR4 and TLR2 agonists were particularly effective in stimulating the proliferation of B-1 cells and generation of IgM, observations similar to that reported by Genestier et al. (14). This response to TLR4 stimulation was only partly dependent on MyD88 signaling, while TLR2 stimulation was completely MyD88 dependent. Interestingly, TLR4 deficiency did not fully block $KdO_2$-lipidA stimulation of B-1 cells. KdO2-lipid A is a highly purified bioactive form of LPS, and appears to be fully TLR4 dependent in macrophages (31). This suggests that there is an additional pathway for $KdO_2$-lipidA signaling in B-1 cells. In contrast, deficiency in TLR2 completely blocked TLR2 agonist induced activation of B-1 cells, suggesting that regardless of the roles of TLR1 and TLR6, TLR2 is an essential component for Pam3CSK4 and FSL-1 mediated activation of B-1 cells.

The stimulation of B-1 cells also induced increased secretion of cytokines. IL-10 levels in the supernatant were dramatically increased after TLR stimulation.

B-1 cells have been reported to secrete IL-10 (30), and IL-10 appears to have an autocrine effect on B-1 cells, as continuous administration of anti-IL-10 antibody to mice from birth blocked the development of B-1a cells and resulted in a depletion of B-1a cell population in these mice (35,36). Therefore, IL-10 is essential during B-1 development. However, the increased levels of IL-10 after TLR activation appear to have an opposite effect. When IL-10 was co-administered with $KdO_2$-lipidA to B-1 cells *in vitro*, $KdO_2$-lipidA induced proliferation and IgM secretion by B-1 cells were inhibited. On the other hand, IL-10 did not inhibit the differentiation of B-1 cells into plasma cells, or increase the rate of apoptosis, suggesting that the decreased production of IgM is probably due to decreased numbers of cells. The same inhibitory effect was observed *in vivo* when C57BL/6 mice were injected with IL-10.

IL-10 is generally considered as an anti-inflammatory cytokine. During infection it inhibits pro-inflammatory activity of Th1 cells, NK cells, and macrophages, all of which are required for pathogen clearance but also contribute to tissue damage if not regulated (37). In atherosclerosis, the overexpression of IL-10 has been shown to reduce the development of advanced atherosclerotic lesions in mice (38). A recent report by Yanaba et al defined a specific subset of B cells, which are $CD1d^{hi}CD5^{+}$ and share cell surface markers with both B-1 and MZ B cells, that can negatively regulate immune responses in mouse autoimmunity and inflammation models through the production of IL-10 (39,40). Therefore, the increased IL-10 production after TLR activation might

serve as a negative feedback signal for B-1 cells to counterbalance the inflammatory response, consistent with the anti-inflammatory role of IL-10.

Aside from IL-10, IL-6 level also increased after TLR activation. In humans, IL-6 has been shown to promote B-cell differentiation and the production of IgM (41). It is possible that IL-6 has a similar effect on TLR activated B-1 cells and induces increased production of IgM. However, it has also been reported that IL-6, like IL-10, can inhibit LPS-induced proliferation of peritoneal B cells (42). Further study will be needed to determine the effect of IL-6 on TLR activated B-1 cells.

TNF-$\alpha$ and IL-1$\beta$ were also induced after TLR activation. These cytokines are pro-inflammatory in the context of atherosclerosis (43). Their role in B-1 cell biology however is unknown. They might be induced in as an inflammatory response after TLR stimulation. IL-9 and IL-13 were also increased after TLR stimulation. They are thought to have anti-inflammatory effects (44,45), but their role in modulating B-1 cells will require further study.

In conclusion, the above data suggests that the secretion of IgM or oxidation-specific IgM by B-1 cells can be regulated directly by TLR activation of B-1 cells, or indirectly, via the cytokines that B-1 cells secrete after TLR activation, reflecting an integrated innate immune response to inflammation and infection.

Importantly, my data also confirmed that oxidation-specific IgMs are B-1 cell derived NAbs. Remarkably, my studies further suggest that these clones are more responsive to activation by TLRs, since oxidation-specific IgM increased to

a greater extent than total IgM or IgM to $\alpha$1,3-Dextran after TLR stimulation. This implies that evolutionary pressure might link the activation of innate PRRs to the generation of NAbs to oxidation-specific epitopes. This is intriguing as our lab and others have provided data suggesting that aside from products of exogenous pathogens, there are endogenous antigens capable of stimulating such TLRs, including OxLDL and specifically oxidized cholesteryl esters (10,46). However, further study will be needed to explore such speculation.

Now that I have demonstrated that oxidation-specific IgMs can be produced by B-1 cells *in vitro*, the next step would be to confirm whether the same holds true *in vivo* and test the hypothesis if oxidation-specific epitopes are a dominant target for NAbs secreted by B-1 cells. Immunodeficient mouse models replenished with B-1 cells will be utilized for this purpose and these studies will be described in Chapter 4.

**Acknowledgement**

Chapters 3, in part, has been accepted for publication in “Oxidation-specific Epitopes are a Dominant Target for Innate Natural Antibodies”, Journal of Clinical Investigation, 2009, by MY Chou, L. Fogelstrand, K. Hartvigsen, LF Hansen, D. Woelkers, PX Shaw, J. Choi, T. Perkmann, F Bäckhed, YI Miller, S. Hörkkö, M. Corr, JL Witztum and CJ Binder. The dissertation author was the primary investigator and author of this paper.

## References


1. **Horkko, S., E. Miller, D. W. Branch, W. Palinski, and J. L. Witztum**. 1997. The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). Proc. Natl. Acad. Sci. U. S. A **94**:10356-10361.

2. **Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum**. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J. Exp. Med. **200**:1359-1370.

3. **Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin**. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. **156**:1177-1185.

4. **Chang, M. K., C. J. Binder, M. Torzewski, and J. L. Witztum**. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. Proc. Natl. Acad. Sci. U. S. A **99**:13043-13048.

5. **Boullier, A., K. L. Gillotte, S. Horkko, S. R. Green, P. Friedman, E. A. Dennis, J. L. Witztum, D. Steinberg, and O. Quehenberger**. 2000. The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. J. Biol. Chem. **275**:9163-9169.

6. **Gillotte-Taylor, K., A. Boullier, J. L. Witztum, D. Steinberg, and O. Quehenberger**. 2001. Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. J. Lipid Res. **42**:1474-1482.

7. **Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum**. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc. Natl. Acad. Sci. U. S. A **96**:6353-6358.

8. **Sambrano, G. R., S. Parthasarathy, and D. Steinberg**. 1994. Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low density lipoprotein. Proc. Natl. Acad. Sci. U. S. A **91**:3265-3269.

9. **Beutler, B.** 2004. Inferences, questions and possibilities in Toll-like receptor signalling. Nature **430**:257-263.

10. **Miller, Y. I., S. Viriyakosol, C. J. Binder, J. R. Feramisco, T. N. Kirkland, and J. L. Witztum**. 2003. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J. Biol. Chem. **278**:1561-1568.

11. **Boullier, A., P. Friedman, R. Harkewicz, K. Hartvigsen, S. R. Green, F. Almazan, E. A. Dennis, D. Steinberg, J. L. Witztum, and O. Quehenberger**. 2005. Phosphocholine as a pattern recognition ligand for CD36. J. Lipid Res. **46**:969-976.

12. **Kawahara, T., H. Ohdan, G. Zhao, Y. G. Yang, and M. Sykes**. 2003. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. J. Immunol. **171**:5406-5414.

13. **Murakami, M., T. Tsubata, R. Shinkura, S. Nisitani, M. Okamoto, H. Yoshioka, T. Usui, S. Miyawaki, and T. Honjo**. 1994. Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. J. Exp. Med. **180**:111-121.

14. **Genestier, L., M. Taillardet, P. Mondiere, H. Gheit, C. Bella, and T. Defrance**. 2007. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J. Immunol. **178**:7779-7786.

15. **Takeda, K., T. Kaisho, and S. Akira**. 2003. Toll-like receptors. Annu. Rev. Immunol. **21**:335-376.

16. **Curtiss, L. K. and P. S. Tobias**. 2007. The toll of Toll-like receptors, especially toll-like receptor 2, on murine atherosclerosis. Curr. Drug Targets. **8**:1230-1238.

17. **Tobias, P. and L. K. Curtiss**. 2005. Thematic review series: The immune system and atherogenesis. Paying the price for pathogen protection: toll receptors in atherogenesis. J. Lipid Res. **46**:404-411.

18. **Palsson-McDermott, E. M. and L. A. O'Neill**. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology **113**:153-162.

19. **Kaisho, T. and S. Akira**. 2006. Toll-like receptor function and signaling. J. Allergy Clin. Immunol. **117**:979-987.

20. **Xu, X. H., P. K. Shah, E. Faure, O. Equils, L. Thomas, M. C. Fishbein, D. Luthringer, X. P. Xu, T. B. Rajavashisth, J. Yano, S. Kaul, and M. Arditi**. 2001. Toll-like receptor-4 is expressed by macrophages in murine and

human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. Circulation **104**:3103-3108.

21. **Michelsen, K. S., M. H. Wong, P. K. Shah, W. Zhang, J. Yano, T. M. Doherty, S. Akira, T. B. Rajavashisth, and M. Arditi**. 2004. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc. Natl. Acad. Sci. U. S. A **101**:10679-10684.

22. **Mullick, A. E., P. S. Tobias, and L. K. Curtiss**. 2005. Modulation of atherosclerosis in mice by Toll-like receptor 2. J. Clin. Invest **115**:3149-3156.

23. **Miller, Y. I., S. Viriyakosol, D. S. Worrall, A. Boullier, S. Butler, and J. L. Witztum**. 2005. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. Arterioscler. Thromb. Vasc. Biol. **25**:1213-1219.

24. **Podrez, E. A., E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P. J. Finton, L. Shan, B. Gugiu, P. L. Fox, H. F. Hoff, R. G. Salomon, and S. L. Hazen**. 2002. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. J. Biol. Chem. **277**:38503-38516.

25. **Pasare, C. and R. Medzhitov**. 2005. Control of B-cell responses by Toll-like receptors. Nature **438**:364-368.

26. **Ruprecht, C. R. and A. Lanzavecchia**. 2006. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur. J. Immunol. **36**:810-816.

27. **Ng, L. G., C. H. Ng, B. Woehl, A. P. Sutherland, J. Huo, S. Xu, F. Mackay, and K. P. Lam**. 2006. BAFF costimulation of Toll-like receptor-activatedB-1 cells. Eur. J. Immunol. **36**:1837-1846.

28. **Ray, A., P. Karmakar, and T. Biswas**. 2004. Up-regulation of CD80-CD86 and IgA on mouse peritoneal B-1 cells by porin of Shigella dysenteriae is Toll-like receptors 2 and 6 dependent. Mol. Immunol. **41**:1167-1175.

29. **Moon, B. G., S. Takaki, K. Miyake, and K. Takatsu**. 2004. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. J. Immunol. **172**:6020-6029.

30. **O'Garra, A., R. Chang, N. Go, R. Hastings, G. Haughton, and M. Howard**. 1992. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. Eur. J. Immunol. **22**:711-717.

31. **Raetz, C. R., T. A. Garrett, C. M. Reynolds, W. A. Shaw, J. D. Moore, D. C. Smith, Jr., A. A. Ribeiro, R. C. Murphy, R. J. Ulevitch, C. Fearns, D. Reichart, C. K. Glass, C. Benner, S. Subramaniam, R. Harkewicz, R. C. Bowers-Gentry, M. W. Buczynski, J. A. Cooper, R. A. Deems, and E. A. Dennis**. 2006. Kdo2-Lipid A of Escherichia coli, a defined endotoxin that activates macrophages via TLR-4. J. Lipid Res. **47**:1097-1111.

32. **Friguet, B., A. F. Chaffotte, L. Djavadi-Ohaniance, and M. E. Goldberg**. 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods **77**:305-319.

33. **Horkko, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum**. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J. Clin. Invest **103**:117-128.

34. **Kearney, J. F.** 2005. Innate-like B cells. Springer Semin. Immunopathol. **26**:377-383.

35. **Ishida, H., R. Hastings, and M. Howard**. 1992. Multiple IL-10 antibody treatment blocks the development of Ly-1 lineage B cells. Ann. N. Y. Acad. Sci. **651**:264-265.

36. **Ishida, H., R. Hastings, J. Kearney, and M. Howard**. 1992. Continuous anti-interleukin 10 antibody administration depletes mice of Ly-1 B cells but not conventional B cells. J. Exp. Med. **175**:1213-1220.

37. **Couper, K. N., D. G. Blount, and E. M. Riley**. 2008. IL-10: the master regulator of immunity to infection. J. Immunol. **180**:5771-5777.

38. **Pinderski, L. J., M. P. Fischbein, G. Subbanagounder, M. C. Fishbein, N. Kubo, H. Cheroutre, L. K. Curtiss, J. A. Berliner, and W. A. Boisvert**. 2002. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. Circ. Res. **90**:1064-1071.

39. **Yanaba, K., J. D. Bouaziz, K. M. Haas, J. C. Poe, M. Fujimoto, and T. F. Tedder**. 2008. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity. **28**:639-650.

40. **Matsushita, T., K. Yanaba, J. D. Bouaziz, M. Fujimoto, and T. F. Tedder**. 2008. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J. Clin. Invest **118**:3420-3430.

41. **Bertolini, J. N. and E. M. Benson**. 1990. The role of human interleukin-6 in B-cell isotype regulation and differentiation. Cell Immunol. **125**:197-209.

42. **O'Garra, A. and M. Howard**. 1992. Cytokines and Ly-1 (B1) B cells. Int. Rev. Immunol. **8**:219-234.

43. **Tedgui, A. and Z. Mallat**. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev. **86**:515-581.

44. **Wynn, T. A.** 2003. IL-13 effector functions. Annu. Rev. Immunol. **21**:425-456.

45. **Demoulin, J. B. and J. C. Renauld**. 1998. Interleukin 9 and its receptor: an overview of structure and function. Int. Rev. Immunol. **16**:345-364.

46. **Harkewicz, R., K. Hartvigsen, F. Almazan, E. A. Dennis, J. L. Witztum, and Y. I. Miller**. 2008. Cholesteryl ester hydroperoxides are biologically active components of minimally oxidized low density lipoprotein. J. Biol. Chem. **283**:10241-10251.

# CHAPTER 4

## OXIDATION-SPECIFIC EPITOPES ARE DOMINANT TARGETS OF INNATE NATURAL ANTIBODIES

**Abstract**

Oxidation of LDL can generate a wide variety of neo-self determinants, which we termed “oxidation-specific epitopes”. There is now ample evidence that oxidation-specific epitopes are not only restricted to OxLDL and atherosclerotic lesions, but are ubiquitously present on apoptotic cells and in inflamed tissues found in many different diseases. Therefore, I hypothesize that B-1 cell clones directed to oxidation-specific epitopes might be conserved by natural selection due to evolutionary pressure. Thus, oxidation-specific epitopes might be an important target for B-1 cell derived NAbs. In this chapter, I provide multiple lines of evidence showing that oxidation-specific epitopes constitute a dominant, previously unrecognized target for NAbs in mice. Utilizing a B-1 cell reconstituted mouse model that expresses solely NAbs, I show that natural IgM Abs to oxidation-specific epitopes are present in vivo and that about 30% of all NAbs bind to model oxidation epitopes. These oxidation-specific IgM Abs might play an important role in mediating homeostatic functions consequent to apoptosis and inflammation.

## Introduction

### Oxidation-specific epitopes are evolutionarily important epitopes

Our lab first demonstrated that the oxidation of LDL can generate a wide variety of neo-self determinants, which we termed “oxidation-specific epitopes”. The generation of these oxidation-specific epitopes were found to lead to robust cellular and humoral responses in atherosclerosis, which in turn led to the consideration of these epitopes of OxLDL as relevant disease-specific antigens (2). Using specific Abs against MDA-lysine, 4-HNE-lysine or PC of OxPL, our lab has demonstrated the presence of oxidation-specific epitopes in lesions ranging from early fatty streaks to advanced lesions (3-5).

Importantly, the presence of oxidation-specific epitopes is not only restricted to OxLDL and atherosclerosis lesions, but are ubiquitously present in inflamed tissues found in many different diseases (see **Table 1-2**). In the case of PC, it’s even present on the surface of bacteria (6). Since oxidative events are a key process in cells undergoing apoptosis, oxidation-specific epitopes can be found on apoptotic cell surfaces as well as microparticles that bud off from apoptotic cells (7-9). Microparticles can be found in plasma and have many bioactive and proinflammatory properties (10). Such inflammatory reactions and oxidative stress occur throughout our life span, therefore we suggest that oxidation-specific epitopes associated with these processes are important self-epitopes that confer a strong evolutionary pressure.

**Repertoire of B-1 cells is selected to bind evolutionarily important epitopes**

LDL is a large particle that is abundant in polyunsaturated fatty acids (PUFA), which are susceptible to oxidation. LDL also contains apoB-100, a 513 KDa protein capable of forming multiple copies of similar adducts on its surface when oxidized. Thus, OxLDL, containing multiple copies of oxidation-specific epitopes on its surface, is capable of stimulating not only classic adaptive response, but also thymus-independent type 2 responses that usually require multivalent ligands (11,12). This renders OxLDL immunogenic and Abs are generated toward a variety of oxidation-specific epitopes present on OxLDL.

Cholesterol-fed apoE$^{-/-}$ mice develop high IgM autoantibody titers to a wide variety of oxidation-specific epitopes. Among the Abs generated toward OxLDL is E06, a B-1 cell derived NAb, which recognizes PC both on OxPL and *S. pneumoniae* (5,13,14). NAbs derived from B-1 cells shows a restricted use of $V_H$ genes that are minimally edited and reflective of germ-line usage (15). Although the exact mechanism of B-1 cell clone selection and expansion is unclear, it is now appreciated that antigen selection during fetal and neonatal periods made by endogenous “self-antigens” can lead to positive selection for B-1 cells (16-18), while in contrast, such encounters with B-2 cells early in life would lead to negative selection via apoptosis (clonal deletion) or anergy. Thus, the repertoire of B-1 cells is selected to bind evolutionarily important epitopes.

As discussed previously, because oxidation-specific epitopes are so ubiquitous and also constitute a class of PAMPs that can be recognized by innate PRRs, we believe that they are evolutionarily important epitopes. B-1 cell

clones toward these epitopes might be conserved by natural selection for beneficial effects. Therefore, my hypothesis is that **oxidation-specific epitopes might be a dominant target for NAbs, consistent with the oxygen-rich environment that we live in**. Furthermore, because many, if not most NAbs have dual specificities (15), typically with molecular epitopes on pathogens, as with the example of E06, an encounter later in life with an external pathogen can serve to further expand a given B-1 cell clone (14,19), providing immediate host defense against pathogens.

**Rationale and study design**

To study B-1 cell derived NAbs *in vivo*, B-1 cells will be isolated from the peritoneum of wild-type C57BL/6 mice and then adoptively transferred into *Rag1*$^{-/-}$ mice, which lack functional B- and T-cells (20). Since B-1 cells have the ability to self-replenish (21), all Abs that are produced in the *Rag1*$^{-/-}$ recipients should be B-1 cell derived NAbs.

In order to validate this mouse model, peritoneal exudate cells (PEC) and splenocytes from *Rag1*$^{-/-}$ B-1 recipients will be analyzed by FACS to verify B-1 cell engraftment. Antibody levels and isotypes that are reconstituted in the plasma of *Rag1*$^{-/-}$ B-1 cell recipients will also be quantified by ELISA.

After validation of the mouse model, the next step will be to demonstrate that natural IgM Abs against oxidation-specific epitopes also exists *in vivo*. IgM titers in the plasma of *Rag1*$^{-/-}$ B-1 cell recipients specific for a variety of oxidation-antigens will be assessed for this purpose. To test my hypothesis that oxidation-specific epitopes are a dominant target for NAbs, the percentage of natural IgM Abs that is targeted towards oxidation antigens will be determined both by examining the percentage of oxidation-specific IgM secreting cells in the spleen of *Rag1*$^{-/-}$ B-1 recipients with ELISpot assay, and by examining the percentage of IgM activity in the plasma of *Rag1*$^{-/-}$ B-1 recipients that bind to oxidation-specific antigens..

Hybridomas will be generated from the spleens of these *Rag1*$^{-/-}$ B-1 recipients. By default, all IgM produced will be B-1 cell derived NAbs. The hybridomas will then be screened for their reactivity to oxidation-specific antigens

to obtain anti-OxLDL monoclonal IgM Abs of B-1 cell origin. The binding specificities and affinities, genetic lineage and biological properties of the mAbs will be studied. The generation of germ-line mAbs against oxidation-specific epitopes will enable us to study the properties of the oxidation-specific NAbs and also serves as another line of evidence for the existence of NAbs against oxidation-specific epitopes *in vivo*.

The main focus of this chapter is first to demonstrate that B-1 cells produce oxidation-specific IgM also *in vivo*, utilizing the B-1 cell mouse model that I have established. Secondly, to test my hypothesis and demonstrate that oxidation-specific epitopes are indeed a dominant target for B-1 cell derived natural IgM Abs.

## Results

### Characterization of Rag1$^{-/-}$ recipients adoptively transferred with B-1 cells

In Chapter 3, I demonstrated that B-1 cells can secrete IgM NAbs against oxidation-specific epitopes *in vitro*. To test whether innate B-1 cells can also secrete oxidation-specific IgM *in vivo*, peritoneal B-1 cells from naïve C57BL/6 mice were isolated as previously described in Chapter 3. Either 0.5x10$^6$ or 1x10$^6$ purified B-1 cells were then adoptively transferred into the peritoneum of each *Rag1*$^{-/-}$ recipient mice. For a typical transfer, about 3 to 4 donor mice were needed for each recipient, and the average yield of pure B-1 cells was about 10% of total peritoneal cavity cells sorted.

To verify the purity of B-1 cells before transfer, purified B-1 cells were analyzed by FACS. Before sorting, both B-2 cells (CD19$^+$/CD23$^+$) and B-1 cells (CD19$^+$/CD23$^-$) were present. Cells were sorted for the CD19$^+$/CD23$^-$ population. After sorting, 99% of the cells were B-1 cells (**Fig.4-1, upper panels**). Further staining of CD5 showed that the sorting process did not alter the percentage of B-1a cells (CD19$^+$/CD5$^+$) versus B-1b cells (CD19$^+$/CD5$^-$), the ratio remained 1:1 after FACS sorting (**Fig.4-1, lower panels**). To avoid possible T cell contamination, CD3 staining was also included in most experiments to actively exclude T cells.

The sorted B-1 cells were then adoptively transferred into *Rag1*$^{-/-}$recipients. The adoptive transfer led to selective reconstitution of only B-1 cells in the

peritoneum of *Rag1*$^{-/-}$ recipients, and both B-1a (CD5$^{+}$/CD19$^{+}$/CD11b$^{+}$) and B-1 b cells (CD5$^{-}$/CD19$^{+}$/CD11b$^{+}$) were detected (**Fig.4-2 lower panels**).

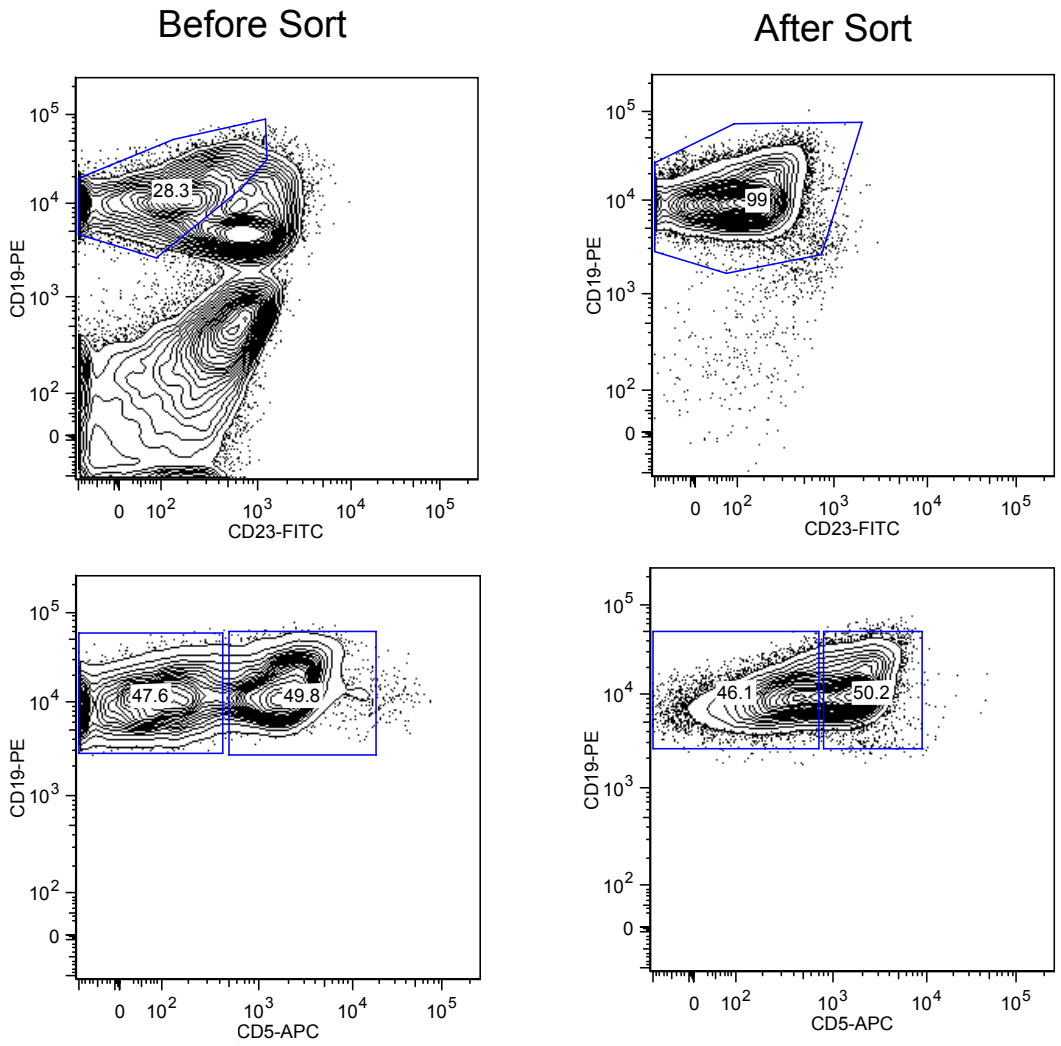



**Fig.4-1 Characterization of B-1 cells after FACS sorting.** Peritoneal exudate cells (PEC) were isolated from donor C57BL/6 mice and sorted for B-1 cells as the CD19$^{+}$/CD23$^{-}$ population. PEC were analyzed by FACS before and after FACS sorting. 99% of the sorted cells are CD19$^{+}$/CD23$^{-}$ (**After sort**). The percentages of B-1a (CD19$^{+}$/CD5$^{+}$) versus B-1b (CD19$^{+}$/CD5$^{-}$) cells were also analyzed (**lower panel**). In this analysis the cells were first gated on live cells based on forward and side scatter.

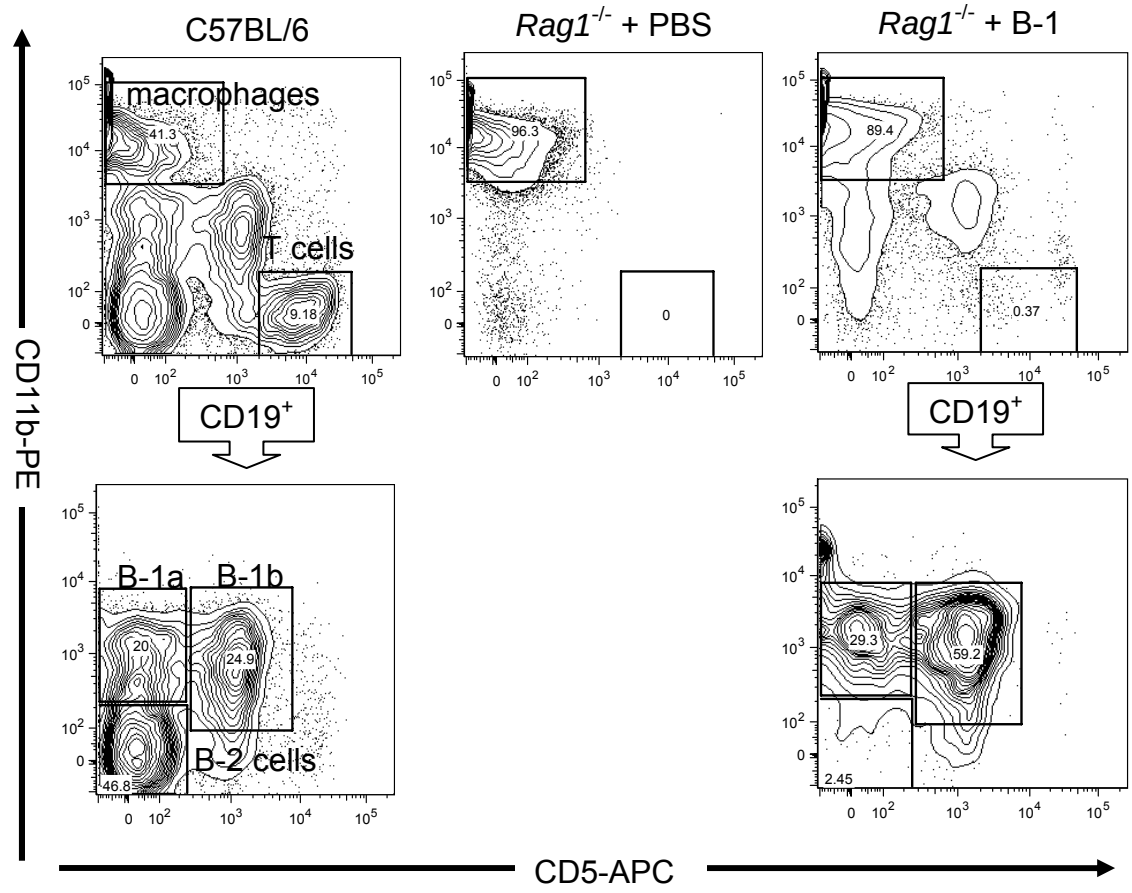



**Fig.4-2 Adoptive transfer of B-1 cells into *Rag1*$^{-/-}$ mice replenishes peritoneal cavity B-1 cell population.** *Rag1*$^{-/-}$ mice were injected with PBS (*Rag1*$^{-/-}$ + PBS) or with B-1 cells (*Rag1*$^{-/-}$ + B-1). After 10 weeks, peritoneal exudate cells (PEC) were isolated and analyzed by FACS**.** In Wild-type C57BL/6 mice, peritoneal macrophage (CD11b$^{high}$/CD5$^{-}$) and T cells (CD11b$^{-}$/CD5$^{high}$) are intact **(upper left panel)**. B-1 cells (CD19$^{+}$) can be divided into B-1a (CD19$^{+}$/CD11b$^{int}$/CD5$^{int}$), B-1b (CD19$^{+}$/CD11b$^{int}$/CD5$^{-}$) and B-2 cells (CD19$^{+}$/CD11b$^{-}$/CD5$^{-}$) **(lower left panel)**. In PBS injected *Rag1*$^{-/-}$ recipients, all lymphocyte populations were absent **(upper middle panel)**. 10 weeks after transfer, B-1 cell populations were reconstituted in the peritoneal cavity of *Rag1*$^{-/-}$ recipients injected with B-1 cells without contamination of B-2 cell or T cells (**right panel**). Cells were first gated on live cells based on forward and side scatter.

