Innate Apoptotic Immunity: Characterization of Macrophage Responses

BY

Goutham Pattabiraman

Master of Science, University of California, Irvine (2007)

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology and Immunology
in the Graduate College of the
University of Illinois at Chicago, 2013
Chicago, Illinois

Defense Committee:

David Ucker, Chair and Advisor
Alan McLachlan, Microbiology and Immunology
William Walden, Microbiology and Immunology
Nancy Freitag, Microbiology and Immunology
Jerrold Levine, Nephrology, Department of Medicine
Pradip Raychaudhuri, Biochemistry and Molecular Genetics

ACKNOWLEDGEMENTS

I would like to thank Dr. David Ucker, my thesis advisor for being a great mentor and providing me with a great opportunity to work with him and grow as a scientist. David has always been willing to explore new ideas, provided the thought was based on a solid background and rationale. This has shaped me into becoming as critical a researcher as I stand today.

I also would like to thank all my thesis committee members – Dr. Alan McLachlan, Dr. William Walden, Dr Pradip Raychaudhuri, Dr. Nancy Freitag, and Dr. Jerrold Levine; who have supported me and guided me throughout my graduate career. I would like to especially mention Dr. Freitag and Dr. Levine, the offices of whom I have frequented many times with questions related to my projects and other issues. I am especially grateful to Lanfei Feng and Dr. Lavanya Visvabharathy for their help with my *in vivo* work, without whom I would have had a hard time completing my thesis.

Finally, I would like to thank my family and friends – without their support and encouragement I would not have been able to complete my thesis. I would especially like to thank my mother and father - who have always given me encouragement at times when I most needed it, as well as my brother-in-law and sister who have always been supportive and kept reminding me of the passion with which I started the PhD program.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
ACRE	Apoptotic Cell-Regulatory Element
APC	AlloPhycoCyanin
CAD	Caspase Activated DNase
cAMP	cyclic Adenosine Mono-Phosphate
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD44	Cluster of Differentiation 44
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CFSE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
CREB	cAMP Response Element-Binding protein
DNA-PK	DNA-dependent Protein Kinase
eNOS	endothelial Nitric Oxide Synthase
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phospahate dehydrogenase
HGF	Hepatocyte Growth Factor
HKLM	Heat Killed *Listeria monocytogenes*
IAI	Innate Apoptotic Immunity
ICE	Interleukin-1β Converting Enzyme
IFN-α	Interferon – alpha
IL-1β	Interleukin 1 