Conventional B-2 cells and T-cells were typically not found in the recipient mice (**Fig.4-2**). The average percentages of B-1a and B-1b cells among total cells analyzed in the peritoneum of *Rag1*$^{-/-}$ B-1 recipients were 11.0%±2.6% and 8.5%±1.0%, respectively (n=12), compared to 18.2%±3.2% and 8.3%±2.3% (n=4) in age-matched wild-type C57BL/6 mice.

The adoptive transfer also reconstituted B-1 cell population in the spleens of *Rag1*$^{-/-}$ B-1 recipients. In wild-type C57BL/6 mice, B-1 cells (IgM$^{+}$/CD43$^{+}$) averaged about 2.7% of total splenocytes (n=3), agreeing with previous literature that B-1 cells constitute about 2-5% of total splenocytes (21) (**Fig.4-3,** C57BL/6). In the reconstituted *Rag1*$^{-/-}$ B-1 recipients, B-1 cells constituted an average of about 1% of total splenocytes analyzed (**Fig. 4-3,** Rag$^{-/-}$ + B-1) (data ranged from 0.4% to 1.4%, n=9). Moreover, reconstitution of B-1 cells in the spleen was also demonstrated by the number of splenic IgM secreting cells (ISC) as measured by ELISpot: 60±15 ISC vs. 152±21 ISC per 200,000 splenocytes in RAG$^{-/-}$ B-1 recipients (n=9) and C57BL/6 mice (n=7), respectively (**Fig.4-8**).

The data above establish the successful engraftment of B-1 cells into the peritoneum of the recipient Rag1-/- mice and migration of B-1 cells into the spleen, where antibody secretion is primarily believed to occur. To document that antibody secretion also occurred, I quantified the levels of each Ab isotype in the plasma of donor C57BL/6 mice and *Rag1*$^{-/-}$ B-1 recipients 10 weeks after the transfer of B-1 cells from 2 separate transfer experiments. NAbs are mainly of the IgM and IgG3 isotype (22), and both IgM and IgG3 were detected in *Rag1*$^{-/-}$ B-1

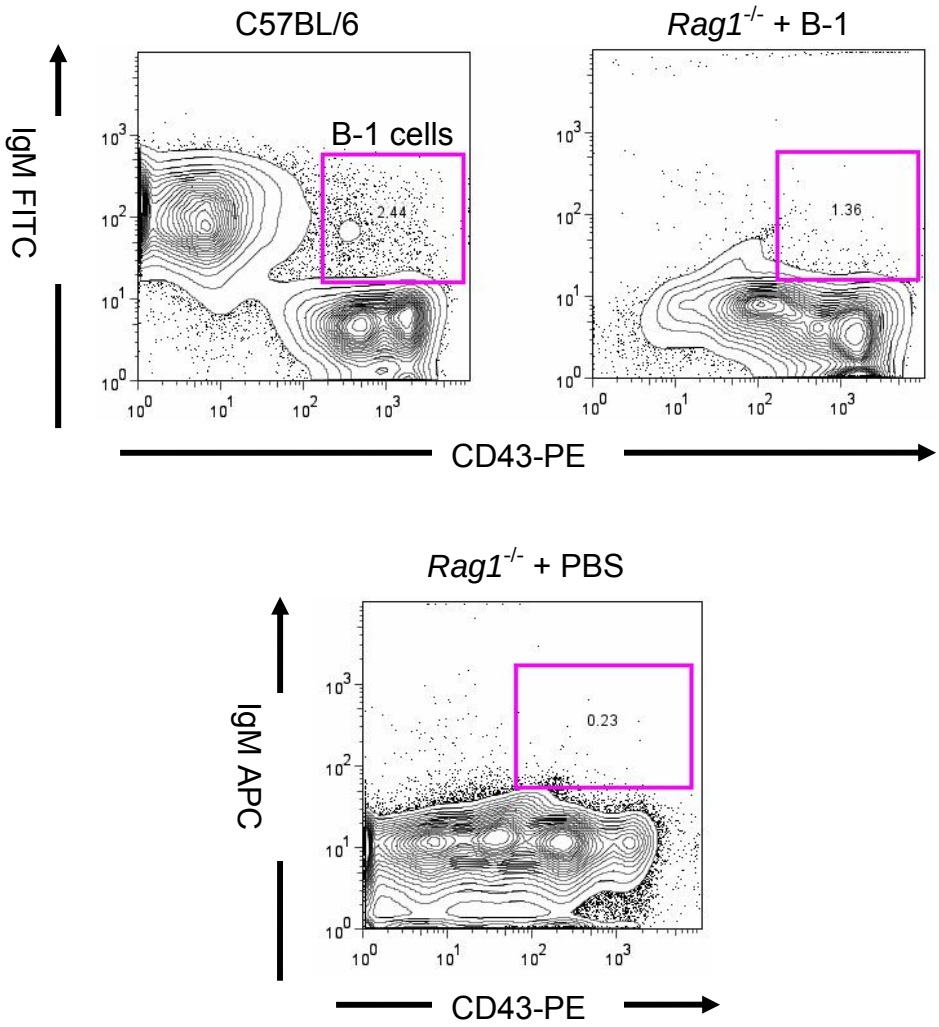



**Fig.4-3 B-1 cells can be detected in the spleen after adoptive transfer of B-1 cells into *Rag1*$^{-/-}$ mice.** Splenocytes were also analyzed by FACS 10 weeks after the adoptive transfer of B-1 cells into *Rag1*$^{-/-}$ mice. In C57BL/6 mice, B-1 cells (IgM$^{+}$/CD43$^{+}$) in the spleen constitute about 2.4% of total splenocytes **(upper left panel)**. In B-1 cells injected *Rag1*$^{-/-}$ recipients (*Rag1*$^{-/-}$ + B-1), B-1 cells were about 1.4% of total splenocytes **(upper right panel)**. B-1 cells were absent from the spleen of *Rag1*$^{-/-}$ mice injected with PBS **(lower panel)**. The cells were first gated on live cells based on forward and side scatter.

**Table 4-1 Levels of each antibody isotype in the plasma of wild-type C57BL/6 and B-1 reconstituted *Rag1*$^{-/-}$ mice**

| | C57BL/6 | Rag1$^{-/-}$ + B-1 | Rag1$^{-/-}$ + PBS |
|---|---|---|---|
| **IgM** | 91.6±18.85 | 18 ± 3.94 | ND |
| **IgG1** | 352.2±57.26 | ND | ND |
| **IgG2a/c** | 4.3±1.35 | ND | ND |
| **IgG2b** | 436.4±126.02 | 7 ± 4.75 | ND |
| **IgG3** | 163.8±84.52 | 122 ± 35.54 | ND |
| **IgA** | 0.28±0.05 | ND | ND |

Plasma were collected from *Rag1*$^{-/-}$ recipients 10 weeks after transfer of either B-1 cells (*Rag1*$^{-/-}$+B-1, n=6) or PBS (*Rag1*$^{-/-}$+PBS, n=7), and age matched C57BL/6 mice (n=5). The amount of each Ab isotype in plasma were tested by ELISA. Values are in μg/ml. ND: not detected

recipients as expected (**Table 4-1**). From these 2 experiments, the IgM levels in donor C57BL/6 mice were about 5 fold higher than in *Rag1*$^{-/-}$ B-1 recipients. However, IgG3 levels in *Rag1*$^{-/-}$ B-1 recipients were reconstituted to about 70% of the levels in donor C57BL/6 mice. Another dominant isotype of NAbs is IgA, which is mainly secreted by the mucosal linings and is present only in small amounts in the plasma. Therefore, IgA could not be detected in the plasma of *Rag1*$^{-/-}$ B-1 recipients. Other IgG isotypes, such as IgG1, IgG2a/c, IgG2b were either absent or barely detectable in *Rag1*$^{-/-}$ B-1 recipients. None of the Ab isotype were detected in *Rag1*$^{-/-}$ PBS recipients, as expected.

**B-1 cells secrete IgM NAbs against oxidation-specific epitopes in vivo**

Consistent with data shown in **Table 4-1**, by the 10$^{th}$ week after transfer, B-1 cell reconstituted *Rag1*$^{-/-}$ mice developed significant plasma titers of IgM, (**Fig.4-4, (a)**). *Rag1*$^{-/-}$ mice that received only PBS did not have any plasma IgM. In a series of eight similar transfer experiments, the extent of reconstitution, as indicated by the levels of plasma IgM, varied and in part appeared to be related positively to the number of B-1 cells transferred and the time after transfer studied. For example, in transfer experiments in which 0.5x10$^6$ cells were transferred, and then the plasma examined after 10 weeks, the percent of IgM reconstitution compared to donor B6 mice ranged from 20-40%, whereas in experiments which 1x10$^6$ cells were transferred and then the plasma examined after 15 weeks, the percent of IgM reconstitution increased to about 60%.

Remarkably, in all of the transfers, the recipient mice exhibited robust IgM titers against oxidation-specific epitopes (**Fig.4-4, (b-c)(e-f)**), including MDA-LDL, CuOx-LDL, PC-BSA, and MAA-BSA but not to BSA or native LDL (data not shown). IgM to the B-1 prototypic antigen, $\alpha$1,3-Dextran, was present in *Rag1*$^{-/-}$ B-1 recipients as well. These specific titers were typically similar to the ones found in naïve wild-type mice, although lower in general. Moreover, consistent with the fact that T15-idiotypic Abs are predominantly secreted by B-1 cells (13), I found that adoptive B-1 cell transfer gave rise to T15-idiotypic IgM in *Rag1*$^{-/-}$ mice as well (**Fig. 4-4,(h)**). The experiment shown in **Fig.4-4** is the antibody binding dilution curves to oxidation-specific epitopes and other antigens for a typical transfer of ~99% pure B-1 cells. The actual titers are shown in the upper right

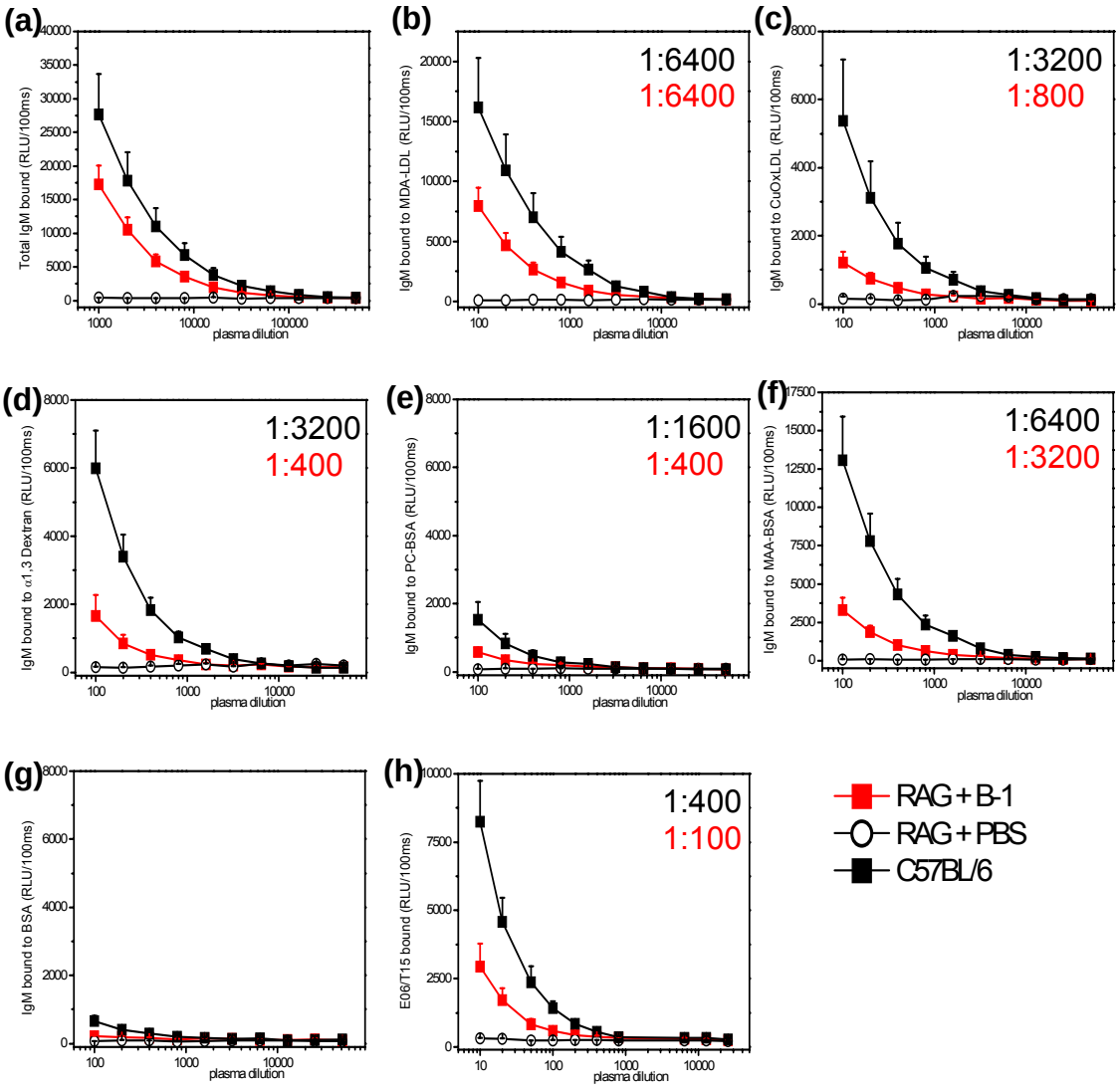



**Fig.4-4 IgM to oxidation-specific epitopes are present in the plasma of B-1 reconstituted *Rag1*$^{-/-}$ mice free of T cells.** Plasma collected from *Rag1*$^{-/-}$ recipients 15 weeks after transfer of either B-1 cells (*Rag1*$^{-/-}$ + B-1, n=8) or PBS (*Rag1*$^{-/-}$ + PBS, n=6), and age matched C57BL/6 mice (n=7) were tested for specific IgM binding by ELISA. In these experiments the purity of transferred B-1 cells was >99% and no T cells were detected in the recipients. Data shown are from one representative experiment out of 6 experiments. T15-idiotypic IgM, E06, can be detected in *Rag1*$^{-/-}$ + B-1 mice as well. A T15-specific anti-idiotype Ab (AB1-2) (1) was used as capture Ab, followed by incubation with AP-labeled goat anti-mouse IgM. Values are means and SEM.

hand corner of each individual graph in **Fig. 4-4**; red represents the actual IgM titers in *Rag1*$^{-/-}$ B-1 recipients, and black represents the actual IgM titers in donor C57BL/6 mice.

In one particular experiment, where a small contamination of T cells (estimated to be < 3% of cells) was present in the adoptively transferred cell population, the IgM levels were similar to that of wild-type C57BL/6 at 10 weeks after transfer (**Fig.4-5**). In all subsequent transfer experiments, in which T cells were actively excluded with CD3 staining, and B-1 cells constituted ~99% of reconstituted lymphocytes, the extent of IgM measured at 10 weeks was generally lower compared to wild-type mice (**Table 4-1** and **Fig.4-4**). Therefore, co-transferred T-cells appears to promote IgM secretion in the recipient mice, possibly in part by secretion of cytokines such as IL-5, which our laboratory has previously shown augments secretion of OxLDL specific IgM by B-1 cells (**Fig.3-1, 3-2** and (23)).

Again, similar to the results observed following *in vitro* stimulation of B-1 cells, oxidation-specific IgM were a major fraction of the total IgM in these *Rag1*$^{-/-}$ B-1 reconstituted recipients as well. Competition immunoassays with pooled plasma of recipient mice demonstrated high specificity of the MDA-specific IgM Abs, as neither OxLDL nor native LDL competed for binding to MDA-LDL (**Fig. 4-6**).

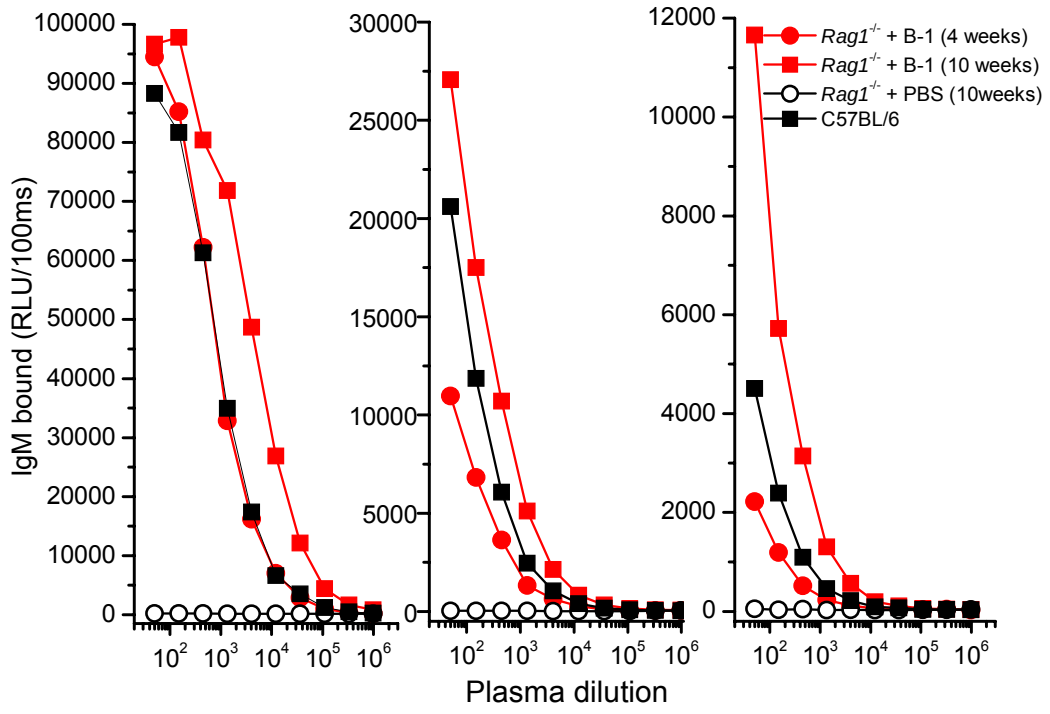



**Fig.4-5 T cells increased IgM levels to oxidation-specific epitopes in B-1 reconstituted *Rag1*$^{-/-}$ mice.** Pooled plasma collected from *Rag1*$^{-/-}$ recipients 4 and 10 weeks after transfer of either B-1 cells (*Rag1*$^{-/-}$ + B-1, n=5) or PBS (*Rag1*$^{-/-}$ + PBS, n=6) were tested for specific IgM binding by ELISA. Plasma from age-matched C57BL/6 mice (n=3) were also included. Plasma IgM Abs specific for MDA-LDL and CuOx-LDL could be detected after 4 weeks of transfer and increased with time. In this experiment minor contamination of T cells (< 3% of transferred cells) was detected. Each value is the mean of triplicate determinations.

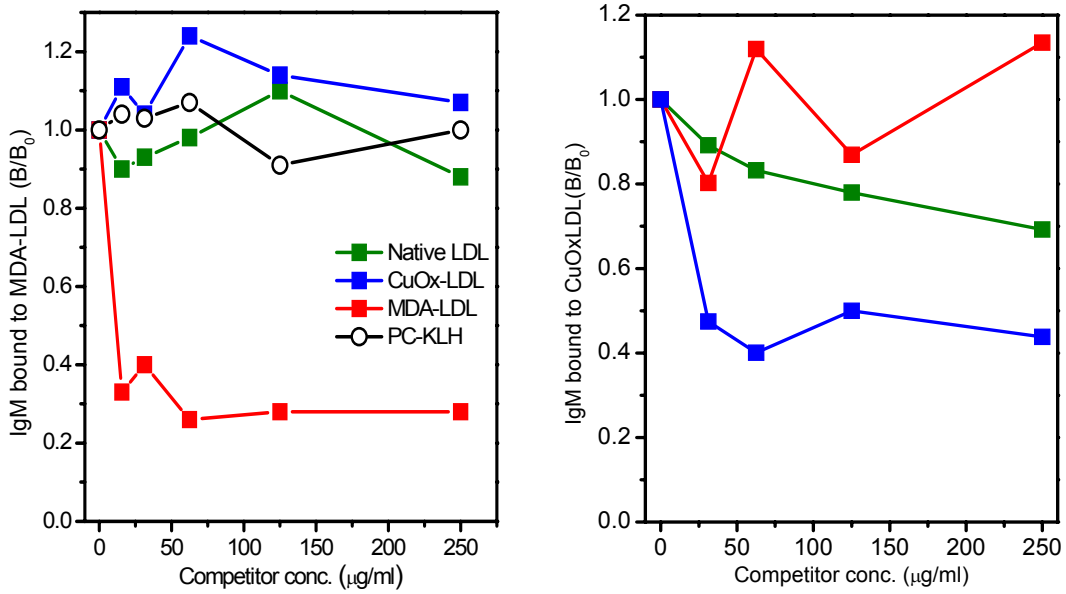



**Fig.4-6 Natural IgM Abs produced *in vivo* show specificity to MDA-LDL and CuOxLDL**. For competition immunoassays, plasma from B-1 cell recipient *Rag1*$^{-/-}$ mice (n=3-8) were pooled and diluted to 1:100 for MDA-LDL and 1:200 for CuOx-LDL and incubated in the absence or presence of indicated concentrations of competitors overnight. After incubation, Ab-competitor complexes were pelleted by centrifugation, and supernatants tested for IgM binding to indicated antigen. Data are the mean of triplicate determinations, expressed as ratio of IgM binding to MDA-LDL or CuOx-LDL in the presence or absence of competitor ($B/B_0$). Data shown are from one representative experiment.

I also calculated binding avidities for the IgM in the *Rag1*$^{-/-}$ B-1 recipient plasmas for oxidation epitopes using the Klotz method as described previously in Chapter 3 (24). The dissociation constants (Kds) for MDA-LDL and OxLDL in *Rag1*$^{-/-}$ B-1 recipient plasma were $9.9 \times 10^{-9}$ and $1.42 \times 10^{-7}$ mol/L, respectively. These values are within the same range as the Kds determined for IgM in the supernatants of cultured B-1 cells for MDA-LDL and OxLDL.

Only low titers of IgG against oxidation-specific epitopes were found in the plasma of *Rag1*$^{-/-}$ B-1 recipient mice, agreeing with the data shown in **Table 4-1**. The IgG Ab against oxidation-specific epitopes were predominantly of the IgG3 isotype, which are known to be secreted by B-1 cells in a T-cell independent fashion (22) (data not shown).

**Oxidation epitopes are a dominant target of natural IgM antibodies.**

To directly address the extent to which oxidation-specific IgM contribute to the total IgM NAb pool, I performed absorption studies using pooled plasma from *Rag1*$^{-/-}$ mice reconstituted with B-1 cells. In these experiments, specific IgM were absorbed from plasma with selected oxidation-specific model antigens and the amount of remaining IgM measured. Strikingly, both MDA-LDL as well as OxLDL, which contains a variety of oxidation-specific epitopes, absorbed out ~10% of all IgM, respectively, while native LDL did not compete (**Fig. 4-7**). Furthermore, the MDA specific MAA epitope, conjugated to mouse serum albumin (MAA-MSA) was capable of absorbing up to 25% of all IgM. Moreover, a combination of all model antigens removed 35% of the NAbs in these plasmas (**Fig. 4-7**). Thus, a surprisingly large percentage of B-1 cell derived NAbs are directed against various oxidation-specific epitopes.

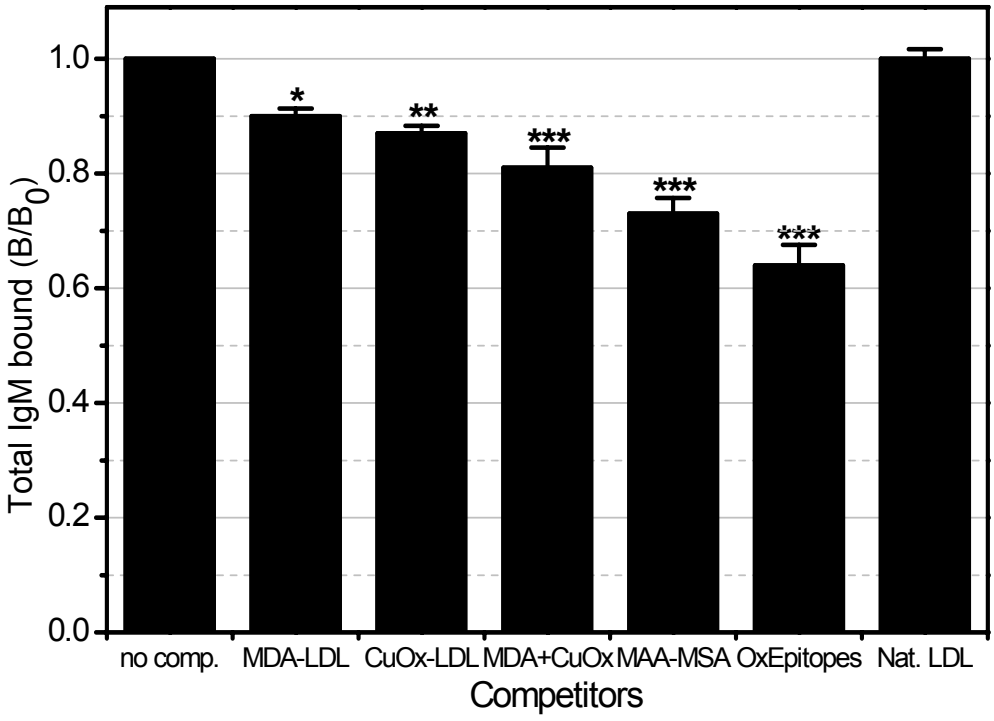



**Fig.4-7 Pre-absorption of plasma of *Rag1*$^{-/-}$ plus B-1 cells with oxidation-specific antigens shows that oxidation-specific epitopes are dominant targets for NAbs.** Plasma from *Rag1*$^{-/-}$ B-1 cell recipients were pre-incubated in the absence or presence of indicated antigens (250ug/ml total antigen) overnight and antigen-immune complexes pelleted by centrifugation. Plasma IgM levels were then tested by ELISA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to native LDL (ANOVA with Tukey-Kramer Multiple Camparisons Test). Data shown are plasma samples from 7 *Rag1*$^{-/-}$ B-1 cell recipients (for MAA-MSA and OxEpitopes, n=3), and each sample was assayed in triplicates. Values are means and SEM.

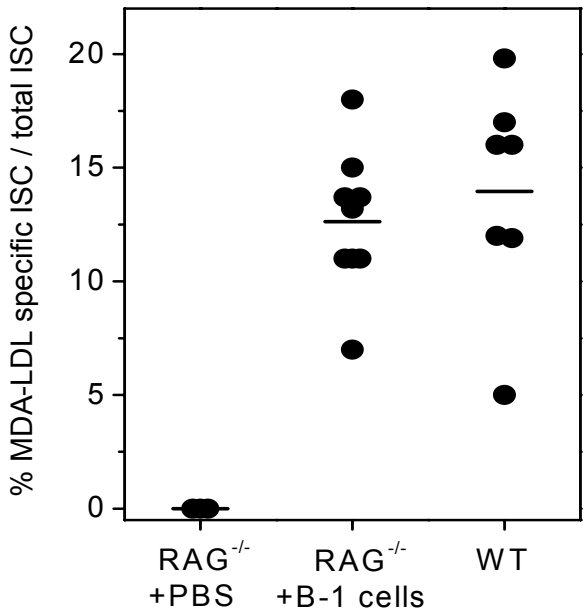



**Fig.4-8 ELISpot assay of frequencies of MDA-LDL specific IgM secreting cells (ISCs).** At time of sacrifice, splenocytes were harvested and frequencies of specific ISCs in the spleens of wild-type C57BL/6 mice (WT), B-1 reconstituted *Rag1*$^{-/-}$ mice (*Rag1*$^{-/-}$ + B-1), and *Rag1*$^{-/-}$ mice that received PBS (*Rag1*$^{-/-}$ + PBS) were determined as described in methods in Chapter 8. Results are from individual mice and data are from 3 separate B-1 cell transfer experiments. Horizontal bar represents the mean for the group. *** $p<0.001$ compared to *Rag1*$^{-/-}$ + PBS (unpaired t test).

The prominent representation of oxidation-specific IgM in plasma was also reflected by the frequency of MDA-specific IgM secreting cells in the spleens of reconstituted *Rag1*$^{-/-}$ mice. As expected, no ISC were detected in the spleens of mice injected with PBS (**Fig. 4-8**). In contrast, in *Rag1*$^{-/-}$ B-1 recipients, ~12% of all ISC were found to have specificity for MDA-LDL (**Fig. 4-8**). Interestingly, the

frequency of MDA-LDL specific ISC in spleens of wild type mice was found to be similarly high (**Fig. 4-8**), demonstrating that a large percentage of all splenic ISCs have specificity for MDA-modifications, and a majority of these ISCs are likely derived from B-1 cells.

**Generation of B-1 cell derived monoclonal antibodies**

The hypothesis that oxidation-specific epitopes are an important target for B-1 cell derived NAbs was further corroborated by the characterization of monoclonal Abs (mAbs) derived from hybridomas prepared from the spleens of B-1 cell reconstituted *Rag1*$^{-/-}$ mice. The hybridomas were screened for reactivity to oxidation-specific antigens such as MDA-LDL by ELISA. From two separate fusions, 20-30% of all IgM secreting hybridomas were found to have reactivity for MDA-LDL (data not shown), demonstrating again the dominance of oxidation-specific IgM in B-1 cell derived natural IgMs. One such mAb, NA-17, was characterized in more detail. NA-17 is an IgM that recognizes MDA-related modifications on LDL such as MDA-LDL, MAA-LDL, and MAA-BSA (**Fig. 4-9**).

The apparent Kds of NA-17 for MAA-BSA was 6.85 x $10^{-8}$ mol/L; this value is similar to the Kds determined for IgMs specific for MDA-LDL and CuOx-LDL in the plasma of *Rag1*$^{-/-}$ B-1 recipients as well as in culture supernatants of B-1 cells, suggesting similar binding avidities for these B-1 cell derived oxidation-specific natural IgM Abs.

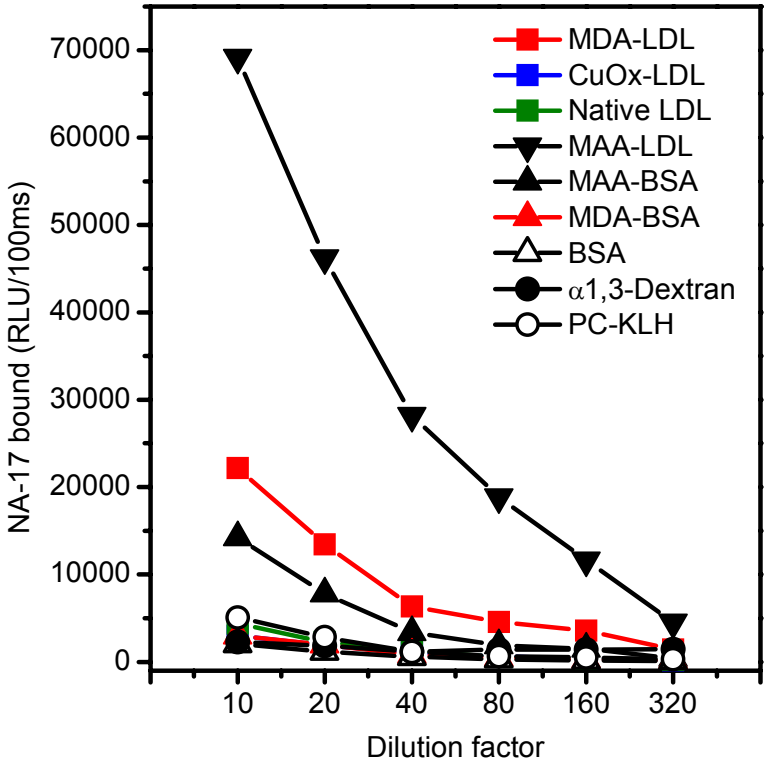



**Fig.4-9 NA-17 recognizes MDA-related epitopes.** Culture supernatant of NA-17 hybridoma was tested by ELISA for binding to various oxidation antigens. NA-17 recognizes MDA-related epitopes including MAA-LDL, MDA-LDL and MAA-BSA but not MDA-BSA. Values are the means of triplicate determinations. The experiment shown is one representative of 3 separate experiments.

Heavy chain

```
                 ____FR3____ _______CDR3________ ____________________FR4_________________
J558.3.90   TAT TAC TGT ACA AGA
DFL16.1j                    GA TGG
DSP2.9                      GA TGG
JH4                            G GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
Clone17     TAT TAC TGT ACA AGA TGG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
             Y   Y   C   T   R   W   D   Y   W   G   Q   G   T   S   V   T   V   S   S
```

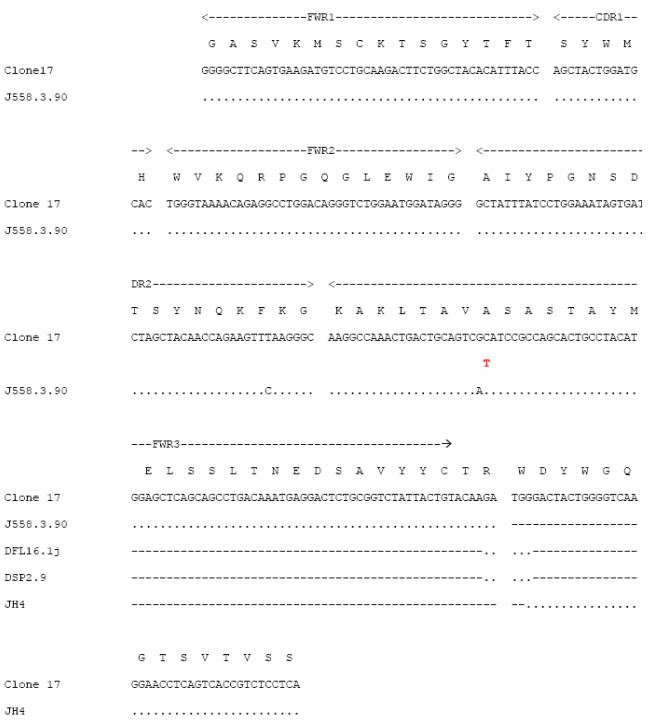
Light chain

```
           _____FR3_____ _____________CDR3_____________ _____________________FR4_____________________
Kh4        ACT TAT TAC TGT CAA CAG TGG AAT AGT TAC CCA C
JH5                                                  C ACG  TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGT
Clone17    ACT TAT TAC TGT CAA CAG TGG AAT AGT TAC CCA CCC ACG  TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA GGT
            T   Y   Y   C   Q   Q   W   N   S   Y   P   P   T   F   G   A   G   T   K   L   E   L   K   G
```

**Fig.4-10 B-1 cell derived natural monoclonal antibody NA-17.** DNA sequences of VDJ splice sites of the $V_H$ and $V_L$ rearrangements expressed in NA-17 B-1 cell hybridoma and their relationship to the most homologous germ-line V, D, J gene segments. Sequence analysis of NA-17 $V_H$ rearrangement did not reveal nucleotide variation to germ-line genes. Sequence analysis of $V_L$ rearrangement revealed one nucleotide insertion between $V_L$ and $J_L$ germ-line gene segments.

The antigen binding region of NA-17 was also sequenced to determine if it is a true germ-line encoded NAb. The DNA sequence of the VDJ splice sites of the $V_H$ and $V_L$ rearrangements expressed in NA-17 displayed complete germ line gene usage of the $V_H$ rearrangement and only one nucleotide insertion (C) at the splice site of the $V_L$ and $J_L$ germ line gene segments (**Fig. 4-10**), showing that NA-17 is a true germ-line encoded NAb. This further supports the presence of MDA-LDL specific NAbs. The complete sequence of NA-17 is presented in **Fig.4-11**.

Heavy chain




Light chain

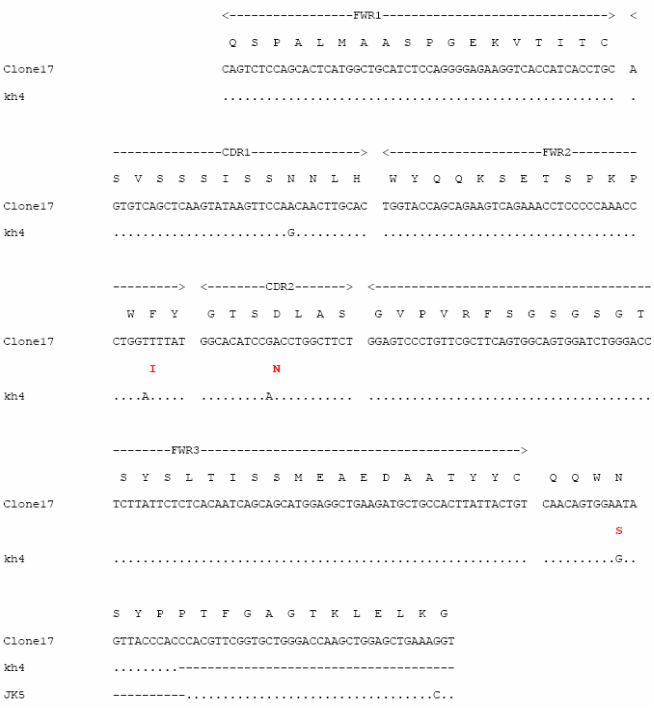



**Fig.4-11 Complete sequence of Clone NA-17.** Genetic sequence and translation of the variable region of the heavy and light chain of monoclonal Ab NA-17. Red Letters indicate amino acids of the germ-line, which differ from the amino acid sequence of NA-17.

## Discussion

In this chapter I established a B-1 cell mouse model, which provides a good system to study B-1 cell derived NAbs and test their potential roles *in vivo*. I validated this mouse model by demonstrating the successful engraftment of B-1 cells in both the peritoneal cavity and the spleen. B-1 cells make up less than 5% of the splenocytes (21), making B-1 cells hard to detect in the spleens of *Rag1*$^{-/-}$ B-1 recipients. In some recipients, B-1 cells constitute less than 1% of total splenocytes. Nevertheless, IgM secreting splenocytes were detected with ELISpot, and Ab isotypes such as IgM and IgG3 could be detected in the plasma of recipients. The above lines of evidence suggest the successful engraftment of B-1 cells in the spleen, since B-1 cells are known to migrate to the spleen and become natural Ab producing plasma cells (25).

Plasma IgM was also partly reconstituted in the B-1 recipients. The extent of IgM reconstitution in *Rag1*$^{-/-}$ B-1 recipients appears to positively correlate with the number of B-1 cells transferred and the time after transfer studied. However, several lines of evidence suggest that the secretion of natural IgM Abs seems to be partially dependent on non-cognate T-cell help as well. The B-1 cell transfer experiment that was contaminated with a small percentage of T-cells resulted in more robust IgM titers in the recipient mice, including titers to OxLDL. Also, in Chapter 2 I showed that the anti-OxLDL IgM titers were lower in age-matched T-cell deficient mice compared to wild type mice (see **Fig.2-6**). Moreover, in Chapter 3 I demonstrated that in culture, T cell cytokine IL-5 stimulated production of IgM to oxidation-epitopes by B-1 cells (see **Fig.3-1**).

However, both T-cell deficient mice as well as recipients of pure B-1 cells exhibited IgM Abs against oxidation-epitopes, indicating that T-cell help were not obligatory, though they have the capacity to modulate IgM generation. Likely, the more robust IgM levels in the recipient mice that had a small percentage of T-cell contamination can be explained in part by the secretion of IL-5 by T cells, which our lab had previously shown to provide non-cognate help for the production of EO6/T15 IgM *in vivo* in atherosclerotic mice (23).

Aside from IgM, decent amounts of IgG3 Ab are also present in *Rag1*$^{-/-}$ B-1 recipients. However, an initial screen of plasma IgG3 from *Rag1*$^{-/-}$ B-1 recipients against oxidation-specific antigens did not show any reactivity of IgG3 to the common antigens used, such as MDA-LDL and CuOx-LDL. More detailed study will be needed to explore if oxidation-specific epitopes are a dominant target for IgG3 as well.

B-1 cells in culture gave rise to a whole array of natural IgM Abs with specificity for various oxidation-specific epitopes. A similar specificity was seen for the IgM found in plasma of *Rag1*$^{-/-}$ mice following adaptive transfer of B-1 cells, demonstrating that B-1 cell derived oxidation-specific IgM are present also *in vivo*.

Here I presented several lines of evidence showing that oxidation-specific epitopes are a dominant target for natural IgM Abs. Absorption studies demonstrated that 20-30% of these B-1 cell derived IgM had specificity for combinations of oxidation epitopes, prominently MDA related epitopes. These data were further corroborated by ELISpot analysis of splenocytes from *Rag1*$^{-/-}$

B-1 recipients, demonstrating that ISCs against MDA-LDL accounted for 10~12% of all ISCs in the spleens of the reconstituted mice, similar to the frequency found in spleens of wildtype mice. Study of hybridomas generated from the spleens of the B-1 cell reconstituted mice indicated that 20-30% of all IgM secreting clones bound to oxidation-specific epitopes, chiefly MDA. Cloning and sequencing of a number of these confirmed their germ-line origin, as exemplified by the MAA-specific B-1 clone NA-17.

NA-17 recognizes MDA related epitopes, including malondialdehyde-acetaldehyde (MAA)-lysine, which is a lysine adduct with 2 MDAs and acetaldehyde (26), a more complex adduct that resulted from advanced oxidation reactions of MDA modifications. However, NA-17 does not bind to MDA-BSA. Possible explanation may be that MDA modified BSA does not contain enough or similar structures that were formed on MDA modified LDL. Nevertheless, the exact MAA-related structure that NA-17 recognizes remains to be identified.

In conclusion, I have demonstrated that in mice, oxidation-specific epitopes are an important target for B-1 cell derived natural IgM Abs. It might not be intuitive why innate immunity should do so, as these IgM Abs are germ-line encoded and selected by evolution. However, there is now ample evidence that oxidation-specific epitopes are present not only in atherosclerosis lesions but in other diseases as well. As described in detail in Chapter 1, apoptotic cells share oxidized cardiolipin, phosphatidylserine, and phosphocholine on their cell surface. Oxidation-specific epitopes were also detected in the pannus of TNF-$\alpha$ transgenic mice, a mouse model for rheumatoid arthritis. Other examples include

Alzheimer's disease, acute lung injury, haemachromatosis or even on bacteria (27-29). A complete list is summarized in **Table 1-2.**

Considering the prevalence of oxidation-specific epitopes, it's not hard to imagine that the repertoire of B-1 cells is positively selected to recognize these "neo-self epitopes" and provide advantageous properties such as immediate defense against pathogens as well as maintaining physiological homeostasis.

To determine the role of oxidation-specific IgM NAb in health and disease, I will test the functions of these natural IgM Abs such as their ability of to clear apoptotic cells and/or neutralize proinflammatory effects, as well as their potential role in atherosclerosis. Such studies will be described in Chapter 5.

**Acknowledgement**

Chapters 4, in part, has been accepted for publication in "Oxidation-specific Epitopes are a Dominant Target for Innate Natural Antibodies", Journal of Clinical Investigation, 2009, by MY Chou, L. Fogelstrand, K. Hartvigsen, LF Hansen, D. Woelkers, PX Shaw, J. Choi, T. Perkmann, F Bäckhed, YI Miller, S. Hörkkö, M. Corr, JL Witztum and CJ Binder. The dissertation author was the primary investigator and author of this paper.

## References


1. **Kearney, J. F., R. Barletta, Z. S. Quan, and J. Quintans**. 1981. Monoclonal vs. heterogeneous anti-H-8 antibodies in the analysis of the anti-phosphorylcholine response in BALB/c mice. Eur. J. Immunol. **11**:877-883.

2. **Horkko, S., C. J. Binder, P. X. Shaw, M. K. Chang, G. Silverman, W. Palinski, and J. L. Witztum**. 2000. Immunological responses to oxidized LDL. Free Radic. Biol. Med. **28**:1771-1779.

3. **Palinski, W., M. E. Rosenfeld, S. Yla-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum**. 1989. Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. U. S. A **86**:1372-1376.

4. **Rosenfeld, M. E., W. Palinski, S. Yla-Herttuala, S. Butler, and J. L. Witztum**. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. Arteriosclerosis **10**:336-349.

5. **Palinski, W., S. Horkko, E. Miller, U. P. Steinbrecher, H. C. Powell, L. K. Curtiss, and J. L. Witztum**. 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J. Clin. Invest **98**:800-814.

6. **Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin**. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. **156**:1177-1185.

7. **Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum**. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc. Natl. Acad. Sci. U. S. A **96**:6353-6358.

8. **Huber, J., A. Vales, G. Mitulovic, M. Blumer, R. Schmid, J. L. Witztum, B. R. Binder, and N. Leitinger**. 2002. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Arterioscler. Thromb. Vasc. Biol. **22**:101-107.

9. **Leroyer, A. S., H. Isobe, G. Leseche, Y. Castier, M. Wassef, Z. Mallat, B. R. Binder, A. Tedgui, and C. M. Boulanger**. 2007. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. J. Am. Coll. Cardiol. **49**:772-777.

10. **Leroyer, A. S., A. Tedgui, and C. M. Boulanger**. 2008. Role of microparticles in atherothrombosis. J. Intern. Med. **263**:528-537.

11. **Mond, J. J., A. Lees, and C. M. Snapper**. 1995. T cell-independent antigens type 2. Annu. Rev. Immunol. **13**:655-692.

12. **Ochsenbein, A. F., D. D. Pinschewer, B. Odermatt, A. Ciurea, H. Hengartner, and R. M. Zinkernagel**. 2000. Correlation of T cell independence of antibody responses with antigen dose reaching secondary lymphoid organs: implications for splenectomized patients and vaccine design. J. Immunol. **164**:6296-6302.

13. **Masmoudi, H., T. Mota-Santos, F. Huetz, A. Coutinho, and P. A. Cazenave**. 1990. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. Int. Immunol. **2**:515-520.

14. **Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum**. 2000. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest **105**:1731-1740.

15. **Baumgarth, N., J. W. Tung, and L. A. Herzenberg**. 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol. **26**:347-362.

16. **Hardy, R. R., C. J. Wei, and K. Hayakawa**. 2004. Selection during development of VH11+ B cells: a model for natural autoantibody-producing CD5+ B cells. Immunol. Rev. **197**:60-74.

17. **Thurnheer, M. C., A. W. Zuercher, J. J. Cebra, and N. A. Bos**. 2003. B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. J. Immunol. **170**:4564-4571.

18. **Haury, M., A. Sundblad, A. Grandien, C. Barreau, A. Coutinho, and A. Nobrega**. 1997. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur. J. Immunol. **27**:1557-1563.

19. **Kearney, J. F.** 2000. Immune recognition of OxLDL in atherosclerosis. J. Clin. Invest **105**:1683-1685.

20. **Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou**. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell **68**:869-877.

21. **Berland, R. and H. H. Wortis**. 2002. Origins and functions of B-1 cells with notes on the role of CD5. Annu. Rev. Immunol. **20**:253-300.

22. **Manz, R. A., A. E. Hauser, F. Hiepe, and A. Radbruch**. 2005. Maintenance of serum antibody levels. Annu. Rev. Immunol. **23**:367-386.

23. **Binder, C. J., K. Hartvigsen, M. K. Chang, M. Miller, D. Broide, W. Palinski, L. K. Curtiss, M. Corr, and J. L. Witztum**. 2004. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. J. Clin. Invest **114**:427-437.

24. **Friguet, B., A. F. Chaffotte, L. Djavadi-Ohaniance, and M. E. Goldberg**. 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods **77**:305-319.

25. **Kawahara, T., H. Ohdan, G. Zhao, Y. G. Yang, and M. Sykes**. 2003. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. J. Immunol. **171**:5406-5414.

26. **Xu, D., G. M. Thiele, M. L. Kearley, M. D. Haugen, L. W. Klassen, M. F. Sorrell, and D. J. Tuma**. 1997. Epitope characterization of malondialdehyde-acetaldehyde adducts using an enzyme-linked immunosorbent assay. Chem. Res. Toxicol. **10**:978-986.

27. **Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum**. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J. Exp. Med. **200**:1359-1370.

28. **Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and G. J. Silverman**. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat. Med. **9**:736-743.

29. **Dei, R., A. Takeda, H. Niwa, M. Li, Y. Nakagomi, M. Watanabe, T. Inagaki, Y. Washimi, Y. Yasuda, K. Horie, T. Miyata, and G. Sobue**. 2002. Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease. Acta Neuropathol. (Berl) **104**:113-122.

# CHAPTER 5

## B-1 CELL DERIVED NATURAL ANTIBODIES RECOGNIZE BIOLOGICALLY RELEVANT OXIDATION-SPECIFIC EPITOPES

**Abstract**

Emerging evidence from my studies suggests that NAbs to neo-epitopes of OxLDL might have been conserved because common oxidation-specific epitopes are present not only in atherosclerotic lesions, but also on apoptotic cells, and display molecular mimicry with the epitopes on common pathogens. However, their role in health and disease is still elusive. In this chapter I studied selected, but important biological functions of natural IgM Abs, such as their ability to effect clearance of apoptotic cells, as well as their ability to recognize pathogens. Plasma from *Rag1*$^{-/-}$ mice reconstituted with B-1 cells from a wild-type donor (*Rag1*$^{-/-}$ B-1 recipients) and monoclonal natural antibody NA-17, both recognized apoptotic cells and facilitated apoptotic cell uptake by macrophages *in vivo*. B-1 cell derived natural IgM and NA-17 also recognized epitopes in atherosclerotic lesions. I also demonstrated that NA-17 has the ability to inhibit the binding of MDA-LDL by macrophages *in vitro*, and thus might prevent foam cell formation. Furthermore, plasma from *Rag1*$^{-/-}$ B-1 recipients, as well as previously cloned oxidation-specific IgM, E06 and E014, which recognize epitopes of oxidized phospholipids and MDA-adducts, respectively, also recognized epitopes on bacteria, demonstrating molecular mimicry between oxidation-specific epitopes on OxLDL and pathogens. The above evidence supports my hypothesis that oxidation-specific NAbs can recognize biologically relevant epitopes and are conserved because they play important roles in maintaining homeostatic functions.

## Introduction

### IgM and its role in atherosclerosis

IgM responses to OxLDL have been found in both animal models of atherosclerosis and in humans (1,2). Although their exact role in atherosclerosis is still elusive, emerging evidence suggest that IgM convey an atheroprotective function. For example, B-cell deficient LDL receptor (*Ldlr*$^{-/-}$) mice developed increased atherosclerosis compared to *Ldlr*$^{-/-}$ mice (3). Splenectomy of *ApoE*$^{-/-}$ mice, which decreased IgM anti-OxLDL Abs, resulted in increased atherosclerosis, and this effect could be reversed by infusion of B-cells from aged *ApoE*$^{-/-}$ mice (4), which was associated with restored IgM titers. Furthermore, emerging evidence suggests that IgM antibody titers to epitopes of OxLDL are inversely related to coronary artery disease in humans (5).

Immunization with OxLDL has been shown to reduce atherosclerosis by nearly 40-60% in various animal models (6-10), and this is likely due to an expansion of anti-OxLDL Abs. For example, immunization with MDA-LDL led not only to a marked stimulation of adaptive IgG antibodies to MDA-LDL, it also led to a robust expansion of T15/E06 natural IgM in plasma. This was shown to occur as a consequence of robust Th2 activation, leading to release of IL-5 and consequent non-cognate stimulation of B-1 cells, leading to enhanced E06/T15 and decreased atherosclerosis in *Ldlr*$^{-/-}$ mice (7). Since T15/E06 binds to the phosphocholine (PC) moiety on oxidized phospholipids (OxPL) in OxLDL as well as the PC linked to the cell wall polysaccharide (CPS) of *Streptococcus*

*pneumoniae*, our lab has shown that immunization of *Ldlr*$^{-/-}$ mice with *S. pneumoniae* expanded the T15/E06 B-1 cell clone and led to atheroprotection in *Ldlr*$^{-/-}$ mice (11). E06 can bind to OxLDL and block scavenger mediated uptake of OxLDL by macrophages *in vitro* (12), thus providing the possibility of preventing foam cell formation. This is one possible mechanism by which E06 mediates its atheroprotective property.

A preliminary report by Dorian Haskard and colleagues from Imperial College London showed that mice that are deficient in both secreted IgM (sIgM$^{-/-}$) and the LDL receptor (*Ldlr*$^{-/-}$), when fed a high-cholesterol diet, had a 7 fold increase in atherosclerotic lesion formation compared to the *Ldlr*$^{-/-}$ mice (Lewis, MJ et al. Hierarchal contributions of IgM and complement C1q in protection against murine atherosclerosis: Exp Biol 2008 Abstract Book, page 123). In that study, they not only reported enhanced lesion formation, but that there was enhanced apoptotic cell number in the sIgM$^{-/-}$ mice. Since NAbs are mainly of the IgM isotype, all the above evidence suggests that NAbs might be protective not only in inhibiting processes that promote atherogenesis, but also that they promote apoptotic cell clearance.

**IgM and its role in apoptotic cell clearance**

Apoptotic cells also share oxidation-specific epitopes on their cell surface that can be recognized by oxidation-specific IgM Abs, including E06 (13). Our lab has shown previously that OxLDL could compete with apoptotic cells and oxidatively damaged red blood cells for macrophage uptake *in vitro* (14). The

membranes of apoptotic cells are enriched in oxidized lipids, including the PC of OxPL that react with E06, but also other oxidized phospholipids including cardiolipin, and phosphtidylserine (15-17). Circulating microparticles are enriched in such epitopes as well (18,19). Apoptotic cells are highly immunogenic and proinflammatory if not promptly cleared, and in fact, our laboratory has shown that mice that are immunized with syngeneic apoptotic cells develop high autoAb titers to a variety of oxidation-specific epitopes (15).

The binding of oxidation-specific autoAbs, including E06, to apoptotic cells has been shown to inhibit apoptotic cell uptake by macrophages *in vitro* (13). This shows that the epitopes to which such Abs bind are ligands binding to scavenger receptors on macrophages. Under *in vivo* settings, however, the opsonization of an antigen by IgM can lead to fixation of complement, which can in turn lead to the uptake of the IgM-complement bound apoptotic cells by complement receptors on phagocytes (20,21). Indeed, Ogden *et al*. have shown that in the presence of complement, E06/T15 has the ability to enhance apoptotic cell clearance by macrophages *in vivo* (22). Therefore, the binding of IgM NAbs to dying cells can lead to subsequent complement binding and thus facilitate the clearance of apoptotic cells *in vivo*.

**IgM and its role in autoimmunity**

In the context of autoimmunity, IgM autoantibodies have often been considered simply as non-pathogenic, and it appears probable that these antibodies are actually protective (23). Using lupus prone (lpr) mice that are also

deficient in secreted IgM, Boes *et al*. showed that compared with regular lpr mice, lpr mice that lack secreted IgM developed elevated levels of IgG autoantibodies to double-stranded DNA and histones and had more abundant deposits of immune complexes in the glomeruli (24). Furthermore, as serum IgM-deficient mice age, there is an increased tendency to produce autoantibodies (25). Thus, IgM seem to have a regulatory role, and in turn, the absence of serum IgM seems to be a mild accelerator of autoimmunity.

The ability of IgM to clear apoptotic cells might confer protection to autoimmunity as well. Apoptotic cells provide a potential source of immunogens for the development of autoimmunity. As described in the previous section, NAbs have been suggested to play a role in the clearance of senescent cells and other self-antigens, and therefore might be protective against autoimmunity (26).

**Molecular mimicry between OxLDL and pathogens**

A traditional view for the function of natural IgM Abs is to protect against pathogen invasion and to prime the IgG response that ensues (27). Experts in the field of B-1 cell biology and NAbs have postulated that most, if not all NAbs have dual-specificity, against neo-self epitopes of modified self, as well as against immunologically similar epitopes present on pathogens (28). The example of E06 nicely illustrates this concept, which binds to both OxLDL and *S. pneumoniae.* Since such a large portion of NAbs are directed against oxidation-specific epitopes, our lab believes that E06 would not be an exception, but rather

one of the many oxidation-specific NAbs that would recognize similar epitopes present on pathogens and provide protection against infection.

Based on the multiple roles that have been discussed for NAbs so far, my hypothesis is that **NAbs to oxidation-specific epitopes are evolutionarily important clones that are conserved because they provide advantageous properties in maintaining homeostasis, such as their crucial role in providing homeostatic host responses to oxidative and inflammatory events, as well as immediate host defense against pathogens.**

**Rationale and study design**

Specific IgM Abs against oxidation-specific epitopes have been found in animal models and humans, but their exact role in health and disease is still elusive. To determine some of the potential biological functions of B-1 cell derived IgM Abs, first I will test the ability of plasma IgM from *Rag1*$^{-/-}$ B-1 recipients and NA-17 to bind apoptotic cells by immunofluorescence microscopy and flow cytometry.

After demonstrating that NAb IgM bind to apoptotic cells, I will attempt to demonstrate that this binding to apoptotic cells will effect the enhanced clearance of apoptotic cells *in vivo* using the phagocytosis assay described by Taylor *et al* (29). In this assay *Rag1*$^{-/-}$ mice that received either B-1 cells or PBS will be injected with 3% thioglycollate intraperitoneally (i.p) to induce sterile peritonitis. After 3 days, labeled apoptotic congenic mouse thymocytes will be injected i.p, the peritoneal exudate cells are then collected after 40min and the percentage of macrophages that have taken up the labeled thymocytes will then be determined by flow cytometry analysis. The effect of NA-17 will be tested as well by pre-incubation with apoptotic thymocytes before injection into *Rag1*$^{-/-}$ mice.

Next I wanted to test whether natural IgM are present in atherosclerotic lesions. B-1 cells were transferred into *Ldlr*$^{-/-}$*Rag*$^{-/-}$ mice that had been fed a high-cholesterol diet for 10 weeks to induce atherosclerotic lesions. The mice were maintained on the atherogenic diet for a further six weeks and then sacrificed and atherosclerotic lesions assessed for the deposition of endogenous Abs.

We have shown previously that the NAb E06 can inhibit the uptake of OxLDL by macrophages in cell culture. I will also test the ability of a new NAb directed against a different oxidation-specific epitopes, NA-17 - directed at MDA-LDL - to also inhibit uptake of MDA-LDL by macrophages in culture. Because OxLDL has multiple oxidation epitopes that can bind to scavenger receptors, demonstration that different NAbs block this uptake would provide important insights as to how natural IgM Abs might affect atherogenesis.

Finally, to demonstrate molecular mimicry between epitopes on pathogens and oxidation-specific structures, I will screen plasma IgM from *Rag1*$^{-/-}$ B-1 recipients and oxidation-specific mAbs for the ability to bind to a panel of pathogens by ELISA. Strains of common pathogens of humans such as *Streptococcus*, *Staphylococcus, Salmonella, Psuedomonas* will be used for initial screening.

The main focus of this chapter is to demonstrate the physiologically relevant properties of oxidation-specific NAbs by studying selected biological functions of natural IgM Abs, such as the clearance of apoptotic cells, functions that might impact lesion formation, and the ability to recognize pathogen. The result from these studies will support my hypothesis that NAbs to oxidation-specific epitopes are evolutionarily important clones that are conserved possibly due to their beneficial role in maintaining physiological homeostasis.

## Results

### B-1 cell derived natural IgM Abs recognize oxidation-specific epitopes on apoptotic cells

To demonstrate that B-1 cell derived IgM Abs can recognize oxidation-specific epitopes on apoptotic cells, plasma from B-1 cell reconstituted *Rag1*$^{-/-}$ mice and NA-17 were incubated with apoptotic mouse thymocytes. As shown in **Fig.5-1**, plasma IgM from B-1 cell reconstituted *Rag1*$^{-/-}$ mice recognized surface epitopes on apoptotic cells, as demonstrated by immunocytochemistry using deconvolution microscopy. Similarly, the MDA-epitope specific NAb NA-17 strongly stained apoptotic cells. Neither plasma from PBS injected *Rag1*$^{-/-}$ mice, nor a trinitrophenol (TNP) specific control IgM bound to apoptotic thymocytes.

I also examined the ability of these NAbs to bind to apoptotic cells in relationship to measures of cellular apoptosis using flow cytometry (**Fig. 5-2**). Plasma IgM from *Rag1*$^{-/-}$ B-1 recipients bound to apoptotic thymocytes with early (Q2) and late (Q3) stages of apoptosis, but did not bind to viable thymocytes (Q1). Monoclonal NA-17, which is specific for MDA-related epitopes, stained almost the entire population of late apoptotic cells, but did not bind normal cells. Thus, similar to the ability of NAb E06/T15 to bind to apoptotic cells via the PC of oxidized phospholipids (12), apoptotic cells contain a variety of oxidation-specific epitopes that are targets for NAbs, including NA-17.

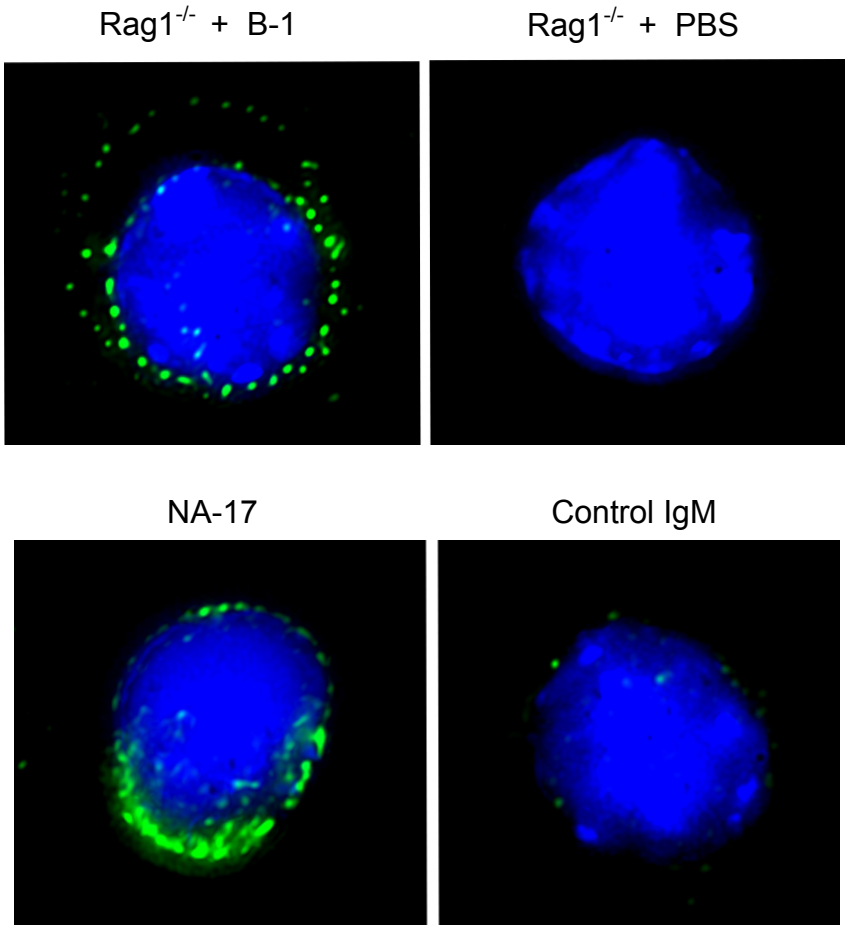



**Fig.5-1 Natural IgM Abs bind to apoptotic thymocytes.** Thymocytes harvested from wild-type C57BL/6 mice were treated with phorbol 12-myristate 13-acetate (PMA) to induce apoptosis and then incubated with plasma from *Rag1*$^{-/-}$ recipients that received either B-1 cells (*Rag1*$^{-/-}$ + B-1) or PBS (*Rag1*$^{-/-}$ + PBS) at 1:10 dilution, monoclonal Ab NA-17 at 2.5μg/ml, or control IgM (G155-228, anti-TNP, BD Pharmingen) at 5μg/ml. Abs bound to apoptotic thymocytes were then detected by FITC-conjugated anti-mouse IgM. DNA staining with 2μg/ml Hoechst dye shows fragmented nuclei of apoptotic cells. Deconvolution microscopy shows that NAbs in (*Rag1*$^{-/-}$ + B-1) as well as NA-17 bound to apoptotic thymocytes.

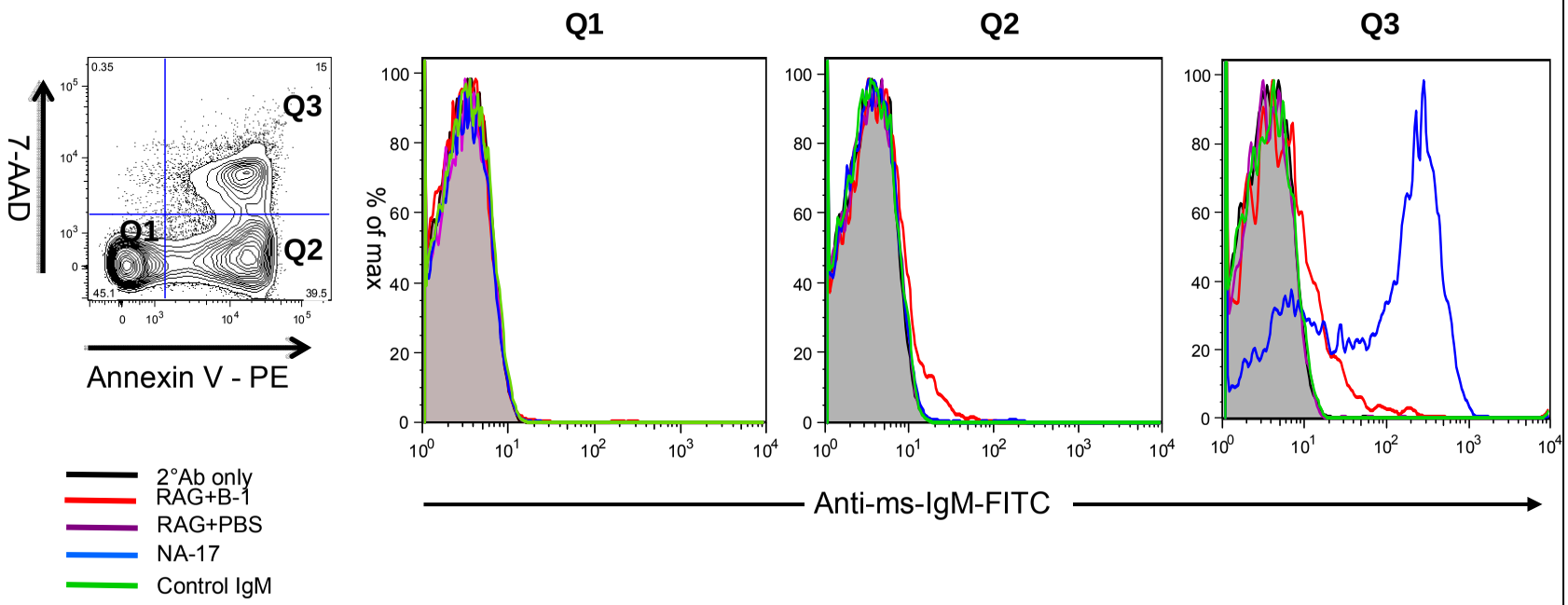



**Fig.5-2 Natural IgM Abs bind to apoptotic thymocytes but not normal thymocytes.** Thymocytes were treated as previously described Fig.5-1 and analyzed by flow cytometry. Apoptotic cells were determined by Annexin-V and 7-AAD staining. None of the IgM bound to normal thymocytes (Q1, Annexin-V$^-$, 7-AAD$^-$). NAbs in plasma from *Rag1*$^{-/-}$ + B-1 bound both early (Q2, Annexin-V$^+$, 7-AAD$^-$) and late apoptotic thymocytes (Q3, Annexin-V$^+$, 7-AAD$^+$), while NA-17 bound prominently to late apoptotic cells (Q3).

**Natural antibodies facilitate apoptotic cell uptake by macrophages in vivo**

The data above show that natural IgM Abs and the cloned monoclonal NA-17 recognize oxidation-specific epitopes on clearance of apoptotic cells (**Fig. 5-1** and **5-2**). Next, I addressed the effect of these NAbs on apoptotic cells. When not promptly cleared, apoptotic cells can be immunogenic and proinflammatory (15,30). Therefore, NAbs might help to maintain homeostasis against oxidatively modified structures by enhancing the clearance of apoptotic cells in vivo. To test this hypothesis, I examined the ability of NAbs to enhance the uptake of apoptotic thymocytes by macrophages in the peritoneum of mice that either have IgM NAbs, or that are completely lacking IgM. This was accomplished by injecting apoptotic thymocytes into the peritoneum of *Rag1*$^{-/-}$ reconstituted with B-1 cells or with PBS, and determining the impact of the IgM on uptake by endogenous macrophages. In a preliminary experiment, I confirmed that IgM were present in the peritoneal cavity of the reconstituted *Rag1*$^{-/-}$ mice (RAG + B-1) mice, but not in the *Rag1*$^{-/-}$ mice injected with PBS.

Fluorescently labeled apoptotic thymocytes were injected into the peritoneal cavity 4 days after the induction of sterile peritonitis with thioglycollate. The ability of peritoneal macrophages to take up apoptotic cells was significantly enhanced in B-1 reconstituted *Rag1*$^{-/-}$ mice (RAG + B-1) compared to PBS controls (RAG + PBS) (33% vs. 27%, $p<0.05$, **Fig. 5-3**).

The experiment above demonstrated that the endogenous IgM in the reconstituted *Rag1*$^{-/-}$ mice bound to the apoptotic cells and were able to enhance macrophage uptake. Next, to directly assess the impact of a purified oxidation-

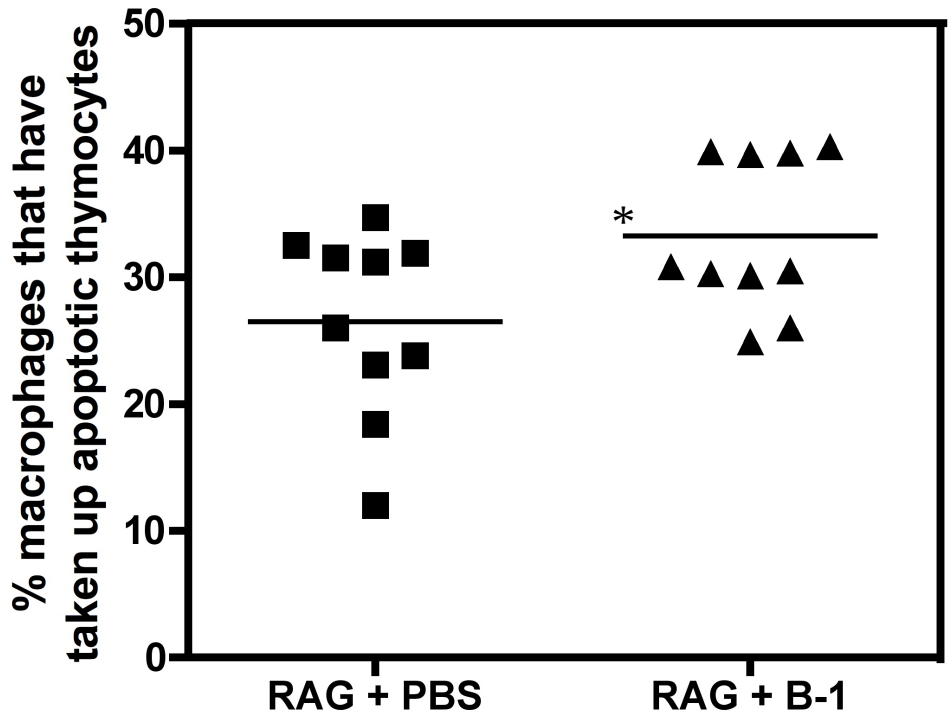



**Fig.5-3 Natural IgM antibodies facilitate apoptotic cell uptake by macrophages *in vivo*.** The graph shows the percentage of macrophages that contained fluorescently-labeled apoptotic thymocytes following injection intraperitoneally. Each point represents an individual mouse. In *Rag1*$^{-/-}$ mice that received PBS injection (RAG + PBS), about 27% of macrophages phagocytosed apoptotic cells. The percentage was significantly increased to 33% in *Rag1*$^{-/-}$ mice that received B-1 cells (RAG + B-1) (*$p<0.05$, Unpaired student's t test). Horizontal bar denotes means.

specific monoclonal IgM on the uptake of apoptotic cells, I pre-incubated the apoptotic cells with NA-17 or with a control IgM (specific for KLH) before

injection into *Rag1*$^{-/-}$ mice. The percentage of macrophages that had taken up

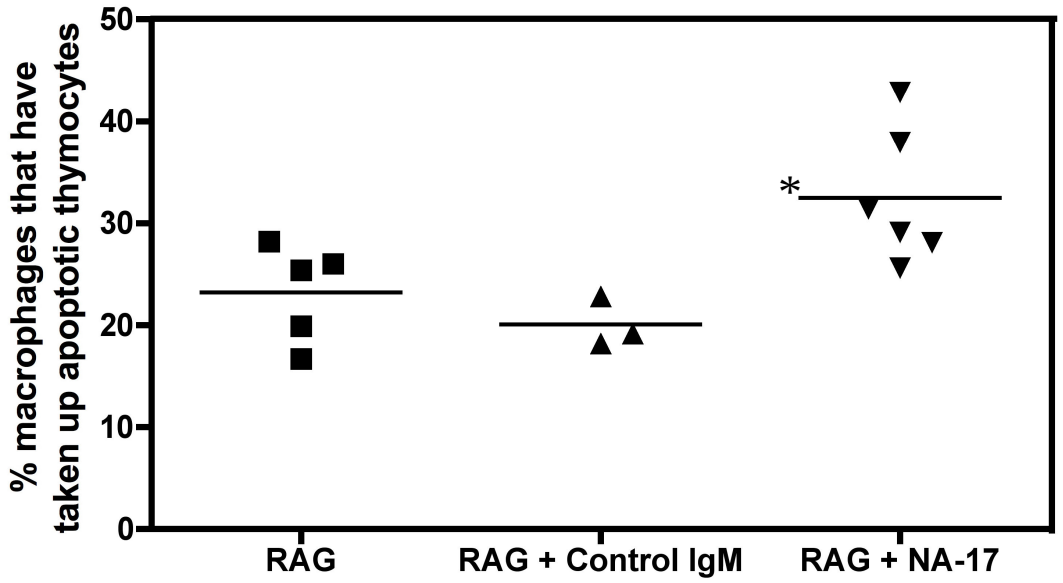



**Fig.5-4 NA-17 facilitates apoptotic cell uptake by macrophages *in vivo*.** Apoptotic thymocytes were pre-incubated with NA-17 or a control IgM that does not bind apoptotic cells before injection into *Rag1*$^{-/-}$ mice**.** The phagocytic uptake was significantly different between the three groups ($P$ = 0.01, one-way ANOVA). The percentage of macrophages taking up apoptotic cells was significantly enhanced when the apoptotic cells were pre-incubated with NA-17 (RAG + NA-17), compared to control IgM (RAG + Control IgM) or without pre-incubation (RAG) (32% vs 20% vs 23%, *p<0.05, Bonferroni multiple comparison test). Horizontal bars denote means.

apoptotic thymocytes was similar in *Rag1*$^{-/-}$ mice receiving apoptotic thymocytes pre-incubated with control IgM (RAG + Control IgM) or untreated apoptotic thymocytes (RAG) (20% vs. 23%, p=NS, **Fig.5-4**). In contrast, the phagocytic uptake increased to 32% when apoptotic thymocytes were pre-incubated with NA-17 (p<0.05), demonstrating that binding of NA-17 facilitated apoptotic cell uptake by macrophages *in vivo*.

**B-1 cell derived natural IgM Abs recognize oxidation-specific epitopes in atherosclerotic lesions**

To further test whether these IgM NAbs recognize their cognate epitopes *in vivo*, I transferred B-1 cells into *Ldlr*$^{-/-}$*Rag*$^{-/-}$ mice that had been fed a high-cholesterol diet for 10 weeks to induce atherosclerotic lesions, which accumulate apoptotic cells and OxLDL (31,32). The mice were maintained on the atherogenic diet for a further six weeks and then sacrificed and atherosclerotic lesions assessed for the deposition of endogenous Abs. As expected, neither IgM nor IgG Abs were present in lesions of *Ldlr*$^{-/-}$*Rag1*$^{-/-}$ mice injected with PBS alone (**Fig. 5-5, top row**). In contrast, IgM accumulated in lesions of B-1 cell reconstituted mice, at sites likely reflecting the edges of lesions at the time of engraftment of the transferred B-1 cells (**Fig. 5-5, bottom row**). The presence of endogenous MDA epitopes in the lesions is demonstrated by immunostaining of adjacent sections using MDA2, a MDA-specific murine monoclonal IgG we previously cloned (33) (**Fig. 5-5**). MDA2 staining and IgM staining occurred in sites comparable to that of the endogenous IgM but were not identical. There is only partial overlap of staining for IgM and MDA2 specific staining, presumably because the IgM were introduced after the formation of lesions began, and the large IgM were only able to penetrate the lesions and bind to the leading edges of atherosclerotic lesion formation, most adjacent to the lumen. Moreover, even though IgG3 Abs were detected in the plasma of B-1 cell recipients, no IgG Abs were found in the lesions of these mice.

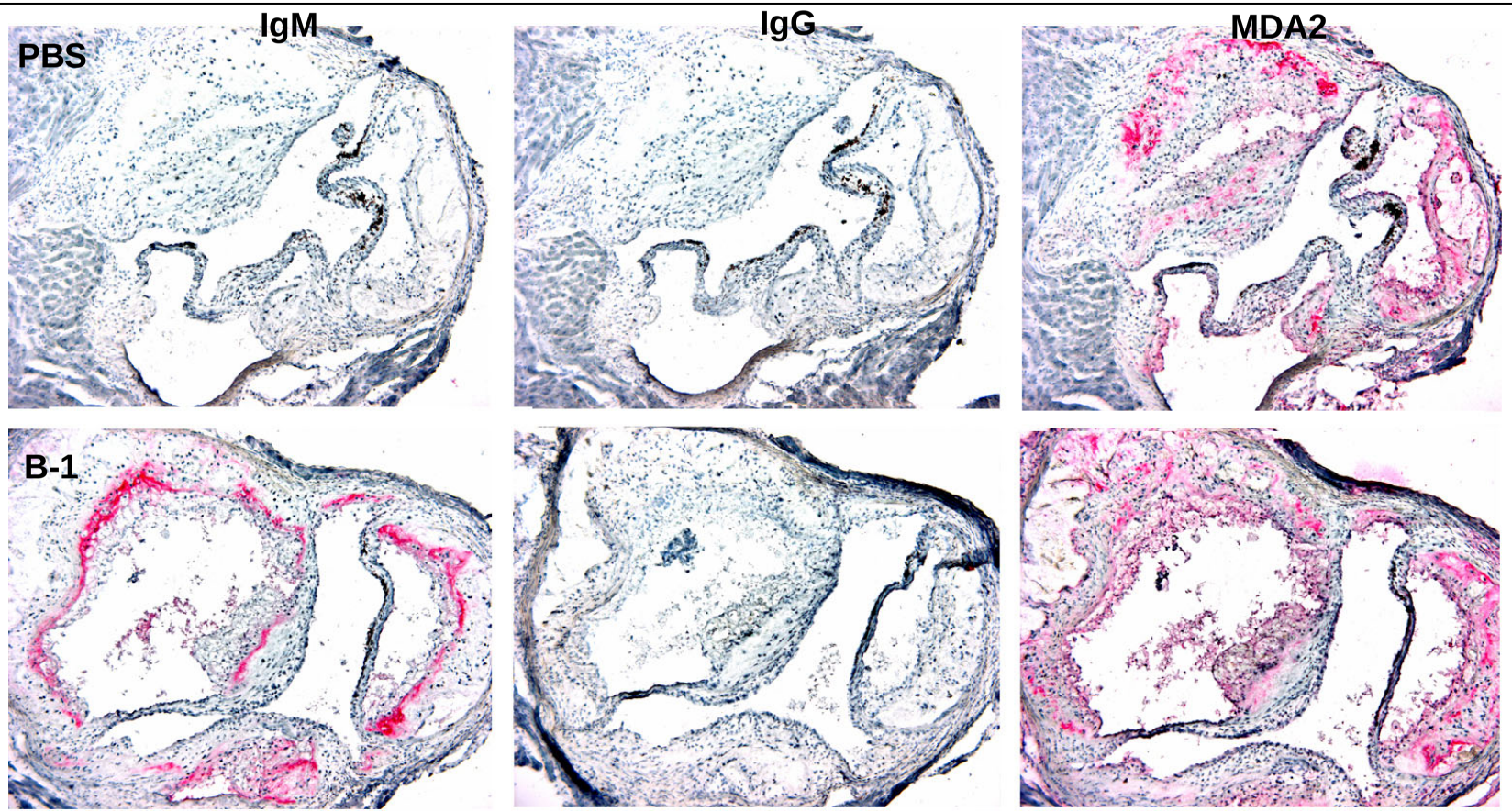



**Fig.5-5 Natural IgM antibodies are present in atherosclerotic lesions.** Sections of atherosclerotic aortas from cholesterol-fed control *Ldlr*$^{-/-}$*Rag1*$^{-/-}$ mice which received PBS (**top row**) and *Ldlr*$^{-/-}$*Rag1*$^{-/-}$ mice that received B-1 cells (**bottom row**) were stained with anti-mouse-IgM or anti-mouse-IgG Abs to detect endogenous NAb deposition in the lesions. Sections were also stained with monoclonal MDA2 (5μg/ml) to detect the presence of MDA-epitopes in the lesions.

B-1 cell derived natural IgM NA-17 also recognized epitopes in the atherosclerotic lesions of *Ldlr*$^{-/-}$*Rag*$^{-/-}$ mice (**Fig. 5-6**). This shows yet another example of oxidation-specific IgM NAb recognizing their cognate epitopes *in vivo*. A control natural IgM, EN-2, which was also cloned in our lab, that did not show any binding to apoptotic cells, did not recognize epitopes in the atherosclerotic lesions either.

To assess the possible roles of NAbs in atherosclerosis, the ability of NA-17 to inhibit macrophage binding to a model oxidized LDL was tested *in vitro*. NA-17 recognizes MDA-LDL as shown in **Fig. 4-9**, thus the ability of NA-17 to inhibit MDA-LDL binding to macrophages was tested by ELISA. As shown in **Fig.5-7,** NA-17 can inhibit specific binding of biotinylated MDA-LDL to mouse J774 macrophages in a dose-dependent fashion. At the highest concentration of NA-17, 80% of biotinylated MDA-LDL binding to mouse J774 macrophages were inhibited, while a control KLH-specific IgM showed only non-specific inhibition. This demonstrated the ability of natural IgM Abs to inhibit OxLDL binding to macrophages and thus suggests the possibility of NAbs to interfere with OxLDL uptake by macrophages, which may eventually reduce foam cell formation, and thus might be beneficial in terms of atherosclerosis.

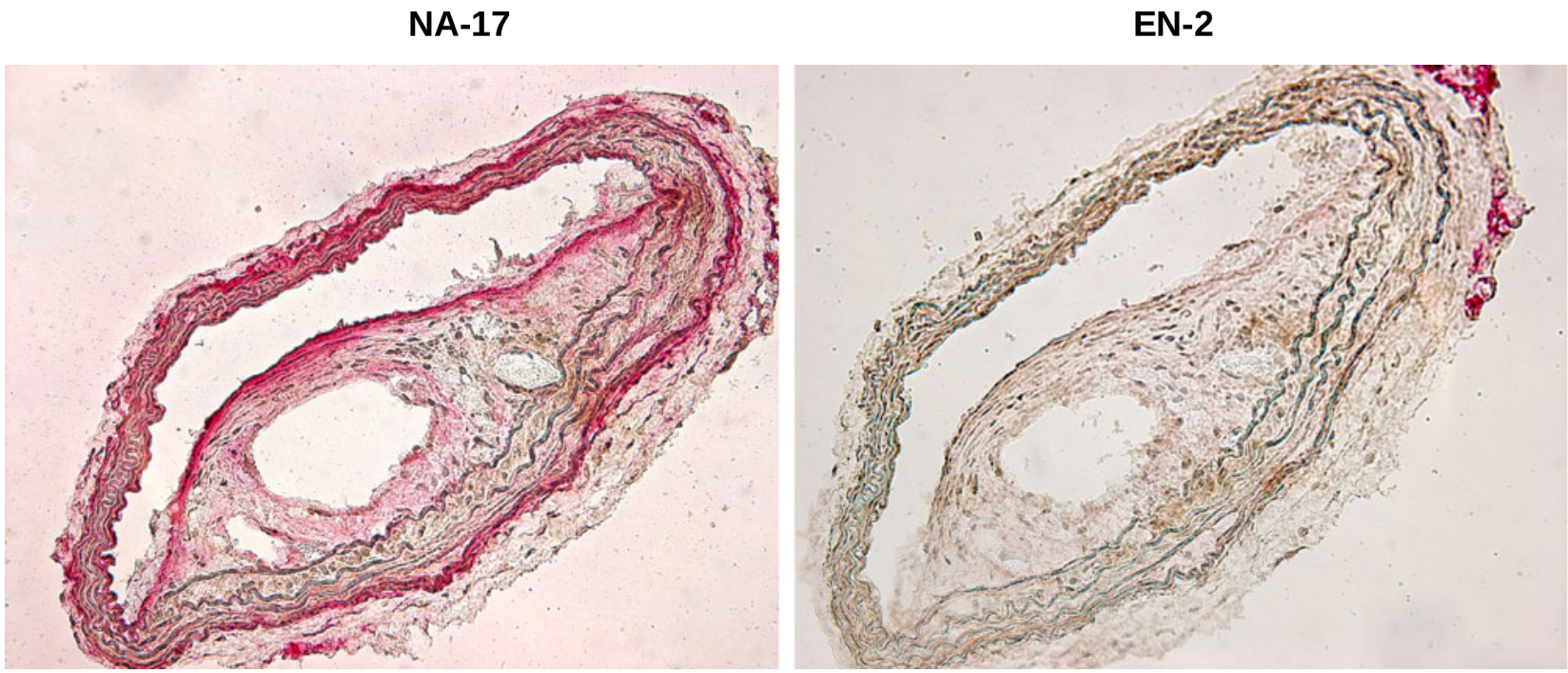



**Fig.5-6 NA-17 recognizes oxidation-specific epitopes present in atherosclerotic lesions.** Sections of atherosclerotic aortas from cholesterol-fed *Ldlr*$^{-/-}$*Rag1*$^{-/-}$ mice were stained with NA-17 (0.85μg/ml) or a control natural IgM Ab EN-2 (1.6μg/ml). Positive staining is indicated by red color.

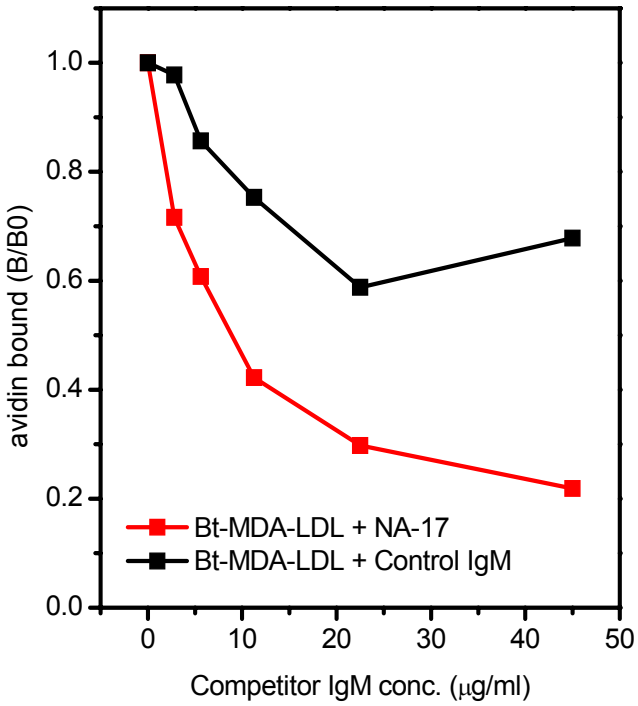



**Fig.5-7 NA-17 inhibits MDA-LDL binding to macrophages.** The specific binding of biotinylated MDA-LDL to mouse J774 macrophages was detected by ELISA. To determine the capacity of NA-17 to inhibit MDA-LDL binding, increasing concentrations of NA-17 and a control IgM (specific for KLH) were added, together with biotinylated MDA-LDL, to macrophages. NA-17 competed about 80% of biotinylated MDA-LDL binding to macrophages. Control IgM only showed non-specific inhibition. Data is expressed as ratio of biotinylated antigen binding macrophages in the presence or absence of IgM ($B/B_0$).

**B-1 cell derived natural IgM Abs recognize pathogens**

NAbs have been postulated to possess dual specificity, like in the case of E06, which recognizes neo-self epitopes such as PC on oxidized LDL, and recognizes similar epitopes present on *Streptococcus pneumoniae* (12,28). To demonstrate that a variety of oxidation-specific IgM can also recognize epitopes on pathogens, I tested the binding of plasma IgM from *Rag1*$^{-/-}$ B-1 recipients to a panel of pathogens by ELISA. However, only the strains that had the highest level of binding are shown in **Fig.5-8**. B-1 cell derived natural IgM Abs showed reactivity towards both gram-positive bacteria such as *S pneumoniae*, *Staphylococcus aureus* and *Group A Streptococcus* (GAS); and gram-negative bacteria such as *Psuedomonas aeruginosa*. Plasma IgM from donor C57BL/6 mice , which contain a full complement of IgM, seemed to show an even stronger response toward these pathogens. However, when the amount of IgM in the plasma of *Rag1*$^{-/-}$ B-1 recipients and donor C57BL/6 were quantified, there was about 3 times more plasma IgM in donor C57BL/6 compared to *Rag1*$^{-/-}$ B-1 recipients (314 vs. 90 μg/ml). Therefore, plasma from donor C57BL/6 mice were diluted 3 times more than *Rag1*$^{-/-}$ B-1 recipients and tested again for binding to bacteria. At comparable IgM concentration, the levels of plasma IgM to binding to bacteria were actually similar between *Rag1*$^{-/-}$ B-1 recipients and donor C57BL/6 mice (data not shown), suggesting that IgM Abs that recognize the pathogens listed above in a naïve C57BL/6 mice are mostly B-1 cell derived NAbs.

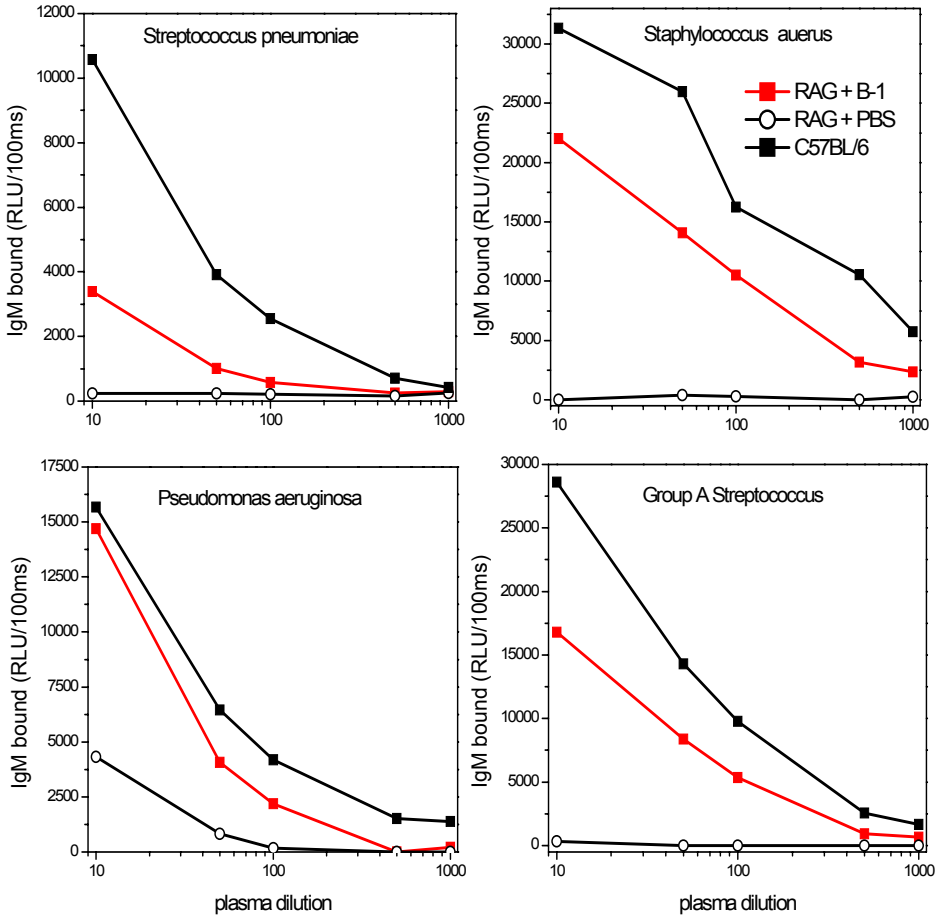



**Fig.5-8 Natural IgM Abs recognizes various pathogens.** A pool of plasma collected from *Rag1*$^{-/-}$ recipients 10 weeks after transfer of either B-1 cells (*Rag1*$^{-/-}$ + B-1) or PBS (*Rag1*$^{-/-}$ + PBS), and donor C57BL/6 mice were tested for IgM binding to indicated pathogen by ELISA. Plasma from both donor and *Rag1*$^{-/-}$ B-1 recipients contain IgM Abs that binds to indicated pathogens while *Rag1*$^{-/-}$ PBS recipients did not show any reactivity.

Next, I tested the monoclonal IgMs specific for oxidation-specific epitopes to see if these mAbs can recognize bacteria as well. E06 has been well characterized to bind to PC epitope on *S. pneumoniae* and provide optimal protection to mice against lethal infection with *S. pneumoniae* infection (34). Here I show that E06 can also bind to *S. aureus* (**Fig.5-9, A.**). This binding is specific too, as shown in **Fig.5-9, B.** Note that *S. aureus* in solution competed for E06 binding to plated *S. aureus* (**Fig.5-9, B. left panel**), while *P. aeruginosa* had no effect. *S. aureus* was also able to compete for E06 binding to CuOx-LDL (**Fig.5-9, B. right panel**), demonstrating that E06 binds to *S. aureus* and CuOx-LDL through the same oxidation-specific epitope. Thus, OxLDL and *S. aureus* and *S. pneumoniae* all share similar if not identical molecular epitopes.

E014, a monoclonal IgM that recognizes MDA-LDL (35), was also tested for binding to bacteria and was found to bind to *Group A Streptococcus* (GAS) (**Fig. 5-10, A.**). Again, this binding is specific, as only GAS was able to compete for E014 binding to itself and MDA-LDL, while *Lactococcus Lactis* did not show any competition (**Fig.5-10, B.**). Even though E014 is not a germ-line encoded NAb, this still illustrated that pathogens can share oxidation-specific epitopes other than PC on their cell surface, therefore serves as an additional piece of evidence showing the molecular mimicry between epitopes on OxLDL and epitopes on bacteria, which is an important target for NAbs.

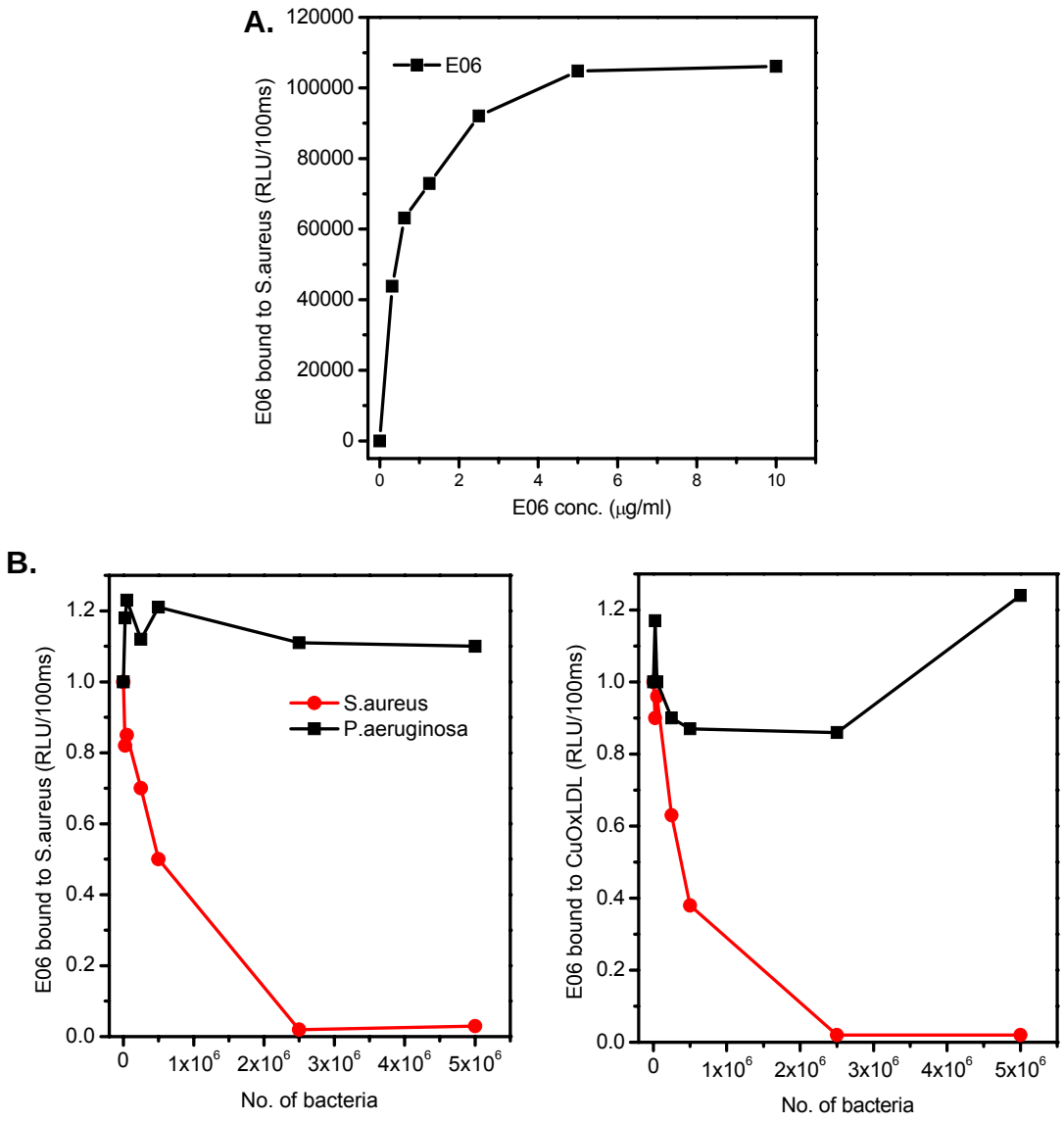



**Fig.5-9 E06 recognizes PC present on *Staphylococcus aureus*.** **(A.)** Binding curve of E06 to *S. aureus* by ELISA. (**B.**) For competition immunoassays, 0.5ug/ml of E06 was incubated in the absence or presence of indicated number of bacteria overnight. After incubation, Ab-bacteria complexes were pelleted by centrifugation, and supernatants tested for IgM binding to indicated antigen. Data are the mean of triplicate determinations, expressed as ratio of IgM binding to *S.aureus* or CuOx-LDL in the presence or absence of competitor ($B/B_0$).

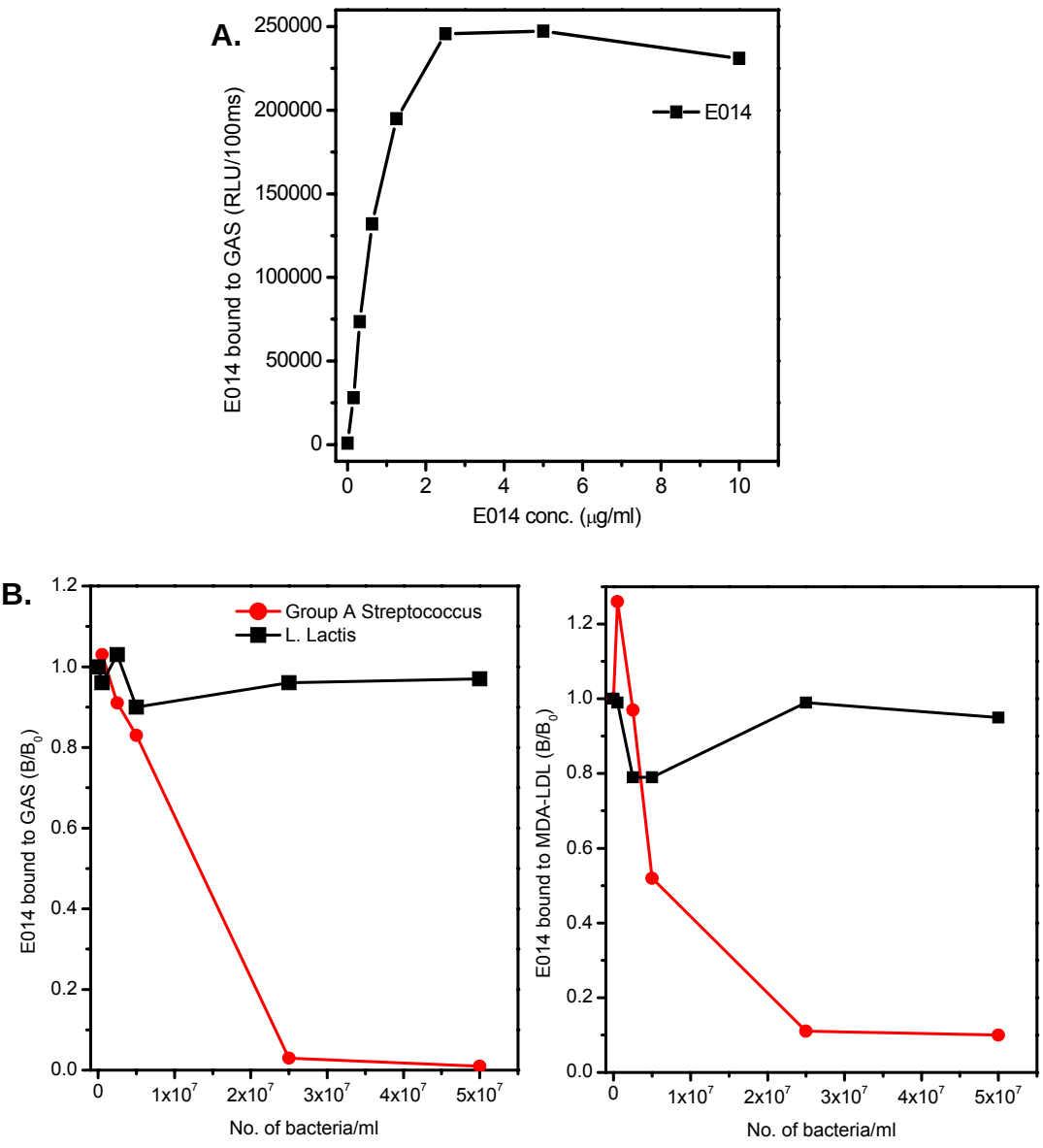



**Fig.5-10 E014 recognizes MDA-epitopes on Group A Streptococcus** **(A.)** Binding curve of E014 to GAS by ELISA. (**B.**) Competition immunoassays were performed as previously described in Fig.5-9. 1 and 0.25 μg/ml of E014 was used for competition immunoassay against GAS and MDA-LDL, respectively. Data are the mean of triplicate determinations, expressed as ratio of IgM binding to GAS or MDA-LDL in the presence or absence of competitor ($B/B_0$).

## Discussion

In previous chapters I demonstrated the existence of oxidation-specific NAbs both *in vitro* and *in vivo*, and that oxidation-specific epitopes constitute a class of epitopes that is a dominant target for NAbs. In this chapter, I show the possible functional implications of NAbs under physiological conditions.

Since apoptotic cells share oxidation-specific epitopes on their cell surface and can be recognized by oxidation-specific IgM Abs previously cloned in our lab (13), it is not surprising that B-1 cell derived IgM in the plasma of the reconstituted *Rag1*$^{-/-}$ mice and NA-17 also recognized apoptotic cells. Interestingly, B-1 cell derived IgM in the plasma of the reconstituted mice bound both early and late apoptotic cells, while NA-17 bound predominantly to the late apoptotic cells. Since plasma from *Rag1*$^{-/-}$ B-1 recipients contain a variety of different IgM Abs, it is not hard to imagine that it has a broader spectrum and recognizes different epitopes on both early and late apoptotic cells. On the other hand, NA-17 is specific for MDA-related epitopes which appears to be present in abundance on late apoptotic cells.

Earlier, we showed that oxidation-specific IgM Abs that bound to apoptotic cells inhibited their uptake by macrophages *in vitro* (13), which demonstrates that the epitopes recognized by such IgMs are ligands mediating binding to macrophage scavenger receptors. However, here I show that *in vivo*, B-1 cell derived IgM Abs can facilitate the uptake of apoptotic cells by macrophages. This can be explained by the known ability of IgM to bind complement. Thus, once an antigen is opsonized by IgM, it can subsequently bind complement, which then

mediates macrophage uptake by binding to complement receptors. For example, the deficiency of complement factor C1q has been widely shown to impair the clearance of apoptotic cells (29,36). Furthermore, the clearance of apoptotic cells through the activation of C1q and thus the deposition of C3 required the help of IgM (20-22,29). Other complement molecules that have been reported to bind to apoptotic cells and affect their clearance include complement factors H, C4BP, and mannose binding lectin (MBL) (37). However, whether IgM acts together with these complement factors in clearing apoptotic cells is still unknown and will require further investigation.

I also demonstrated that natural IgM Abs are present in the atherosclerotic lesions, and endogenous B-1 derived IgM in the reconstituted mice bound to such atherosclerotic lesions at sites comparable to, but not identical to sites stained *ex vivo* by the MDA-specific IgG, MDA2. There might be two explanations. First, some atherosclerotic lesions were already formed prior to the transfer of B-1 cells to the $Rag1^{-/-}Ldlr^{-/-}$ mice, and thus, the large endogenous IgM could not penetrate to MDA epitopes deep in the lesion. Second, as many of the MDA epitopes in the lesion are bound by endogenous IgM, these epitopes were unavailable for MDA2 staining.

We previously showed that the NAb E06, which binds the PC of OxPL, binds to OxLDL and inhibits binding and uptake of OxLDL by macrophages. I now showed that NA-17, which binds to MDA-LDL, also recognized epitopes in the lesions. NA-17 was able to inhibit MDA-LDL binding to macrophages, which suggests a possible role for MDA-specific NAbs in the interference of foam cell

formation, agreeing with accumulating evidence that NAbs might be atheroprotective. However, further study will be needed to determine the exact role of NAbs in atherosclerosis.

B-1 cell derived IgM in the plasma of the reconstituted *Rag1*$^{-/-}$ mice and oxidation-specific monoclonal IgMs were tested for their ability to bind to bacteria. B-1 cell derived IgM show strong reactivity towards common pathogens that are associated with human infections. *S. aureus* and *P. aeruginosa* are both leading causes of hospital acquired infections (38,39). GAS infection usually leads to pharyngitis or skin infections (39), and *S. pneumoniae* is the most common cause of community-acquired pneumonia (40). Interestingly, B-1 cell derived IgM only showed weak binding towards non-pathogenic strains of bacteria such as *E. coli* MC1061 and *L. Lactis* (data not shown). This is not unexpected, since natural IgM have been shown to play a critical role in defense against viral and bacterial infections (27,41,42). However, whether this recognition of bacteria by B-1 cell derived IgM is mediated through oxidation-specific epitopes still needs to be confirmed.

Nevertheless, I demonstrated that E06 and E014 bound specifically to *S. aureus* and GAS, respectively. Even though E014 is not a full germ-line NAb, these examples still illustrated our paradigm that epitopes on pathogens share molecular mimicry with oxidation-specific epitopes. The cell wall of *S. aureus*, like *S. pneumoniae*, contains lipoteichoic acid and teichoic acid which possibly has covalently linked PC residues (43), and thus might provide a possible explanation for the binding of E06 to *S. aureus.* Further investigation is needed still to

determine the exact epitope that E06 and E014 are binding to on the bacteria, and whether these Abs will provide protection towards bacterial infection.

However, the observation that B-1 cell derived IgM and oxidation-specific IgM can bind to pathogens supports my hypothesis that NAbs towards oxidation-specific epitopes might be conserved for their beneficial effects, in this case, recognition and defense against invasive pathogens.

In conclusion, these data strongly support the hypothesis that oxidation-specific epitopes are a dominant target of NAbs in mice, which in turn bind to biologically relevant "self-antigens" and an encounter later in life with external pathogens that bear oxidation-specific epitopes could serve to further expand a given B-1 cell clone (12,44). A recent study by Catera *et al*. reported that monoclonal Abs cloned from certain human chronic lymphocytic leukemia cells, which represents the outgrowth of $CD5^+$ B cells, recognized conserved epitopes associated with apoptosis and oxidation (45). A separate study by Myhrinder *et al*. showed that aside from apoptotic cells and OxLDL, certain chronic lymphocytic leukemia immunoglobulins in human also bound to polysaccharides of *S. pneumoniae* (46). These studies corroborated my observations and further supported my hypothesis that oxidation-specific epitopes are conserved because of their crucial role in immediate host defense and also in mediating host responses to oxidative and inflammatory events.

Now that I've shown the presence of oxidation-specific NAbs in mice and their possible functional roles, the next step is to see if these oxidation-specific NAbs are present in humans as well. Such studies will be described in Chapter 6.

**Acknowledgement**

Chapters 5, in part, has been accepted for publication in “Oxidation-specific Epitopes are a Dominant Target for Innate Natural Antibodies”, Journal of Clinical Investigation, 2009, by MY Chou, L. Fogelstrand, K. Hartvigsen, LF Hansen, D. Woelkers, PX Shaw, J. Choi, T. Perkmann, F Bäckhed, YI Miller, S. Hörkkö, M. Corr, JL Witztum and CJ Binder. The dissertation author was the primary investigator and author of this paper.

## References


1. **Witztum, J. L. and D. Steinberg**. 2001. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? Trends Cardiovasc. Med. **11**:93-102.

2. **Binder, C. J., M. K. Chang, P. X. Shaw, Y. I. Miller, K. Hartvigsen, A. Dewan, and J. L. Witztum**. 2002. Innate and acquired immunity in atherogenesis. Nat. Med. **8**:1218-1226.

3. **Major, A. S., S. Fazio, and M. F. Linton**. 2002. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler. Thromb. Vasc. Biol. **22**:1892-1898.

4. **Caligiuri, G., A. Nicoletti, B. Poirier, and G. K. Hansson**. 2002. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. J. Clin. Invest **109**:745-753.

5. **Fraley, A. E. and S. Tsimikas**. 2006. Clinical applications of circulating oxidized low-density lipoprotein biomarkers in cardiovascular disease. Curr. Opin. Lipidol. **17**:502-509.

6. **Freigang, S., S. Horkko, E. Miller, J. L. Witztum, and W. Palinski**. 1998. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. Arterioscler. Thromb. Vasc. Biol. **18**:1972-1982.

7. **Binder, C. J., K. Hartvigsen, M. K. Chang, M. Miller, D. Broide, W. Palinski, L. K. Curtiss, M. Corr, and J. L. Witztum**. 2004. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. J. Clin. Invest **114**:427-437.

8. **Zhou, X., G. Caligiuri, A. Hamsten, A. K. Lefvert, and G. K. Hansson**. 2001. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. Arterioscler. Thromb. Vasc. Biol. **21**:108-114.

9. **Ameli, S., A. Hultgardh-Nilsson, J. Regnstrom, F. Calara, J. Yano, B. Cercek, P. K. Shah, and J. Nilsson**. 1996. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler. Thromb. Vasc. Biol. **16**:1074-1079.

10. **George, J., A. Afek, B. Gilburd, H. Levkovitz, A. Shaish, I. Goldberg, Y. Kopolovic, G. Wick, Y. Shoenfeld, and D. Harats**. 1998. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. Atherosclerosis **138**:147-152.

11. **Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and G. J. Silverman**. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat. Med. **9**:736-743.

12. **Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum**. 2000. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest **105**:1731-1740.

13. **Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum**. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc. Natl. Acad. Sci. U. S. A **96**:6353-6358.

14. **Sambrano, G. R. and D. Steinberg**. 1995. Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. Proc. Natl. Acad. Sci. U. S. A **92**:1396-1400.

15. **Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum**. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J. Exp. Med. **200**:1359-1370.

16. **Kagan, V. E., G. G. Borisenko, Y. Y. Tyurina, V. A. Tyurin, J. Jiang, A. I. Potapovich, V. Kini, A. A. Amoscato, and Y. Fujii**. 2004. Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. Free Radic. Biol. Med. **37**:1963-1985.

17. **Tuominen, A., Y. I. Miller, L. F. Hansen, Y. A. Kesaniemi, J. L. Witztum, and S. Horkko**. 2006. A natural antibody to oxidized cardiolipin binds to oxidized low-density lipoprotein, apoptotic cells, and atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. **26**:2096-2102.

18. **Huber, J., A. Vales, G. Mitulovic, M. Blumer, R. Schmid, J. L. Witztum, B. R. Binder, and N. Leitinger**. 2002. Oxidized membrane vesicles and

blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Arterioscler. Thromb. Vasc. Biol. **22**:101-107.

19. **Leroyer, A. S., A. Tedgui, and C. M. Boulanger**. 2008. Role of microparticles in atherothrombosis. J. Intern. Med. **263**:528-537.

20. **Kim, S. J., D. Gershov, X. Ma, N. Brot, and K. B. Elkon**. 2002. I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. J. Exp. Med. **196**:655-665.

21. **Quartier, P., P. K. Potter, M. R. Ehrenstein, M. J. Walport, and M. Botto**. 2005. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. Eur. J. Immunol. **35**:252-260.

22. **Ogden, C. A., R. Kowalewski, Y. Peng, V. Montenegro, and K. B. Elkon**. 2005. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. Autoimmunity **38**:259-264.

23. **Manson, J. J., C. Mauri, and M. R. Ehrenstein**. 2005. Natural serum IgM maintains immunological homeostasis and prevents autoimmunity. Springer Semin. Immunopathol. **26**:425-432.

24. **Boes, M., T. Schmidt, K. Linkemann, B. C. Beaudette, A. Marshak-Rothstein, and J. Chen**. 2000. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. Proc. Natl. Acad. Sci. U. S. A **97**:1184-1189.

25. **Ehrenstein, M. R., H. T. Cook, and M. S. Neuberger**. 2000. Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. J. Exp. Med. **191**:1253-1258.

26. **Montecino-Rodriguez, E. and K. Dorshkind**. 2006. New perspectives in B-1 B cell development and function. Trends Immunol. **27**:428-433.

27. **Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel**. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science **286**:2156-2159.

28. **Baumgarth, N., J. W. Tung, and L. A. Herzenberg**. 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol. **26**:347-362.

29. **Taylor, P. R., A. Carugati, V. A. Fadok, H. T. Cook, M. Andrews, M. C. Carroll, J. S. Savill, P. M. Henson, M. Botto, and M. J. Walport**. 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J. Exp. Med. **192**:359-366.

30. **Savill, J., I. Dransfield, C. Gregory, and C. Haslett**. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat. Rev. Immunol. **2**:965-975.

31. **Tabas, I.** 2005. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler. Thromb. Vasc. Biol. **25**:2255-2264.

32. **Yla-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg**. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest **84**:1086-1095.

33. **Palinski, W., S. Yla-Herttuala, M. E. Rosenfeld, S. W. Butler, S. A. Socher, S. Parthasarathy, L. K. Curtiss, and J. L. Witztum**. 1990. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis **10**:325-335.

34. **Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin**. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. **156**:1177-1185.

35. **Palinski, W., S. Horkko, E. Miller, U. P. Steinbrecher, H. C. Powell, L. K. Curtiss, and J. L. Witztum**. 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J. Clin. Invest **98**:800-814.

36. **Botto, M., C. Dell'Agnola, A. E. Bygrave, E. M. Thompson, H. T. Cook, F. Petry, M. Loos, P. P. Pandolfi, and M. J. Walport**. 1998. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. **19**:56-59.

37. **Trouw, L. A., A. M. Blom, and P. Gasque**. 2008. Role of complement and complement regulators in the removal of apoptotic cells. Mol. Immunol. **45**:1199-1207.

38. **Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja**. 1983. Infections caused by Pseudomonas aeruginosa. Rev. Infect. Dis. **5**:279-313.

39. **Nizet, V.** 2007. Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. J. Allergy Clin. Immunol. **120**:13-22.

40. **Musher, D. M.** 1992. Infections caused by Streptococcus pneumoniae: clinical spectrum, pathogenesis, immunity, and treatment. Clin. Infect. Dis. **14**:801-807.

41. **Boes, M., A. P. Prodeus, T. Schmidt, M. C. Carroll, and J. Chen**. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. Exp. Med. **188**:2381-2386.

42. **Jayasekera, J. P., E. A. Moseman, and M. C. Carroll**. 2007. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. J. Virol. **81**:3487-3494.

43. **Fischer, W.** 1994. Lipoteichoic acid and lipids in the membrane of Staphylococcus aureus. Med. Microbiol. Immunol. **183**:61-76.

44. **Kearney, J. F.** 2000. Immune recognition of OxLDL in atherosclerosis. J. Clin. Invest **105**:1683-1685.

45. **Catera, R., G. J. Silverman, K. Hatzi, T. Seiler, S. Didier, L. Zhang, M. Herve, E. Meffre, D. G. Oscier, H. Vlassara, R. H. Scofield, Y. Chen, S. L. Allen, J. Kolitz, K. R. Rai, C. C. Chu, and N. Chiorazzi**. 2008. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol. Med. **14**:665-674.

46. **Lanemo, M. A., E. Hellqvist, E. Sidorova, A. Soderberg, H. Baxendale, C. Dahle, K. Willander, G. Tobin, E. Backman, O. Soderberg, R. Rosenquist, S. Horkko, and A. Rosen**. 2008. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood **111**:3838-3848.

# CHAPTER 6

## NATURAL IGM ANTIBODIES IN HUMAN CORD BLOOD RECOGNIZE OXIDATION-SPECIFIC EPITOPES

**Abstract**

Our lab has previously shown that there are high titers of Abs, including IgM, to oxidation-specific epitopes in human plasma, but it is unknown whether these are NAbs. IgM found in the umbilical cord blood are exclusively from the infant and are considered to represent the human equivalent of naïve NAbs. Therefore, to study human NAbs, umbilical cord plasma and their respective maternal samples were analyzed. Compared to maternal plasma, the titers of oxidation-specific IgM were enriched in umbilical cord blood. The umbilical cord plasma IgM also bound to apoptotic cells in part through the recognition of MDA-related epitopes on apoptotic cells. In analogy to murine data, human monoclonal IgM Abs, cloned from lymphocytes of umbilical cord blood, also bound to late apoptotic cells, atherosclerotic lesions and were able to inhibit MDA-LDL binding to macrophages. The above lines of evidence suggest that oxidation-specific epitopes are an important target of NAbs in humans as well.

## Introduction

### B-1 cells in humans

The amount of information on B-1 cells in humans in the literature is rather limited. In fact, it is still controversial whether a distinct B-1 cell population exists in humans (1). An opinion held by some is that the $IgM^+$ memory B cells in humans have the same function as the B-1a cells in mice (2). There are a lot of similarities between human surface $IgM^+$ ($sIgM^+$) B cells and mouse B-1a cells. For example, a significant portion of the $sIgM^+$ B cells in the human peritoneal cavity are $CD5^+$ (3). Such $CD5^+$ B cells account for up to 40% of B cells in 15-24 week old human embryos (4). Another similarity is the presence of the $CD5^+$ $sIgM^+$ B cells in the human omentum, where about 40% of the mature B cells are $CD5^+$ (5). Finally, the spleen has been implicated in mouse B-1-cell development (6), and $CD5^+$ $sIgM^+$ B cells are a prominent population in the human fetal spleen as well. These splenic $CD5^+$ $sIgM^+$ B secrete autoreactive IgM (7), which is also a characteristic of mouse B-1 cells (8). Also, $IgM^+$ memory B cells are lacking in patients without a spleen and this is associated with impaired immune response to *Streptococcus pneumoniae* (9).

Of interest is also the origin of leukemia in mice and humans. In mice, the transfer of peritoneal cells from 15-month-old mice into newborn recipient mice led to the development of chronic $CD5^+$ leukemias of B-1 cell origin in the recipients six months later (10). In humans, because some B cells from patients

diagnosed with chronic lymphocytic leukemia (CLL) expressed CD5, it has been proposed that human CLL is a malignancy of B-1 cells (11).

However, whether these $CD5^+$ B cells are the equivalent of true human B-1 cells, similar to B-1 cells in mice, remains to be determined, because CD5, even when co-expressed with sIgM, is not sufficient to assign a cell to the B-1 cell lineage.

**Natural Antibody in humans**

Although studies of NAbs in humans are also very limited, there is evidence that such “naïve” IgM Abs that are low affinity and polyreactive exist in humans and bind to a variety of “self-antigens” (12,13). The functions of these autoreactive NAbs are still unclear, but they have been widely implicated to play an important role in autoimmune diseases (14). The specific molecules recognized by these natural autoantibodies appear to predict the development of future autoimmune disease; some mark resistance to the disease while others increase the susceptibility to a future autoimmune disease (15). It has been proposed that natural autoantibodies and autoreactive T cells in healthy individuals might have been selected to recognize a specific and limited set of self-antigens (16-18). My focus would be to determine whether OxLDL and related oxidation-specific epitopes are one of these sets of “self-antigens” that are recognized by human NAbs.

IgM in human newborn cord blood are of fetal origin since IgM cannot cross the placenta and are though to represent human NAbs. Bobrzynski *et al*. generated a Fab display library from human cord blood and demonstrated the presence of germ-line encoded Abs (19). A recent study by Merbl *et al*. also utilized human cord blood to study antigens that the autoreactive NAbs bind to with an antigen microarray (20). Remarkably, the most common antigen to which the cord blood IgM bound to was LDL. The LDL used in the studies of Merbl *et al*., was commercially purchased, and it may have been modified, for example by oxidation, at the time of use. Thus, their data may support my hypothesis that OxLDL might be a dominant target for human NAbs as well.

**Rationale and study design**

To test if NAbs against oxidation-specific epitopes also exist in humans, umbilical cord plasma from 10 newborns and the respective maternal samples will be tested for IgM titers against oxidation-specific antigens. IgM found in the umbilical cord blood are exclusively from the infant and are considered to represent the human equivalent of naïve NAbs (20).

In analogy to the murine data, I will also test the ability of umbilical cord plasma IgM to bind to apoptotic cells, and how the binding will be affected in the presence of oxidation-specific antigens such as MDA-LDL and MAA-BSA.

To obtain monoclonal NAbs from humans, our lab has successfully generated human Fabs using lymphocytes from newborn cord blood with the phage display technique described previously (21). The human Fabs that have specificity for MDA-LDL or MAA-BSA will be tested for their ability to bind apoptotic cells and epitopes in the atherosclerotic lesions, as well as their ability to inhibit oxidation-antigen binding to macrophages.

The main focus of this chapter is to demonstrate the presence of oxidation-specific NAbs in humans. The result from these studies will provide evidence for whether oxidation-specific NAbs in humans share similar characteristics with those in mice and preliminary information on the properties of these NAbs.

## Results

### Natural IgM Abs in human cord blood recognize oxidation-specific epitopes

Our lab previously showed that IgG and IgM titers to oxidation-specific epitopes are present in nearly all adult human plasmas tested (22). However, whether the human IgM NAb repertoire displays similar binding to oxidation-specific epitopes in analogy to the findings in mice is still unknown. Therefore, I characterized the binding properties of IgM in umbilical cord blood, and compared them to the IgM titers in the respective maternal plasma samples. Umbilical cord blood IgM are exclusively from the infant and are considered to represent the human equivalent of naïve NAbs (20). Both umbilical cord plasma and maternal plasma contained prominent IgM titers against MDA-LDL and CuOxLDL, but not native LDL (**Fig. 6-1, (A)**). Maternal plasma also contained IgM against Keyhole Limpet Hemocyanin (KLH), whereas such IgMs were not detected in the umbilical cord plasma, suggesting the titers in mothers were presumably due to exogenous antigen exposure (**Fig. 6-1, (A)**). Unlike IgM titers, IgG titers were found to be similar in maternal and umbilical cord plasma (data not shown), consistent with the ability of maternal IgG to be actively transferred across the placenta, whereas IgM cannot. As the total IgM titers were found to be generally higher in maternal samples, I calculated the ratios of oxidation-specific IgM to total IgM in individual maternal-cord blood samples. This revealed that MDA-LDL and OxLDL-specific IgM are relatively enriched in umbilical cord plasma compared to maternal

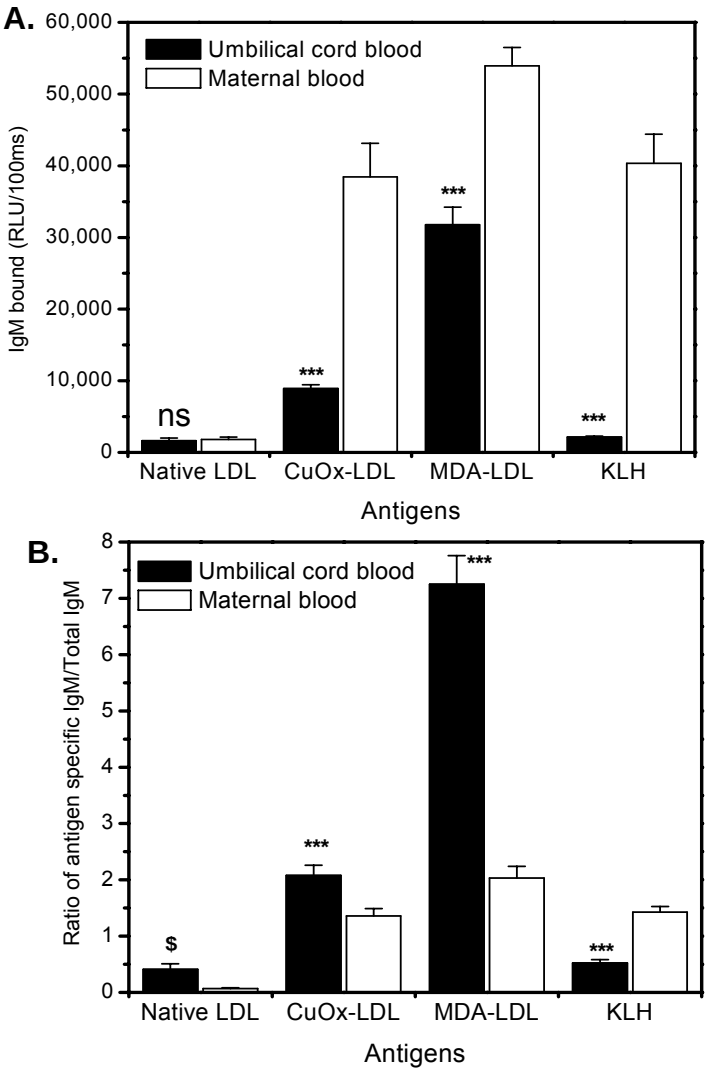



**Fig. 6-1 Human umbilical cord blood contains natural IgM antibodies to oxidation-specific antigens. (A)** Plasma titers of IgM in maternal and umbilical cord plasma to native LDL, KLH, and oxidation-specific antigens measured by ELISA. **(B)** Data are plotted as ratio of antigen specific IgM to total IgM. $^{***}p<0.002$ compared to maternal blood (Wilcoxon matched pairs test and paired t test). Data shown are from 10 paired maternal-infant samples, and each sample was assayed in triplicate. Values are means and SEM.

plasma (**Fig. 6-1, (B)**), which typically also contain adaptive IgM Abs (e.g. against KLH). Thus, oxidation-specific epitopes also appear to be a dominant target for natural IgM Abs in humans.

Although it is thought that humans are not exposed to mollusk proteins and therefore do not have responses to KLH, our lab have used KLH as a control for many years and have repeatedly seen some (but not all) plasmas of adult human subjects having specific IgM that bind to KLH like shown here in **Fig. 6-1**. Competition study with pooled maternal plasma showed that the IgM binding to plated KLH can be effectively competed by soluble KLH (**Fig. 6-2**), demonstrating the specificity of this binding.

**Natural IgM Abs in human cord blood recognize oxidation-specific epitopes on apoptotic cells**

In analogy to the murine data shown before, I further showed that human umbilical cord IgM also bound to apoptotic cells (**Fig. 6-3** and **6-4**). This binding was at least in part mediated through the recognition of MDA-related epitopes, as shown in **Fig.6-3**, MDA-LDL competed for up to 45% of the binding of the umbilical cord IgM to apoptotic cells, while native LDL only had a minimal effect. A similar effect was observed with MAA-BSA. MAA-BSA inhibited umbilical cord IgM binding to apoptotic cells by a dramatically 70%, while BSA alone had no effect (**Fig.6-4**). Umbilical cord IgM did not recognize normal cells, similar to the murine data shown in the previous chapter.

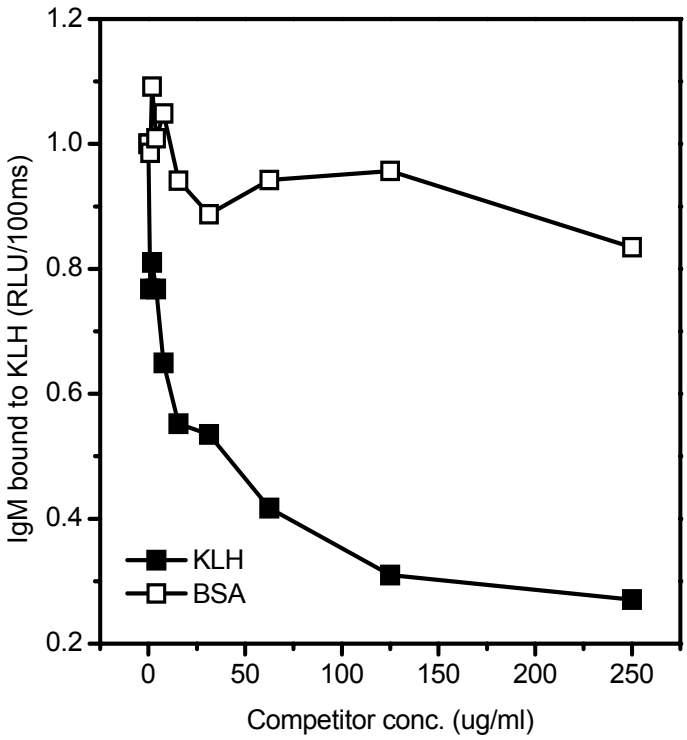



**Fig. 6-2 Human maternal plasma IgM antibodies show specificity to KLH.** For competition immunoassay, pooled maternal plasma samples (n=3) were diluted to 1:200 and incubated in the presence of indicated concentrations of competitors overnight. After incubation, IgM binding to KLH was then tested by ELISA. Data are the mean of triplicate determinations, expressed as ratio of IgM binding to KLH in the presence of competitor to the binding in the absence of competitor ($B/B_0$).

**Human Fab Abs recognize oxidation-specific epitopes on apoptotic cells**

To obtain and study the characteristic of individual human NAbs, Dr. Lotte Hansen from our laboratory generated a phage display library with RNA from lymphocytes pooled from seven newborn umbilical cord blood plasmas, using techniques previously described by Barbas *et al.* (21). Human Fab Ab fragments

were generated from the phage display library and were screened for their reactivity against MDA-LDL and MAA-BSA. Since lymphocytes in the umbilical cord plasma cannot cross the placental barrier, these lymphocytes are of fetal origin and the Fabs that are generated can only originate from the newborns. Out

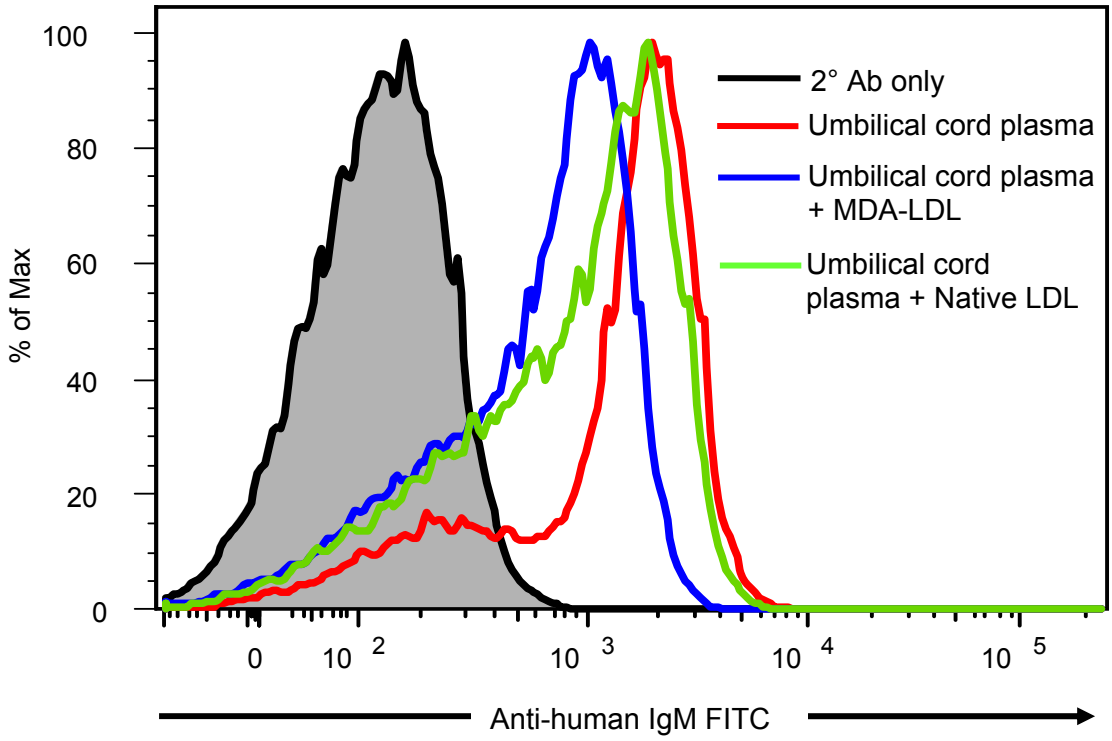



**Fig.6-3 Umbilical cord IgM binds to apoptotic cells in part via binding to MDA-related adducts.** Apoptotic Jurkat cells, induced by UV exposure, were incubated with a representative umbilical cord plasma (1:50 dilution) in the absence and presence of MDA-LDL and Native LDL (1mg/ml). Abs bound were detected by anti-human IgM FITC. Umbilical cord IgM binding to apoptotic Jurkat cells (median fluorescence intensity (MFI) =1917) was inhibited 45% by MDA-LDL (MFI=1047) while minimally affected by Native LDL (MFI=1514).

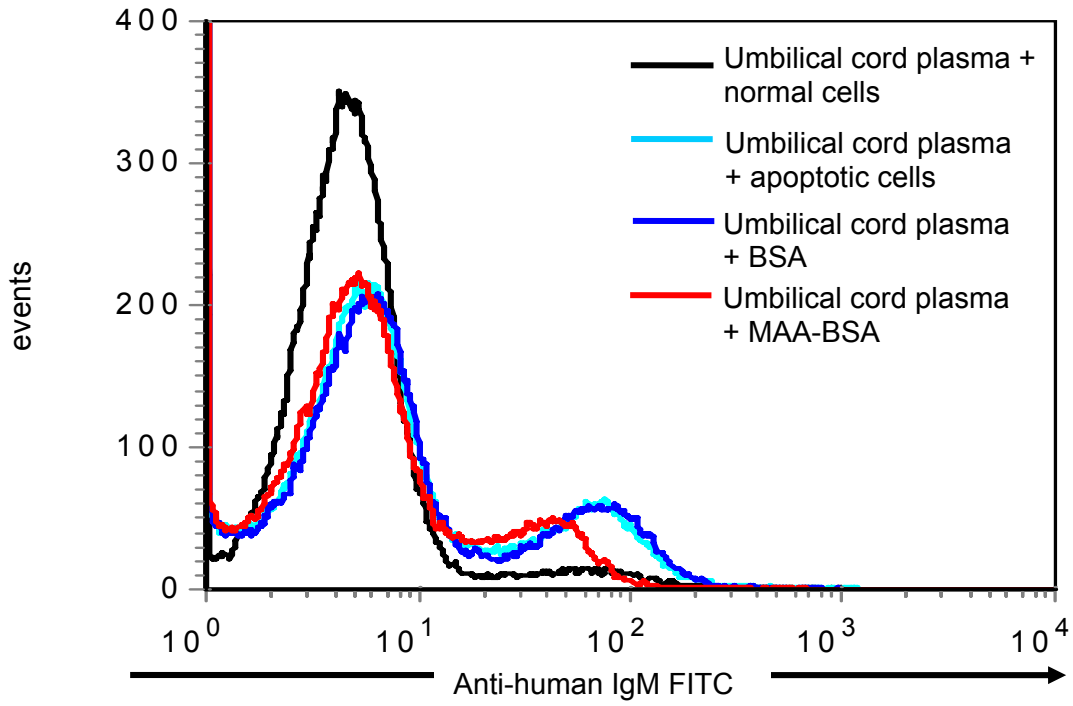



**Fig.6-4 MAA-BSA also inhibits umbilical cord IgM binding to apoptotic cells.** Apoptotic Jurkat cells were incubated with a representative umbilical cord plasma in the absence and presence of latex beads coated with BSA or MAA-BSA. Like MDA-LDL, MAA-BSA could compete about 70% of umbilical cord IgM binding to apoptotic Jurkat cells, while BSA has no effect.

of the 100 individual clones that were grown from the phage display library, HA-2, HA-25, and HA-24 were selected as representative examples. HA-2 has specificity to both MDA-LDL and MAA-BSA. HA-25 binds specifically to oxidation-specific antigens that contain the MAA adduct. On the other hand, HA-24 binds only to MAA-BSA but interestingly not to other any MAA-modified proteins, and not to any modified LDL. HA-2 is germ-line encoded while both HA-24 and HA-25 had a few mutations in their CDR3 region (Hansen *et al*. unpublished data). In

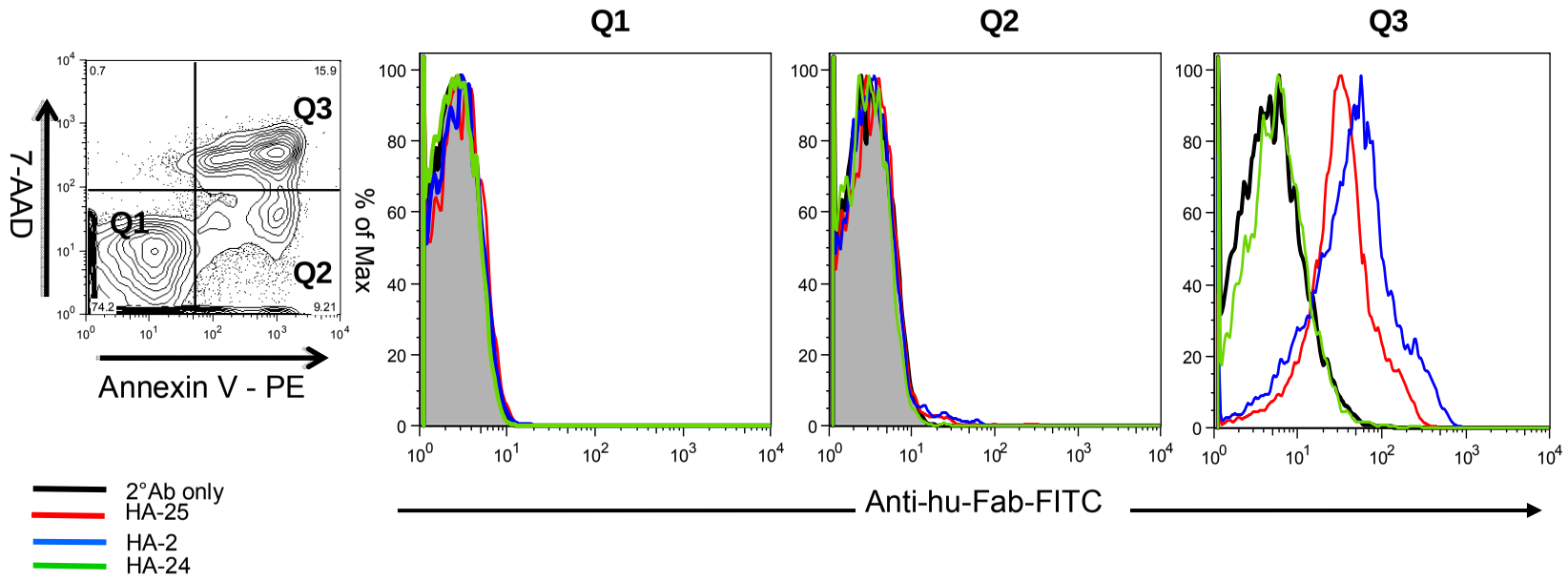



**Fig.6-5 human Fab HA2 and HA25 bind to late apoptotic cells but not early apoptotic cells or normal cells.**

The ability of human Fabs to bind to were apoptotic Jurkat cells were analyzed by flow cytometry. Apoptotic cells were determined by Annexin-V and 7-AAD staining. None of the Fabs bound to normal thymocytes (Q1, Annexin-$V^-$, 7-$AAD^-$) or early apoptotic cells (Q2, Annexin-$V^+$, 7-$AAD^-$), while both HA-2 and HA-25 bound to late apoptotic thymocytes (Q3, Annexin-$V^+$, 7- $AAD^+$). HA-24 did not recognize epitopes on apoptotic cells.

collaboration with Dr. Hansen, I tested the ability of these human Fabs to recognize apoptotic cells. As shown in **Fig.6-5**, both HA-2 and HA-25 were able to recognize epitopes on late apoptotic cells (Q3, Annexin-$V^+$, 7- $AAD^+$), but not the early apoptotic cells (Q2, Annexin-$V^+$, 7-$AAD^-$), or normal cells (Q1, Annexin-$V^-$, 7-$AAD^-$). HA-24, on the other hand, did not bind to any population of cells. The same observation was made for HA-25 using deconvolution microscopy. HA-25 only bound to apoptotic Jurkat cells but not normal cells (**Fig.6-6**).

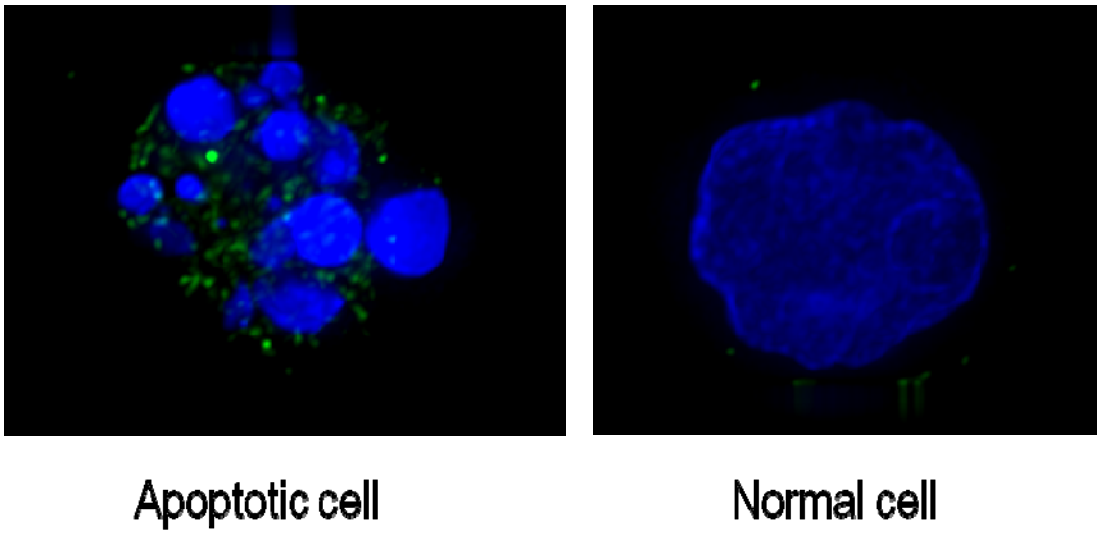



**Fig. 6-6 Deconvolution microscopy shows binding of human Fab HA-25 to apoptotic cells but not normal cells.** Apoptotic and normal Jurkat cells were incubated with human Fab HA-25 and then detected with FITC-conjugated anti-human IgG (Fab specific, green). DNA staining with 1μg/ml Hoechst dye shows fragmented nuclei of apoptotic cells (blue).

**Human Fab Abs recognize oxidation-specific epitopes in atherosclerotic lesions**

In addition to apoptotic cells, the ability of human Fabs to recognize epitopes in atherosclerotic lesions was also tested. Aortic lesions from hypercholesterolemic rabbits were stained with both HA-2 and HA-24. As shown in **Fig. 6-7**, HA-2 stained for the intermediate lesion that was present in the rabbit aorta while HA-24 did not recognize any epitopes in the lesions.

HA-2 has specificity to both MDA-LDL and MAA-BSA. Thus, the ability of HA-2 to inhibit specific binding of biotinylated MAA-LDL and MDA-LDL to mouse J774 macrophages was tested as well. Increasing concentrations of HA-2 and HA-24 were added together with biotinylated MAA-LDL or MDA-LDL to J774 macrophages. As shown in **Fig.6-8**, HA-2 competed about 90% of biotinylated MAA-LDL and MDA-LDL binding to mouse J774 macrophages, while HA24 had no effect. In analogy to the murine data, this demonstrated the possibility of human natural IgM Abs to inhibit OxLDL binding to macrophages and thus supports our hypothesis that natural IgM Abs might be beneficial in terms of atherosclerosis.

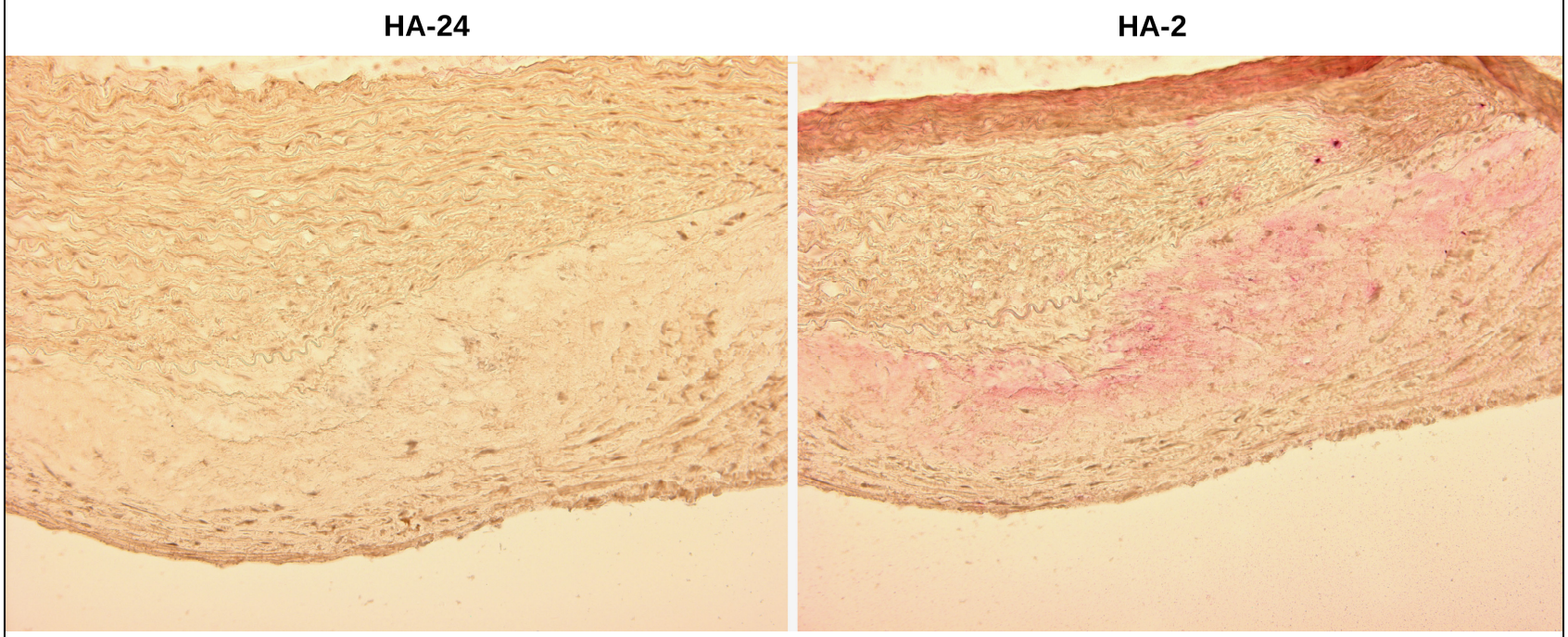



**Fig.6-7 Staining of atherosclerotic lesions from rabbit aorta with human Fab clones.** Aortic lesions from hypercholesterolemic rabbit were stained with HA-24 (1μg/ml) and HA-2 (0.5μg/ml). HA-2 stained for MDA-related epitopes in the lesions. In contrast, HA-24 did not show any staining. Positive staining is indicated by red color.

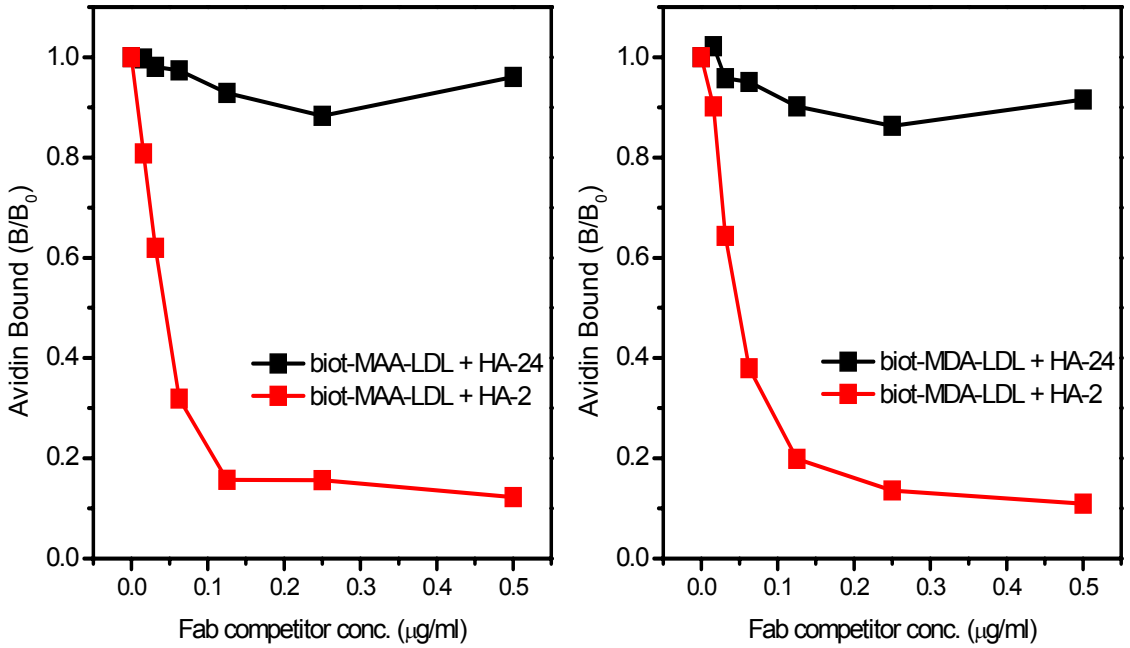



**Fig.6-8 HA-2 inhibits oxidation-specific antigen binding to macrophages.** The specific binding of biotinylated MAA-LDL and MDA-LDL to mouse J774 macrophages was detected by ELISA. To determine the capacity of HA-2 and HA-24 to inhibit MAA-LDL and MDA-LDL binding, increasing concentrations of NA-17 and HA-24 were added, together with biotinylated antigens, to macrophages. HA-2 competed about 90% of biotinylated MAA-LDL and MDA-LDL binding to macrophages, while HA-24 had no effect. Data is expressed as ratio of biotinylated antigen binding to macrophages in the presence or absence of IgM ($B/B_0$).

## Discussion

Compared to the knowledge of NAbs in mice, much less is known about NAbs in humans and of the cells responsible for their origin. However, here I demonstrated the presence of oxidation-specific IgM in human umbilical cord plasma, the human equivalent of NAbs. Compared to maternal plasma, the titers of oxidation-specific IgM are enriched in umbilical cord blood, suggesting that oxidation-specific epitopes are an important target of NAbs in humans as well.

Maternal plasma also contained IgM against KLH. Although it is thought that humans are not exposed to mollusk proteins and therefore do not have responses to KLH, the maternal IgM binding to plated KLH can be effectively competed by soluble KLH, demonstrating the specificity. Furthermore, there are in fact numerous reports in the literature demonstrating the presence of IgM anti-KLH antibodies. For example, a study by Korver et al. reported the common occurrence of natural IgM anti-KLH in humans, which they attributed in part to binding to polysaccharide determinants (23). Thornton et al. also demonstrated the presence of “natural IgM” to KLH in all 11 patients tested as part of a study to show that complement mediated such antigen presentation (24). Husby et al studied the ability of oral KLH in humans to induce tolerance to subcutaneous immunization. Inspection of their data show that most of the volunteers in this study had low levels of anti-KLH IgM at baseline (25).

Moreover, in my studies, IgM binding to KLH were not detected in the umbilical cord plasma, which was studied in the same assay with the same KLH preparation, which further addresses the specificity of my assay. The fact that

only the adults had IgM to KLH suggests that these were adaptive Abs which required exogenous antigen exposure.

The umbilical cord plasma IgM, like NAbs from the plasma of *Rag1*$^{-/-}$ B-1 recipients, also bound to apoptotic cells. This binding is in part mediated through the recognition of MDA-LDL and MAA-related epitopes, suggesting again that oxidation-specific epitopes might be a dominant target for human NAbs. Furthermore, human Fabs specific for MDA-LDL and MAA-BSA that were generated from the lymphocytes of umbilical cord blood, can also recognize apoptotic cells. HA-2 and HA-25 both recognized late apoptotic cells but not the early apoptotic cells, a similar property that was seen with the mouse monoclonal Ab NA-17 (see **Fig.5-2**). HA-2, HA-25 and NA-17 all have specificity towards MAA-related epitopes. Therefore, MAA-related epitopes must be enriched on the surface of late apoptotic cells. However, the exact structures that these NAbs are binding to still need to be determined with further studies.

HA-2 also recognized epitopes in the lesions and inhibited modified LDL binding to J774 macrophages. On the other hand, HA-24, which is specific for MAA-BSA but not other MAA-modified proteins, did not recognize epitopes on apoptotic cells or in the atherosclerotic lesions. Neither did it have the ability to inhibit MAA-LDL binding to macrophages. One possible explanation might be that HA-24 only recognizes a specific MAA structure (or conformation) on MAA-BSA that is not found on other MAA-modified proteins. Therefore, HA-24 can't recognize MAA-adducts that are present on apoptotic cells or OxLDL in the lesions, though possibly it recognizes different conformational MAA epitopes in a

different biological setting. However, HA-24 was generated, selected, grown and purified in parallel to HA-2 and HA-25, and thus serves as a useful negative control for the other clones in the settings tested.

The above evidence strongly suggests that oxidation-specific epitopes are an important target for human NAbs. Interestingly, in the recent report by Merbl et al., they reported that the most common antigen to which human NAbs bound was native LDL (20). However, in my studies, I only observed low levels of IgM binding to native LDL. “Native LDL” is routinely used in our lab as controls, but as noted in Chapter 3, though we often find low levels of binding to plated LDL, this cannot be competed by the same LDL in solution (see **Fig.3-4**), suggesting that the LDL undergoes modification during the plating procedure (either structural, e.g. oxidation, or even conformational). Indeed, the LDL used in the studies of Merbl et al., was bought from a commercial source, and it may have been modified, for example by oxidation, at the time of use. Thus, their data may be consistent with my observations that oxidation-specific epitopes are a dominant target of NAbs found in humans.

It has been proposed that NAbs in healthy individuals might have been selected to recognize a specific and limited set of self-antigens (16-18). Based on above evidence, these autoantibodies might actually be recognizing “altered-self” antigens, such as OxLDL. In fact, in a study by Brändlein et al., tumor-reactive human monoclonal Abs were isolated from both cancer patients and healthy donors and were found to have specificity to carbohydrates on modified tumor-specific receptors (26). Remarkably, all these Abs were IgM Abs secreted by

$CD5^+$ B-cells and expressed only few or no mutations at all. These Abs had the ability to inhibit cell growth and induce apoptosis.

Moreover, monoclonal Abs cloned from human chronic lymphocytic leukemia cells, which represents the outgrowth of $CD5^+$ B cells, recognized conserved epitopes associated with apoptosis and oxidation (27). Aside from apoptotic cells and OxLDL, chronic lymphocytic leukemia immunoglobulins in human were also reported to bind to polysaccharides of *S. pneumoniae* (28). Thus, natural IgM antibodies may recognize “altered-self” and play an important role not only in defense against pathogens but also in immunosurveillance mechanisms against “altered-self” such as apoptotic cells, OxLDL and tumors in humans.

In conclusion, oxidation-specific IgMs are present and enriched in human umbilical cord blood, suggesting that oxidation-specific epitopes are an important target of NAbs in humans as well. In analogy to the murine NAbs, oxidation-specific human NAbs can also recognize biologically relevant epitopes on apoptotic cells and in atherosclerotic lesions, which possibly function as an immunosurveillance mechanism against “altered-self”. Further studies will be needed to prove this speculation.

**Acknowledgement**

Chapters 6, in part, has been accepted for publication in “Oxidation-specific Epitopes are a Dominant Target for Innate Natural Antibodies”, Journal of Clinical Investigation, 2009, by MY Chou, L. Fogelstrand, K. Hartvigsen, LF Hansen, D. Woelkers, PX Shaw, J. Choi, T. Perkmann, F Bäckhed, YI Miller, S. Hörkkö, M. Corr, JL Witztum and CJ Binder. The dissertation author was the primary investigator and author of this paper

**References**


1. **Dorshkind, K. and E. Montecino-Rodriguez**. 2007. Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. Nat. Rev. Immunol. **7**:213-219.

2. **Carsetti, R., M. M. Rosado, and H. Wardmann**. 2004. Peripheral development of B cells in mouse and man. Immunol. Rev. **197**:179-191.

3. **Donze, H. H., C. Lue, B. A. Julian, W. H. Kutteh, A. Kantele, and J. Mestecky**. 1997. Human peritoneal B-1 cells and the influence of continuous ambulatory peritoneal dialysis on peritoneal and peripheral blood mononuclear cell (PBMC) composition and immunoglobulin levels. Clin. Exp. Immunol. **109**:356-361.

4. **Bofill, M., G. Janossy, M. Janossa, G. D. Burford, G. J. Seymour, P. Wernet, and E. Kelemen**. 1985. Human B cell development. II. Subpopulations in the human fetus. J. Immunol. **134**:1531-1538.

5. **Solvason, N., X. Chen, F. Shu, and J. F. Kearney**. 1992. The fetal omentum in mice and humans. A site enriched for precursors of CD5 B cells early in development. Ann. N. Y. Acad. Sci. **651**:10-20.

6. **Wardemann, H., T. Boehm, N. Dear, and R. Carsetti**. 2002. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. J. Exp. Med. **195**:771-780.

7. **Bhat, N. M., A. B. Kantor, M. M. Bieber, A. M. Stall, L. A. Herzenberg, and N. N. Teng**. 1992. The ontogeny and functional characteristics of human B-1 (CD5+ B) cells. Int. Immunol. **4**:243-252.

8. **Hardy, R. R., C. J. Wei, and K. Hayakawa**. 2004. Selection during development of VH11+ B cells: a model for natural autoantibody-producing CD5+ B cells. Immunol. Rev. **197**:60-74.

9. **Kruetzmann, S., M. M. Rosado, H. Weber, U. Germing, O. Tournilhac, H. H. Peter, R. Berner, A. Peters, T. Boehm, A. Plebani, I. Quinti, and R. Carsetti**. 2003. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. J. Exp. Med. **197**:939-945.

10. **Forster, I., H. Gu, and K. Rajewsky**. 1988. Germline antibody V regions as determinants of clonal persistence and malignant growth in the B cell compartment. EMBO J. **7**:3693-3703.

11. **Damle, R. N., F. Ghiotto, A. Valetto, E. Albesiano, F. Fais, X. J. Yan, C. P. Sison, S. L. Allen, J. Kolitz, P. Schulman, V. P. Vinciguerra, P. Budde, J. Frey, K. R. Rai, M. Ferrarini, and N. Chiorazzi**. 2002. B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. Blood **99**:4087-4093.

12. **Meffre, E. and J. E. Salmon**. 2007. Autoantibody selection and production in early human life. J. Clin. Invest **117**:598-601.

13. **Wardemann, H., S. Yurasov, A. Schaefer, J. W. Young, E. Meffre, and M. C. Nussenzweig**. 2003. Predominant autoantibody production by early human B cell precursors. Science **301**:1374-1377.

14. **Coutinho, A., M. D. Kazatchkine, and S. Avrameas**. 1995. Natural autoantibodies. Curr. Opin. Immunol. **7**:812-818.

15. **Quintana, F. J., P. H. Hagedorn, G. Elizur, Y. Merbl, E. Domany, and I. R. Cohen**. 2004. Functional immunomics: microarray analysis of IgG autoantibody repertoires predicts the future response of mice to induced diabetes. Proc. Natl. Acad. Sci. U. S. A **101 Suppl 2**:14615-14621.

16. **Cohen, I. R. and D. B. Young**. 1991. Autoimmunity, microbial immunity and the immunological homunculus. Immunol. Today **12**:105-110.

17. **Cohen, I. R.** 1992. The cognitive paradigm and the immunological homunculus. Immunol. Today **13**:490-494.

18. **Nobrega, A., M. Haury, A. Grandien, E. Malanchere, A. Sundblad, and A. Coutinho**. 1993. Global analysis of antibody repertoires. II. Evidence for specificity, self-selection and the immunological "homunculus" of antibodies in normal serum. Eur. J. Immunol. **23**:2851-2859.

19. **Bobrzynski, T., M. Fux, M. Vogel, M. B. Stadler, B. M. Stadler, and S. M. Miescher**. 2005. A high-affinity natural autoantibody from human cord blood defines a physiologically relevant epitope on the FcepsilonRIalpha. J. Immunol. **175**:6589-6596.

20. **Merbl, Y., M. Zucker-Toledano, F. J. Quintana, and I. R. Cohen**. 2007. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. J. Clin. Invest **117**:712-718.

21. **Barbas, C. F., III, A. S. Kang, R. A. Lerner, and S. J. Benkovic**. 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. U. S. A **88**:7978-7982.

22. **Binder, C. J., P. X. Shaw, M. K. Chang, A. Boullier, K. Hartvigsen, S. Horkko, Y. I. Miller, D. A. Woelkers, M. Corr, and J. L. Witztum**. 2005. The role of natural antibodies in atherogenesis. J. Lipid Res. **46**:1353-1363.

23. **Korver, K., W. P. Zeijlemaker, P. T. Schellekens, and J. M. Vossen**. 1984. Measurement of primary in vivo IgM- and IgG-antibody response to KLH in humans: implications of pre-immune IgM binding in antigen-specific ELISA. J. Immunol. Methods **74**:241-251.

24. **Thornton, B. P., V. Vetvicka, and G. D. Ross**. 1994. Natural antibody and complement-mediated antigen processing and presentation by B lymphocytes. J. Immunol. **152**:1727-1737.

25. **Husby, S., J. Mestecky, Z. Moldoveanu, S. Holland, and C. O. Elson**. 1994. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. J. Immunol. **152**:4663-4670.

26. **Brandlein, S., T. Pohle, N. Ruoff, E. Wozniak, H. K. Muller-Hermelink, and H. P. Vollmers**. 2003. Natural IgM antibodies and immunosurveillance mechanisms against epithelial cancer cells in humans. Cancer Res. **63**:7995-8005.

27. **Catera, R., G. J. Silverman, K. Hatzi, T. Seiler, S. Didier, L. Zhang, M. Herve, E. Meffre, D. G. Oscier, H. Vlassara, R. H. Scofield, Y. Chen, S. L. Allen, J. Kolitz, K. R. Rai, C. C. Chu, and N. Chiorazzi**. 2008. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol. Med. **14**:665-674.

28. **Lanemo, M. A., E. Hellqvist, E. Sidorova, A. Soderberg, H. Baxendale, C. Dahle, K. Willander, G. Tobin, E. Backman, O. Soderberg, R. Rosenquist, S. Horkko, and A. Rosen**. 2008. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood **111**:3838-3848.

# CHAPTER 7

## CONCLUSION AND FUTURE DIRECTIONS

## Conclusion

Innate immune responses provide a vital and non-redundant role in the initial defense against invading pathogens and in maintaining homeostasis against a variety of “self-antigens”. The mediators of innate immunity are germ-line encoded, present at birth and/or maturated via positive selection during the neonatal period or shortly thereafter.

NAbs are part of the humoral arc of innate immunity that share most of the characteristics described above. They exhibit a genetically determined and stable repertoire that is largely independent of external antigenic stimuli (2-4). NAbs have been defined in various ways, for example, as the IgM present in normal plasma (5), but more stringently, as germ-line encoded Ab that arise without any exogenous immune exposure or bacterial colonization of the gut (6). In normal, uninfected mice, most plasma IgM are NAbs that are derived from innate B-1 cells.

B-1 cells differ from conventional B-2 cells in many ways, including surface phenotype and anatomic location. B-1 cells also utilize a restricted set of $V_H$ genes that are minimally edited and reflective of germline usage. Importantly, B-1 cells have the capacity to self renew (reviewed in (7-9)), which is the basic rationale behind the B-1 cell mouse model that I have developed in this work. However, relatively little is known about B-1 cell ontogeny, development and surprisingly, their function.

Although the mechanisms leading to selection and expansion of B-1 cells are unclear, it is now appreciated that antigen selection during fetal and neonatal period leads to positive selection (7,10,11). Since this appears to occur equally well in mice raised in germ-free environments (2,12,13), this selection is of necessity made by endogenous “self-antigens”. This is in contrast to developing, self-reactive B-2 cells, in which such antigen encounter early in life leads to negative selection via apoptosis, (= clonal deletion) or anergy. **Thus, the repertoire of B-1 cells is selected to bind to evolutionarily important epitopes.**

Oxidation of LDL creates a wide variety of novel epitopes that are important immunogens in the context of atherosclerosis. We have termed these oxidation-specific epitopes and demonstrated that during the course of atherogenesis there is a profound humoral and cellular response to a variety of oxidation-specific epitopes found on OxLDL (14). More importantly, oxidation-specific epitopes are not only restricted to OxLDL and atherosclerotic lesions, but in fact are ubiquitously present in inflamed tissues found in many different diseases. In particular, because oxidative events are a key process in cells undergoing apoptosis, oxidation-specific epitopes are present on apoptotic cell surfaces as well as in apoptotic bodies - often termed microparticles - that bud off from such cells (15-17). Because the generation of apoptotic cells and inflammatory reactions take place throughout life, oxidation-specific epitopes might confer strong evolutionary pressure and drive the selection of NAbs. Thus, my studies revolved around how oxidation-specific neo-epitopes as a class

constitute a major target for B-1 cell derived NAbs, how these B-1 cells might be regulated, as well as the possible physiology function of the oxidation-specific NAbs.

In this dissertation I provided multiple lines of evidence showing that oxidation-specific epitopes are a dominant target of innate NAbs in both mice and humans. In the first part of my studies I characterized murine humoral IgM responses to oxidation-related antigens in mice. Consistent with the view that the IgM pool of uninfected mice represent NAbs (7), I made the key observations that mice maintained under specific pathogen free conditions, as well as germ-free mice raised under strict gnotobiotic conditions contained prominent IgM titers to oxidation-specific epitopes compared to titers against recognized TI-2 antigens, such as $\alpha$1,3-Dextran. Also, there was a high frequency of IgM secreting cells (ISCs) to oxidation-specific epitopes, chiefly MDA, in spleens of wild-type mice. Both suggest the NAb origin of oxidation-specific IgM.

Interestingly, the introduction of normal gut flora into the germ-free mice (conventionalized mice) increased the titers of certain oxidation-specific IgM, including those directed to Cu-OxLDL, PC-BSA, and 4-HNE-LDL, but not MDA-LDL. Thus, differences exist between the different oxidation-specific IgM and some, but not all may be stimulated by commensal bacteria. In mice, NAbs are mainly secreted by B-1 cells, which are thought to typically have very little response to antigenic stimuli. However, some antigens, such as PC and $\alpha$1,3-dextran, have been shown to stimulate antigen-specific NAb production by B-1 cells (7). Thus, the increase in the conventionalized mice of IgM to PC-BSA and

OxLDL, in which PC of OxPL is a dominant epitope (18), may be reflective of such exogenous antigenic stimulation.

Next, to directly demonstrate that B-1 cells can produce oxidation-specific IgM, I showed that B-1 cells in culture, purified from naïve uninfected mice, gave rise to a whole array of IgM secreting cells with specificity for various oxidation epitopes. Indeed, compared to the B-1 cell classic antigen, $\alpha$1,3-Dextran (9), oxidation-specific epitopes were a dominant target of secreted IgMs. Such IgM were already detectable in supernatants of unstimulated B-1 cells, and the production of these IgM in response to stimulation, especially that of TLRs, increased to a greater extent than total IgM or IgM to $\alpha$1,3-dextran. Moreover, in analogy to the robust anti-MDA-responses *in vivo*, MDA-specific IgM were the most dominant set of IgM Abs secreted *in vitro,* constituting up to 30% of total IgM.

I also demonstrated that TLR4 and TLR2 agonists were particularly effective in stimulating the generation of IgM by B-1 cells in culture, an effect that was in large part My88D dependent. A similar observation was reported by Genestier et al (19) while my studies were in progress. Remarkably, my data indicates that TLR activation seems to have preferentially expanded B-1 clones secreting IgM to oxidation-specific epitopes as compared to total IgM or IgM to $\alpha$1,3-dextran. This would imply that B-1 clones secreting such IgM are more responsive to activation by TLRs, suggesting evolutionary pressure linking generation of NAbs to oxidation-epitopes and activation of innate PRRs. This is intriguing as our lab and others have provided data suggesting that aside from

products of exogenous pathogens, there are endogenous antigens capable of stimulating such TLRs, including OxLDL (20).

The stimulation of B-1 cells also induced increased secretion of cytokines, especially IL-10. The increase in IL-10 production after TLR activation might provide a negative feedback mechanism utilized by B-1 cells to control the inflammatory response, since IL-10 was found to inhibit the proliferation and IgM secretion of B-1 cells when co-administered with $KdO_2$-lipidA (a synthetically pure bioactive form of LPS).

To study B-1 cell derived NAbs *in vivo*, I generated a *Rag1*$^{-/-}$ B-1 reconstituted mouse model by adoptively transferring B-1 cells isolated from the peritoneum of wild-type C57BL/6 mice into *Rag1*$^{-/-}$ mice. Because B-1 cells have the capacity to self-replicate, they repopulated the peritoneum and migrated to the spleen, where they secreted IgM into plasma. After only 10 weeks, the plasma IgM titers in the Rag1$^{-/-}$ B-1 mice were restored to 30-50% or more of the wildtype mice. Analyses of the specificity of these IgM revealed that they again bound prominently to oxidation-specific epitopes, similar to that observed in the IgM found in wildtype mice or in the supernatants of B-1 cells in culture.

Absorption studies of the plasma IgM demonstrated that 20-30% of these IgM had specificity for combinations of oxidation epitopes, prominently MDA related epitopes. These data were further corroborated by ELISpot analysis of splenocytes of *Rag1*$^{-/-}$ B-1 recipients, demonstrating that ISCs against MDA-LDL accounted for 10~12% of all ISCs in the spleens of the reconstituted mice, similar to the frequency found in spleens of wild-type mice. Also, 20 to 30% of the IgM

secreting hybridomas generated from the spleens of the B-1 cell reconstituted *Rag1*$^{-/-}$ mice , which by default consisted entirely of hybridomas secreting NAbs, showed binding to oxidation-specific epitopes, chiefly MDA. Cloning and sequencing of a number of these confirmed their germ-line origin, as exemplified by the MDA-specific B-1 clone NA-17. These data strongly support the hypothesis that oxidation-specific epitopes are a dominant target of NAbs in mice.

Next, I demonstrated that these NAbs bound prominently to biologically relevant oxidation-specific epitopes. B-1 cell derived IgM in the plasma of the B-1 reconstituted *Rag1*$^{-/-}$ mice bound prominently to epitopes on apoptotic cells and to atherosclerotic lesions. Furthermore, endogenous B-1 derived IgM in the reconstituted mice co-localized with oxidation-specific epitopes *in vivo* in atherosclerotic lesions in combined *Rag1*$^{-/-}$ *Ldlr*$^{-/-}$ mice that were cholesterol-fed. Furthermore, the monoclonal Ab NA-17, which binds MDA-related epitopes, bound prominently to late apoptotic cells and atherosclerotic lesions. NA-17 was also able to inhibit the binding of MDA-LDL to mouse macrophages. In the context of atherosclerosis, this might be beneficial due to the possible role for NAbs in the interference of foam cell formation.

Previously we showed that E06, which binds to the PC of oxidized phospholipids (OxPL) on OxLDL, was able to inhibit the binding of OxLDL to macrophages in cell culture. This indicates that the epitope to which E06 binds, the PC of OxPL, is a ligand mediating binding of OxLDL to macrophages. In this *in vitro* setting, complement was not present. In my experiments, I also demonstrated that both the IgM from the plasma of the reconstituted Rag1-/-

mice as well as NA-17 bound to apoptotic cells *in vitro*. Because IgM Ab are efficient in binding complement, and have previously been shown to play a role in mediating clearance of apoptotic cells *in vivo*, I also examined the ability of these IgM to effect clearance of apoptotic cells *in vivo*. Using a previously established model that examines the uptake of apoptotic cells *in vivo* in peritoneal macrophages, I demonstrated that IgM NAbs facilitate macrophage uptake of apoptotic cells *in vivo*, and specifically, that the MDA-specific NAb, NA-17, similarly mediates enhanced clearance. Previously, the OxPL-specific NAb E06/T15, was also shown to facilitate efficient complement mediated phagocytosis of apoptotic cells *in vivo* (21). These data demonstrate an important role for oxidation-specific natural IgM in mediating clearance of apoptotic cells.

It has been suggested that most innate NAbs have dual specificities, binding to both an endogenous “self-antigen” as well as to an exogenous epitope on infectious pathogens (7). I have also provided preliminary evidence in support of this hypothesis as a general property of NAbs to oxidation-specific epitopes. The B-1 cell derived IgM showed strong reactivity towards common pathogens that are associated with human infections such as *S. aureus*, *P. aeruginosa,* GAS and *S. pneumoniae.* Monoclonal IgM Abs such as E06, which binds to PC on OxPL and E014, which binds to MDA-LDL, were also found to bind to specifically to *S. aureus* and GAS, respectively. These examples illustrated nicely that epitopes on pathogens share molecular mimicry with oxidation-specific epitopes. These data strongly support the concept that oxidation-specific

epitopes are a dominant target of NAbs in mice, which in turn bind to biologically relevant “self-antigens”.

Much less information is known about NAbs in humans and of the cells responsible for their origin. However, because the IgM found in fetal cord blood are solely of fetal origin, they are thought to represent such naïve NAbs in humans (22). Here I showed that oxidation-specific IgM are enriched in human umbilical cord plasma compared to the maternal plasma, and bind to apoptotic cells, in part mediated by binding to MDA and MAA epitopes, Furthermore, HA-2 and HA-25, which are germ-line (or near germ-line) encoded human Fabs generated from a phage display library prepared from the lymphocytes of umbilical cord blood, also bound to MDA-LDL (and MAA) and apoptotic cells. HA-2 also recognized apoptotic cells. HA-2 also recognized epitopes in atherosclerotic lesions and inhibited MAA-LDL and MDA-LDL binding to macrophages. These data strongly suggests that oxidation-specific epitopes are an important target for NAbs in humans as well. These human NAbs, like mouse NAbs, recognize biologically relevant epitopes on apoptotic cells and in atherosclerotic lesions.

**Fig. 7-1** summarizes the paradigm that my dissertation work is based on. The apparent high prevalence of oxidation-specific epitopes as targets of NAbs in mice and humans likely reflects the ubiquitous presence of these epitopes consequent to oxidative events. Cells undergoing apoptosis and the microparticles shed from them are rich in oxidation epitopes (16,23,24). Furthermore, inflammatory events are associated with enhanced oxidative stress

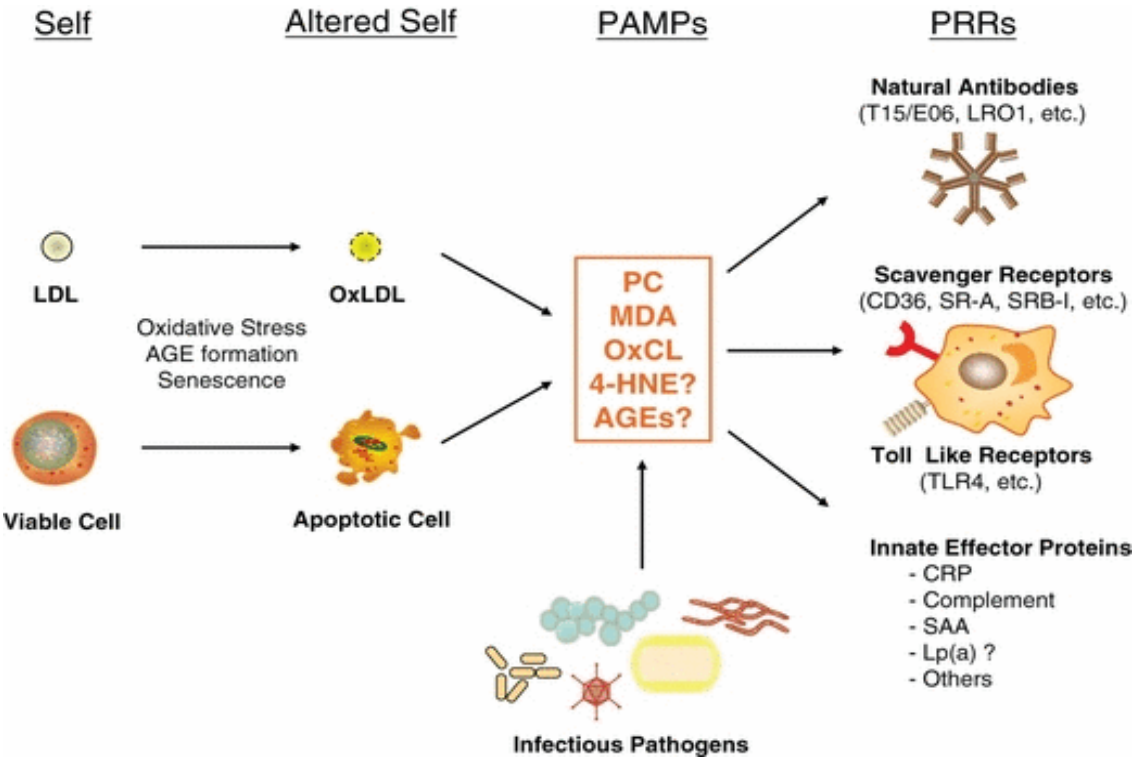



**Fig. 7-1** Oxidation-specific epitopes are a class of pathogen-associated molecular patterns (PAMPs) recognized by multiple arcs of innate immunity, including natural antibodies, scavenger receptors, toll-like receptors (TLRs) as well as a variety of innate effector proteins. Oxidation-specific epitopes (altered self) can be generated by physiological as well as pathophysiological processes on lipoproteins or cell membranes (self). In many cases, there is molecular mimicry of oxidation-specific epitopes on oxidized lipoproteins and apoptotic cells that share molecular identity with structures on infectious pathogens. Many other postsecretory modifications of proteins and lipids may generate similar PAMPs, for example, as occurs during formation of advanced glycation end products (AGE). These will in turn be recognized by the PRRs of innate immunity. In my studies I focused on the NAbs. (Figure adopted from Ref (1)).

and our different oxidation-specific antibodies have been used to demonstrate the presence of these epitopes not only in atherosclerotic tissue, but in a variety of inflammatory settings, including renal and liver disease (25,26), rheumatoid arthritis (unpublished observation), CNS lesions found in multiple sclerosis (27) and Alzheimer's disease (28). Finally, many if not most NAbs also react with microbes that contain the same antigenic self-determinants or closely related molecules, leading to cross-reactivity between self-determinants and microbial antigens. Therefore, we speculate that many of the so-called "self-antigens" that have been reported to be targets of NAbs may well be "altered self" such as oxidation-specific epitopes.

Thus, oxidation-specific epitopes may constitute a previously unrecognized class of pathogen-associated molecular patterns (PAMPs) that can be recognized by innate pattern recognition receptors (PRRs). It is now widely recognized that one class of PRRs, the so-called "scavenger receptors", such as SRA-1,2, CD36, SR-B1, LOX-1, PSOX and others, all recognize various oxidation-specific epitopes present on OxLDL and apoptotic cells (29). Recent evidence suggests that members of the innate TLR family also recognize such epitopes as well (30,31). Another innate protein, CRP, also binds to the PC of OxPL present on OxLDL and apoptotic cells, as well as to *S. pneumoniae* (32).

Natural antibodies are another arc of innate immunity and because their $V_H/V_L$ regions, which form the antigen binding sites, are germline encoded, they are in essence PRRs that bind to evolutionary important antigens. NAbs have been said to be polyreactive and to bind to many "autoantigens" nonspecifically.

However, many of the so-called “self-antigens” that have been reported to be targets of NAbs may well be modified self-antigens and the polyreactivity may be confounded by the fact that a given NAb to a specific oxidation-specific epitope may bind to the same structural modification on many different proteins or even lipids. For example, E06 binds to both the OxPL present in lipid as well as that bound to apoB of OxLDL. EO6 binds to OxPL covalently bound to apo(a) as well as OxPL in the lipid phase of Lp(a) (33). It also binds to the OxPL present on the cell surface of apoptotic cells (15), as well as the PC present on the cell wall of *S.pnemoniae* and *S.aureus*. E06 also recognizes a large number of tissues in which inflammation exists, presumably to the PC epitope generated as result of lipid peroxidation and/or apoptosis. If one did not know the true identity of the epitope of E06, one would say this Ab was “polyreactive”, while in fact, it is binding to the same structural modification on many different proteins or lipids, which is the concept depicted in **Fig. 7-1**.

In this work, I identified an even more prominent set of IgM directed against MDA-related epitopes, which is exemplified by MAA, that are generated as a consequence of lipid peroxidation. These highly reactive aldehydes may similarly modify a wide range of substrates, including proteins, lipids, and even DNA (34), creating “altered-self”. MDA epitopes occur *in vivo* in a variety of pathophysiological events associated with oxidative stress, such as ischemia and reperfusion, diabetes, and atherosclerosis as well as inflammatory events in the brain (35). In my studies, I demonstrated that these MDA-related epitopes are a dominant target for NAbs both in mice and human, and these epitopes are

present on late apoptotic cells as well as in atherosclerotic lesions. Furthermore, I showed that infectious pathogens share these MDA-related epitopes on their surface as well. These data nicely illustrates the paradigm depicted in **Fig.7-1** and added another piece of evidence that oxidation-specific epitopes--as a class--constitute an important PAMP recognized by innate immunity, consistent with the oxygen-centric nature of life itself.

Although the mechanisms of B-1 cell clone selection and expansion are poorly understood, because oxidative events are such a universal process, we speculate that they could be fundamental in providing “selecting antigens” for the positive selection of many B-1 cell clones. Most NAbs have dual specificities (7), typically with molecular epitopes on pathogens. Thus, an encounter later in life with such external pathogens could further serve to expand a given B-1 cell clone (36,37).

Functionally, NAbs play an important role in providing the first line of defense against viral and bacterial pathogens (38-40). NAbs also have important activities in homeostasis (41), including immunoregulatory functions, tumor surveillance, and recognition and removal of senescent cells, cell debris, and other self-antigens, which if persisting would be proinflammatory and immunogenic. Indeed, mice that cannot secrete IgM Abs and thus lack natural IgM have been found to more readily develop autoimmune disease when crossed on a susceptible background (42). Here I demonstrated that both murine natural IgM as well as human umbilical cord IgM bind apoptotic cells, in part through binding to oxidation-specific epitopes on their cell surface. Furthermore,

these IgM NAbs facilitate macrophage uptake of apoptotic cells *in vivo*, suggesting an important role for oxidation-specific natural IgM in mediating clearance of apoptotic cells. IgM in general have been shown to be required in complement mediated clearance of apoptotic cells (43). There are likely many other roles for NAbs. For example, multiple lung pathogens, such as chemical agents, H5N1 avian flu, or SARS cause high lethality due to acute respiratory distress syndrome. In turn, these infections cause lung macrophages to generate large amounts of OxPL, which in turn induced lung injury and cytokine production by lung macrophages. In vitro, the impact of OxPL on macrophages could be ameliorated by E06/T15 (44). As another example, there are a multitude of oxidation-specific epitopes in atherosclerotic lesions and we have previously shown that enhancing the titer of EO6/T15 is atheroprotective (45).

In summary, my studies demonstrated that oxidation-specific epitopes constitute a previously unrecognized but important target of NAbs, and of innate immunity in general. NAbs may be beneficial not only for defense against pathogens but also in identifying “altered-self” that occurs as a result of oxidative stress under inflammatory events. Understanding their role in health and disease may lead to novel diagnostic and possibly therapeutic approaches to deal with consequences of oxidative stress such as atherogenesis.

## Future Directions

These studies raise many questions that remain to be answered. Many detailed studies are needed to explore what regulates B-1 cells and in particular, how the secretion of NAbs and cytokines by B-1 cells can be regulated by the ligation of TLRs. B-1 cells, when stimulated with TLR4 and TLR2 agonists *in vitro*, showed increased IgM production. While we know this effect is partly Myd88 dependent, the downstream genes that are activated in the immunoglobulin (Ig) secretion pathway that led to increased IgM production is still unknown. Blimp-1 has been shown to be required for normal Ig secretion by B-1 cells and is a possible candidate that might be regulated by TLR activation (46).

In addition, a variety of cytokines are also induced with TLR activation. In this work I only studied the impact of IL-10 in further detail. More studies should be done to address the effect of other cytokines on B-1 cells. Importantly, my data indicated the stimulation of B-1 cells with TLR agonists led to an apparent preferential stimulation of B-1 cells secreting oxidation-specific IgMs, at least as compared to the secretion of the classic B-1 cell antigen, $\alpha$1,3-dextran. Because the repertoire of B-1 cells is germ-line encoded, this implies that there is a preferential stimulation of B-1 cells with specificity to oxidation-specific epitopes. One might hypothesize that there has been an evolutionary pressure that links the activation of TLRs to the generation of NAbs to oxidation-specific epitopes. This activation may come from endogenous ligands, such as OxLDL. Therefore,

to test the ability of OxLDL in being an endogenous ligand for TLRs is crucial in validating this hypothesis.

While studying B-1 cell derived NAbs in vivo, I also detected measurable titers of IgG3 in the plasma of B-1 cell reconstituted *Rag1*$^{-/-}$ mice, aside from IgM. However, whether oxidation-specific epitopes are also a target for IgG3 Ab will require more detailed study.

Monoclonal IgM NA-17, which was cloned from the spleens of *Rag1*$^{-/-}$ B-1 recipients, showed high level of binding to MDA-related antigens. Even though NA-17 serves as an example of a true germ-line Ab that has specificity for oxidation-specific epitopes, the exact MDA-related structure that NA-17 is recognizing remains to be identified, but appears to be a highly fluorescent adduct identical too or closely related to MAA. Human monoclonal Fabs HA-2 and HA-25, like NA-17, binds to MDA-related antigens as well as apoptotic cells. Therefore, understanding the chemical structure to which these Abs are binding will be very helpful in identifying the similar or same structures that are present on apoptotic cells or infectious pathogens.

A great deal of work will be needed to assess the role of oxidation-specific NAbs in atherosclerosis in particular and homeostasis in general. In this study I showed that NA-17 was able to inhibit MDA-LDL binding to macrophages, which suggests a possible role for NAbs in the interference of foam cell formation. However, an atherosclerosis study will be needed to evaluate the exact role of NAbs in atherosclerosis.

B-1 cell derived IgM Abs can facilitate the uptake of apoptotic cells by macrophages *in vivo*. IgM are known to opsonize antigens, leading to the binding and activation of complement, which in turn can bind to complement receptors on phagocytes, leading to receptor mediated clearance (47). Indeed, Elkon and colleagues have previously shown the necessity of both IgM and complement C1q binding to the activation of complement and subsequent binding of complement C3, which eventually leads to complement dependent clearance of apoptotic cells (21). In a similar manner, oxidation-specific IgM could mediate the enhanced clearance not only of apoptotic cells, but a variety of structures containing oxidation-specific epitopes on their surface. For example, microparticles, which in large part are derived from apoptotic blebs are quite bioactive and proinflammatory (48). Therefore, it will be exciting to know if oxidation-specific NAbs can inhibit some if their adverse effects.

Another intriguing functional aspect of NAbs is their role in defense against pathogens. I have shown that *Rag1*$^{-/-}$ B-1 recipient plasma IgM can bind to a variety of both gram positive and negative pathogens. However, I have shown using existing monoclonal antibodies that not only are PC epitopes present on pathogens, as is well known, but that MDA epitopes recognized by oxidation-specific epitopes are also present on a variety of pathogens as well. I will test whether other true germline NAbs, such as NA-17 also bind to pathogens, and what are the functional properties of such binding. For example, as with the case for E06/T15, it will be interesting to know if binding to MDA-specific epitopes on pathogens will also provide optimal protection in vivo in murine models of

disease. Because there are readily measured titers of MDA-specific IgM in umbilical cord blood, and in adults, the identification that such NAbs provide protection against pathogens could be of important therapeutic benefit.

These studies will eventually lead to a better understanding of NAbs. They will be essential in elucidating the biology of B-1 cells and may reveal new therapeutic targets that can be exploited to interfere with inflammatory states in general.

## References


1. **Chou, M. Y., K. Hartvigsen, L. F. Hansen, L. Fogelstrand, P. X. Shaw, A. Boullier, C. J. Binder, and J. L. Witztum**. 2008. Oxidation-specific epitopes are important targets of innate immunity. J. Intern. Med. **263**:479-488.

2. **Haury, M., A. Sundblad, A. Grandien, C. Barreau, A. Coutinho, and A. Nobrega**. 1997. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur. J. Immunol. **27**:1557-1563.

3. **Vasconcellos, R., A. Nobrega, M. Haury, A. C. Viale, and A. Coutinho**. 1998. Genetic control of natural antibody repertoires: I. IgH, MHC and TCR beta loci. Eur. J. Immunol. **28**:1104-1115.

4. **Mouthon, L., A. Nobrega, N. Nicolas, S. V. Kaveri, C. Barreau, A. Coutinho, and M. D. Kazatchkine**. 1995. Invariance and restriction toward a limited set of self-antigens characterize neonatal IgM antibody repertoires and prevail in autoreactive repertoires of healthy adults. Proc. Natl. Acad. Sci. U. S. A **92**:3839-3843.

5. **Jayasekera, J. P., E. A. Moseman, and M. C. Carroll**. 2007. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. J. Virol. **81**:3487-3494.

6. **Hooijkaas, H., R. Benner, J. R. Pleasants, and B. S. Wostmann**. 1984. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. Eur. J. Immunol. **14**:1127-1130.

7. **Baumgarth, N., J. W. Tung, and L. A. Herzenberg**. 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol. **26**:347-362.

8. **Hardy, R. R. and K. Hayakawa**. 2005. Development of B cells producing natural autoantibodies to thymocytes and senescent erythrocytes. Springer Semin. Immunopathol. **26**:363-375.

9. **Kearney, J. F.** 2005. Innate-like B cells. Springer Semin. Immunopathol. **26**:377-383.

10. **Hardy, R. R., C. J. Wei, and K. Hayakawa**. 2004. Selection during development of VH11+ B cells: a model for natural autoantibody-producing CD5+ B cells. Immunol. Rev. **197**:60-74.

11. **Qian, Y., K. L. Conway, X. Lu, H. M. Seitz, G. K. Matsushima, and S. H. Clarke**. 2006. Autoreactive MZ and B-1 B-cell activation by Faslpr is coincident with an increased frequency of apoptotic lymphocytes and a defect in macrophage clearance. Blood **108**:974-982.

12. **Bos, N. A., C. G. Meeuwsen, H. Hooijkaas, R. Benner, B. S. Wostmann, and J. R. Pleasants**. 1987. Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. Cell Immunol. **105**:235-245.

13. **Thurnheer, M. C., A. W. Zuercher, J. J. Cebra, and N. A. Bos**. 2003. B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. J. Immunol. **170**:4564-4571.

14. **Horkko, S., C. J. Binder, P. X. Shaw, M. K. Chang, G. Silverman, W. Palinski, and J. L. Witztum**. 2000. Immunological responses to oxidized LDL. Free Radic. Biol. Med. **28**:1771-1779.

15. **Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum**. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc. Natl. Acad. Sci. U. S. A **96**:6353-6358.

16. **Huber, J., A. Vales, G. Mitulovic, M. Blumer, R. Schmid, J. L. Witztum, B. R. Binder, and N. Leitinger**. 2002. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Arterioscler. Thromb. Vasc. Biol. **22**:101-107.

17. **Leroyer, A. S., H. Isobe, G. Leseche, Y. Castier, M. Wassef, Z. Mallat, B. R. Binder, A. Tedgui, and C. M. Boulanger**. 2007. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. J. Am. Coll. Cardiol. **49**:772-777.

18. **Horkko, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum**. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J. Clin. Invest **103**:117-128.

19. **Genestier, L., M. Taillardet, P. Mondiere, H. Gheit, C. Bella, and T. Defrance**. 2007. TLR agonists selectively promote terminal plasma cell

differentiation of B cell subsets specialized in thymus-independent responses. J. Immunol. **178**:7779-7786.

20. **Miller, Y. I., S. Viriyakosol, C. J. Binder, J. R. Feramisco, T. N. Kirkland, and J. L. Witztum**. 2003. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J. Biol. Chem. **278**:1561-1568.

21. **Ogden, C. A., R. Kowalewski, Y. Peng, V. Montenegro, and K. B. Elkon**. 2005. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. Autoimmunity **38**:259-264.

22. **Merbl, Y., M. Zucker-Toledano, F. J. Quintana, and I. R. Cohen**. 2007. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. J. Clin. Invest **117**:712-718.

23. **Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum**. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J. Exp. Med. **200**:1359-1370.

24. **Kagan, V. E., G. G. Borisenko, Y. Y. Tyurina, V. A. Tyurin, J. Jiang, A. I. Potapovich, V. Kini, A. A. Amoscato, and Y. Fujii**. 2004. Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. Free Radic. Biol. Med. **37**:1963-1985.

25. **Houglum, K., G. A. Ramm, D. H. Crawford, J. L. Witztum, L. W. Powell, and M. Chojkier**. 1997. Excess iron induces hepatic oxidative stress and transforming growth factor beta1 in genetic hemochromatosis. Hepatology **26**:605-610.

26. **Horie, K., T. Miyata, K. Maeda, S. Miyata, S. Sugiyama, H. Sakai, S. C. van Ypersole de, V. M. Monnier, J. L. Witztum, and K. Kurokawa**. 1997. Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. J. Clin. Invest **100**:2995-3004.

27. **Newcombe, J., H. Li, and M. L. Cuzner**. 1994. Low density lipoprotein uptake by macrophages in multiple sclerosis plaques: implications for pathogenesis. Neuropathol. Appl. Neurobiol. **20**:152-162.

28. **Dei, R., A. Takeda, H. Niwa, M. Li, Y. Nakagomi, M. Watanabe, T. Inagaki, Y. Washimi, Y. Yasuda, K. Horie, T. Miyata, and G. Sobue**. 2002. Lipid peroxidation and advanced glycation end products in the brain in

normal aging and in Alzheimer's disease. Acta Neuropathol. (Berl) **104**:113-122.

29. **Pluddemann, A., C. Neyen, and S. Gordon**. 2007. Macrophage scavenger receptors and host-derived ligands. Methods **43**:207-217.

30. **Miller, Y. I., S. Viriyakosol, D. S. Worrall, A. Boullier, S. Butler, and J. L. Witztum**. 2005. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. Arterioscler. Thromb. Vasc. Biol. **25**:1213-1219.

31. **Miller, Y. I., M. K. Chang, C. J. Binder, P. X. Shaw, and J. L. Witztum**. 2003. Oxidized low density lipoprotein and innate immune receptors. Curr. Opin. Lipidol. **14**:437-445.

32. **Chang, M. K., C. J. Binder, M. Torzewski, and J. L. Witztum**. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. Proc. Natl. Acad. Sci. U. S. A **99**:13043-13048.

33. **Kiechl, S., J. Willeit, M. Mayr, B. Viehweider, M. Oberhollenzer, F. Kronenberg, C. J. Wiedermann, S. Oberthaler, Q. Xu, J. L. Witztum, and S. Tsimikas**. 2007. Oxidized phospholipids, lipoprotein(a), lipoprotein-associated phospholipase A2 activity, and 10-year cardiovascular outcomes: prospective results from the Bruneck study. Arterioscler. Thromb. Vasc. Biol. **27**:1788-1795.

34. **Blair, I. A.** 2008. DNA-adducts with lipid peroxidation products. J. Biol. Chem.

35. **Esterbauer, H., R. J. Schaur, and H. Zollner**. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. **11**:81-128.

36. **Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum**. 2000. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest **105**:1731-1740.

37. **Kearney, J. F.** 2000. Immune recognition of OxLDL in atherosclerosis. J. Clin. Invest **105**:1683-1685.

38. **Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel**. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science **286**:2156-2159.

39. **Boes, M., A. P. Prodeus, T. Schmidt, M. C. Carroll, and J. Chen**. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. Exp. Med. **188**:2381-2386.

40. **Baumgarth, N., O. C. Herman, G. C. Jager, L. Brown, L. A. Herzenberg, and L. A. Herzenberg**. 1999. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proc. Natl. Acad. Sci. U. S. A **96**:2250-2255.

41. **Lutz, H. U.** 2007. Homeostatic roles of naturally occurring antibodies: an overview. J. Autoimmun. **29**:287-294.

42. **Boes, M., T. Schmidt, K. Linkemann, B. C. Beaudette, A. Marshak-Rothstein, and J. Chen**. 2000. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. Proc. Natl. Acad. Sci. U. S. A **97**:1184-1189.

43. **Quartier, P., P. K. Potter, M. R. Ehrenstein, M. J. Walport, and M. Botto**. 2005. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. Eur. J. Immunol. **35**:252-260.

44. **Imai, Y., K. Kuba, G. G. Neely, R. Yaghubian-Malhami, T. Perkmann, L. G. van, M. Ermolaeva, R. Veldhuizen, Y. H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J. S. Peiris, A. S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C. J. Binder, and J. M. Penninger**. 2008. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell **133**:235-249.

45. **Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and G. J. Silverman**. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat. Med. **9**:736-743.

46. **Savitsky, D. and K. Calame**. 2006. B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. J. Exp. Med. **203**:2305-2314.

47. **Savill, J., I. Dransfield, C. Gregory, and C. Haslett**. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat. Rev. Immunol. **2**:965-975.

48. **Leroyer, A. S., A. Tedgui, and C. M. Boulanger**. 2008. Role of microparticles in atherothrombosis. J. Intern. Med. **263**:528-537.

# CHAPTER 8

# MATERIAL AND METHODS

## Animals

**C57BL/6 and *Rag1*$^{-/-}$ mice** (The Jackson Laboratories), and *Ldlr*$^{-/-}$ *Rag1*$^{-/-}$ mice (a kind gift from Drs. Godfrey Getz and Katherine Reardon, University of Chicago), all on C57BL/6 background, were bred and maintained in our colony under SPF conditions unless otherwise noted. Plasma was obtained from 6 week-old female C57BL/6 T cell receptor $\alpha^{-/-}$ (TcR $\alpha^{-/-}$) mice (courtesy of Dr. Stephen Hedrick, University of California, San Diego).

**Germ-free mice:** Plasma from 14-16 week-old germ-free Swiss Webster mice were kindly provided by F. Bäckhed from a colony of gnotobiotic mice maintained at the University of Göteborg University, Göteborg, Sweden, vivarium under strict gnotobiotic conditions. Sterility was monitored by stool culturing and PCR for bacterial DNA. Conventionally-raised mice were transferred to gnotobiotic isolators at weaning and fed the same autoclaved chow diet. To obtain “conventionalized” mice, 13-14 week old germ-free mice were colonized with cecal content from 12 week-old conventionally-raised Swiss Webster donors for 14 days.

All experimental protocols were approved by the Animal Subjects Committee at University of California, San Diego, or for the germ-free mice, by the Göteborg University Animal Studies Committee.

**B-1 cell isolation**

Peritoneal exudate cells (PEC) from 6 to 15 week old naïve C57BL/6 mice were harvested by peritoneal lavage using ice-cold PBS supplemented with 1% heat-inactivated FCS (Invitrogen) PEC were incubated with an anti Fcγ-receptor monoclonal antibody (clone 2.4G2, BD-Pharmingen) for 15min at 4°C before staining with fluorescently-labeled monoclonal antibodies to block non-specific binding. PEC were stained with R-Phycoerythrin (PE)-labeled anti-CD19 (1D3), FITC-labeled anti-CD23 (B3B4), and in some experiments PE-Cy5-labeled anti-CD3 (145-2C11) (all BD-Pharmingen). B-1 cells were sorted to greater than 99% purity using a FACS Vantage SE cell sorter (Becton Dickinson) as the ($CD3^-$), $CD19^+$ and $CD23^-$ population.

**B-1 cell cultures**

Purified B-1 cells were seeded at $1x10^5$ cells per well in 48 well flat-bottom plates in culture media (RPMI 1640 media containing 10% heat inactivated FCS, 10mM Hepes buffer, 2mM L-glutamine, 0.05mM 2-mercaptoethanol, 50 µg/ml gentamycin) in the presence and absence of the specific TLR4 agonist, $KdO_2$-lipid A (100ng/ml, Avanti Polar Lipids), a combination of the TLR2 agonists Pam3CSK4 (300ng/ml) and FSL-1 (1µg/ml) (Invivogen) or vehicle control in triplicates in a final volume of 500µl. Cells were incubated at 37°C/ 5% $CO_2$ for up to 7 days.

**Adoptive transfer of B-1 cells**

B-1 cells were isolated from the peritoneum of female 6-15 week old donor C57BL/6 mice as described above. Purified B-1 cells were resuspended in PBS and 0.5 or $1x10^6$ cells in 200μl were injected into the peritoneal cavity of 6-15 week old *Rag1*$^{-/-}$ mice. Three to four donor mice were used for each recipient. Control *Rag1*$^{-/-}$ mice received an equal volume of PBS. Blood was collected via the retro-orbital plexus or cheek pouch from recipient mice before and 4 and 10 weeks after transfer.

**Flow Cytometry**

At time of sacrifice, PEC and splenocytes were resuspended in staining buffer (PBS + 1% BSA). After blocking with a specific anti-Fcγ receptor monoclonal antibody (2.4G2) for 15 min at 4°C, $10^6$ cells were stained with fluorescently-labeled monoclonal antibodies specific for various surface markers (FITC-labeled anti-CD11b/Mac-1 [M1/70], PE-labeled anti-CD5, PerCP-Cy5.5-labeled anti-CD19 [1D3]; PE labeled anti-mouse CD43 [S7], and APC or FITC-labeled anti-mouse IgM [II/41]; all BD-Pharmingen) in 100μl volumes of staining buffer for 30 min at 4°C in darkness, followed by extensive washing. Cell populations were analyzed on a BD FACSCalibur or FACScan instrument. More than $0.5x10^5$ cells were analyzed per sample, with dead cells excluded by forward and side scatter. Surface marker analysis was performed using Flow Jo software (Tree Star, Inc).

**Measurement of Antibody Titers**

Specific antibody titers to given antigens in plasma or cell culture supernatants were determined by chemiluminescent ELISA as previously described(1,2). Purified rat anti-mouse IgM (II/41; BD-Pharmingen) was used as capture Ab to measure total IgM levels. AP-labeled goat anti-mouse IgM (μchain specific) and anti-mouse IgG (γ chain specific) (Sigma-Aldrich) were used as detection Abs, as well as biotinylated rat anti-mouse IgM (R6-60.2; BD-Pharmingen). To detect other Ig isotypes, rat anti-mouse IgG1 (A85-3), IgG2a/c (R11-89), IgG2b (R9-91), IgG3 (R2-38), and IgA (C10-3) were used as capture Abs, biotin-conjugated rat anti-mouse IgG1 (A85-1), IgG2a/c (R19-15), IgG2b (R12-3), IgG3 (R40-82), and IgA (C10-1) (all BD-Pharmingen), were used as secondary antibodies. Biotin-conjugated Abs were then detected with AP-conjugated neutravidin (Pierce Biotechnology). Mouse anti human IgG (G18-145) and IgM (G20-127, BD-Pharmingen) were used as capture Ab to measure total IgM and IgG levels in humans. AP-labeled goat anti-human IgG and IgM (A3187 and A3437, Sigma Aldrich) were used as detection Abs. The following antigens were prepared as described(3): copper sulfate oxidized LDL (CuOx-LDL), 4-hydroxynonenal modified LDL (4-HNE-LDL), and malondialdehyde modified LDL (MDA-LDL) prepared from human LDL, 4-hydroxynonenal modified mouse serum albumin (4-HNE-MSA) prepared from MSA. MAA-BSA was prepared by Dr. Karsten Hartvigsen in our laboratory as described (4). α1,3-dextran, and α1,6-

dextran were a kind gift from Dr. John F. Kearney (University of Alabama). PC-BSA and PC-KLH were from Biosearch Technologies, Inc. and KLH from Pierce Biotechnology.

**Immunocompetition Assays**

The specificity of IgM Abs binding to MDA-LDL was determined by competition immunoassays as described(1,2). Diluted plasma samples or B-1 cell supernatants were incubated overnight at 4°C in the presence and absence of increasing concentrations of competitors. Samples were then centrifuged at 15,800g for 45 min at 4°C and supernatants analyzed for binding to the respective antigen by chemiluminescent ELISA.

To determine the percentage of total IgM in plasma binding to specific antigens, plasma samples were diluted in 1% BSA-TBS to yield a limiting dilution for each antigen, as determined in preliminary experiments. Diluted plasma samples were incubated overnight at 4°C in the absence or presence of individual or combinations of antigens at a final concentration of 250μg/ml. Thereafter, samples were centrifuged at 15,800g for 45 min at 4°C to pellet immune complexes, and supernatants analyzed for total IgM content by chemiluminescent ELISA, using a monoclonal rat anti-mouse IgM to capture and a polyclonal AP-labeled goat anti-mouse IgM (μchain specific) for detection. Data were calculated as the amount of IgM remaining in the supernatant after absorption, expressed as a percentage of total IgM.

**Enzyme-linked Immunospot Assay**

The frequencies of immunoglobulin- and antigen-specific secreting splenocytes were quantified by Enzyme-linked Immunospot (ELISpot) assay as described(1). Splenocytes were suspended in culture media at $2x10^5$ and $1x10^5$ cells/100μl and cultured in triplicates in washed and blocked 96-well Multiscreen-HA sterile nitrocellulose plates (Millipore), which had been coated overnight with 4μg/ml of either rat anti-mouse IgM (II/41), MDA-LDL, OxLDL, 4-HNE-LDL, or α1,3-Dextran. After 22 hours incubation at 37°C/ 5% $CO_2$, cells were removed by washing and immunoglobulin secreting cells (ISC) were detected using biotinylated rat anti-mouse IgM, followed by HRP-streptavidin (Zymed Laboratories Inc, South San Francisco, CA, USA). Plates were developed using a tetramethylbenzidine membrane substrate system (Kirkegaard & Perry Laboratories), and spots were quantified using an automated ImmunoSpot Image Analyzer (Cellular Technology).

**Apoptotic cells and Immunofluorescence Microscopy**

Thymocytes harvested from C57BL/6 mice were cultured in cell culture media and induced to undergo apoptosis by 10ng/ml PMA (Sigma-Aldrich) for 16 hours as described (5). Plasma or NA-17 ascites diluted in 1% BSA-PBS were incubated with apoptotic thymocytes for 30min at 4°C. Cells were washed and incubated with FITC-labeled rat anti-mouse IgM (II/41) in 1% BSA-PBS for 30min

and then washed again. For FACS, apoptotic cells were detected with Annexin V-PE apoptosis detection kit (BD Pharmingen) and analyzed by BD FACScanto. Umbilical plasma IgM or human Fab binding to apoptotic Jurkat cells was detected by goat-anti-human IgM FITC or goat anti-human Fab FITC (Jackson ImmunoResearch Laboratories, Inc). Apoptosis of Jurkat cells was induced with UV irradiation at 20mJ/cm$^2$, followed by further incubation of the cells in medium for 14-16 hrs before use. For immunofluorescence microscopy studies, cells were incubated with 2 μg/ml of Hoechst dye (Sigma-Aldrich) for 10 min, fixed with 2% paraformaldehyde, and spun down on glass slides using cytospin (StatSpin In). Images were captured by deconvolution microscopy using a DeltaVision deconvolution microscopic system operated by SoftWorx software (Applied Precision) as described(6).

**Immunocytochemistry**

Frozen-embedded sections of aortic origin, or from the brachiocephalic artery obtained from cholesterol-fed *Ldlr*$^{-/-}$*Rag1*$^{-/-}$ mice were fixed with methanol, blocked with 2% goat serum and stained with 5 μg/ml MDA2, 1.6μg/ml EN2 or 0.85μg/ml NA-17 (29), followed by a biotinylated goat anti-mouse IgG, or anti-mouse IgM (Jackson ImmunoResearch Laboratories) to detect endogenous NAbs in the lesions. For human Fabs, aortic sections of hypercholesterolemic rabbits were used followed by staining of biotinylated goat anti-human Fab (Jackson ImmunoResearch Laboratories). A Vectastain ABC-alkaline

phosphatase kit and a Vector Red alkaline phosphatase chromogenic substrate (Vector Labs, Burlingame, CA, USA) were used to visualize the antibody staining. Slides were counterstained with Weigert's Iron Hematoxylin (Richard-Allan Scientific). Immunostaining of adjacent sections in the absence of primary antibody was used as negative controls.

**Cloning and Genetic Analysis of Hybridoma NA-17**

Spleens of *Rag1*$^{-/-}$ mice reconstituted with B-1 cells were used to prepare B-1 derived hybridomas using techniques established in our laboratory(3). Productive hybridomas, by default, should only secrete NAbs. In brief, splenocytes from 4 *Rag1*$^{-/-}$ B-1 recipients were fused with the P3 × 63Ag8.653.1 myeloma cell line. Primary screening of supernatants was performed after 10 days of growth for the ability to secrete IgM and subsequently for IgM binding to MDA-LDL by chemiluminescent ELISA. Selected hybridomas were then cloned by limiting dilution(7). A more detailed description of these clones will be presented elsewhere. Here I report on clone NA-17, which was confirmed by DNA sequencing to be a full germline NAb. Cloning and sequence analysis of IgM NA-17 hybridoma $V_H$ and $V_L$ was accomplished as previously described(8).

**Human Samples**

Blood samples from healthy pregnant women and their newborn infants delivering at Magee-Womens Hospital (Pittsburgh, PA, USA) were collected as

part of a prospective IRB-approved study of pre-eclampsia by Dr. Doug Woelkers, who kindly provided these samples. Maternal samples were collected in EDTA before delivery (mean 10.5 hours) and cord venous samples by sterile aspiration of the umbilical vein after delivery of the infant. Samples were processed and aliquoted within 3 hours of collection, and stored at -70˚ C until use. Ten uncomplicated nulliparous maternal-infant pairs were selected for this preliminary analysis. The mothers were 23.4 ±5.4 years of age, 39.9 ± 1.2 weeks gestational age, and 50% Caucasian and 50% Black (values mean and SD). There were 6 males and 4 female infants.

**In Vivo Uptake of Apoptotic Cells by Peritoneal Macrophages**

The in vivo clearance of apoptotic cells was assessed using a modification of the method described by Taylor et al (9). *Rag1*$^{-/-}$ mice, adoptively transferred with B-1 cells or with PBS, were injected i.p. with 1 ml of sterile 3% thioglycollate to induce sterile peritonitis 15-19 weeks after B-1 cell transfer. 4 days later, the mice were injected i.p. with $20x10^6$ fluorescently labeled apoptotic thymocytes (using 5-CMFDA, Molecular Probes) in 200ul PBS. The mice were sacrificed 40 min after injection and peritoneal cells were recovered by lavage with 10 ml of ice cold PBS with 1% heat-inactivated FBS/10 mM EDTA. Macrophages were labeled with PE-conjugated F4/80 (BM8, eBioscience) and macrophage specific uptake of apoptotic cells performed by FACS. Phagocytosis was expressed as the percentage of macrophages ingesting apoptotic cells. To test the ability of

NA-17 to mediate enhanced uptake, fluorescent-labeled apoptotic thymocytes were pre-incubated with cultured NA-17 hybridoma supernatant or anti-KLH IgM (C48-6, BD Pharmingen) at 5ug/ml for 1 hour, washed and then injected into 8-10 weeks old *Rag1*$^{-/-}$ mice.

**CFSE Proliferation Assay**

For CFSE proliferation assay, isolated B-1 cells were stained with 2 uM CFSE (Invitrogen) for 20 min at room temperature and then incubated in cold cell culture media (RPMI 1640 media containing 10% heat inactivated FCS, 10mM Hepes buffer, 2mM L-glutamine, 0.05mM 2-mercaptoethanol, 50 µg/ml gentamycin) for 5 min. Cell were then washed twice with culture media and plated in 48 well plates at $1x10^5$ cells/well, and incubated with the indicated reagents.

**Measurement of Cytokines**

The cytokine profile in B-1 cell culture supernatants was measured with xMAP techonology (Luminex Corp, Austin, TX). IL-10 in cell culture supernatants was also analyzed with ELISA using purified anti-IL-10 (JES052A5) as capture antibody and biotinylated anti-IL-10 (both from R&D Systems) followed by AP-conjugated neutravidin (Pierce Biotechnology) for detection.

## Macrophage Binding Assay

Binding of biotinylated MDA-LDL and MAA-LDL to J774 macrophages plated in microtiter wells was assessed by a chemiluminescent binding assay as described (10). Biotinylated MDA-LDL and Biotinylated MAA-LDL (2 ug/ml each) was prepared with the aid of Dr. Karsten Hartvigsen in our lab, using methodology previously described (4). These ligands were incubated in the absence or presence of NA-17 or control IgM at different concentrations. Samples were then added to macrophages for 2 hours and the binding of biotinylated modified LDLs were determined by ELISA.

## Statistical Analysis

Appropriate statistical tests that apply for each experiment are given in the legends to the figures depicting a given set of data or described in the text. Results were analyzed with InStat 3 for Macintosh (GraphPad Software Inc.). $P < 0.05$ was considered significant.

## References


1. **Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and G. J. Silverman**. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat. Med. **9**:736-743.

2. **Binder, C. J., K. Hartvigsen, M. K. Chang, M. Miller, D. Broide, W. Palinski, L. K. Curtiss, M. Corr, and J. L. Witztum**. 2004. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. J. Clin. Invest **114**:427-437.

3. **Palinski, W., S. Yla-Herttuala, M. E. Rosenfeld, S. W. Butler, S. A. Socher, S. Parthasarathy, L. K. Curtiss, and J. L. Witztum**. 1990. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis **10**:325-335.

4. **Xu, D., G. M. Thiele, M. L. Kearley, M. D. Haugen, L. W. Klassen, M. F. Sorrell, and D. J. Tuma**. 1997. Epitope characterization of malondialdehyde-acetaldehyde adducts using an enzyme-linked immunosorbent assay. Chem. Res. Toxicol. **10**:978-986.

5. **Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum**. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J. Exp. Med. **200**:1359-1370.

6. **Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum**. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc. Natl. Acad. Sci. U. S. A **96**:6353-6358.

7. **Palinski, W., S. Horkko, E. Miller, U. P. Steinbrecher, H. C. Powell, L. K. Curtiss, and J. L. Witztum**. 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J. Clin. Invest **98**:800-814.

8. **Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum**. 2000. Natural antibodies with the T15

idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest **105**:1731-1740.

9. **Taylor, P. R., A. Carugati, V. A. Fadok, H. T. Cook, M. Andrews, M. C. Carroll, J. S. Savill, P. M. Henson, M. Botto, and M. J. Walport**. 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J. Exp. Med. **192**:359-366.

10. **Miller, Y. I., S. Viriyakosol, C. J. Binder, J. R. Feramisco, T. N. Kirkland, and J. L. Witztum**. 2003. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. J. Biol. Chem. **278**:1561-1568.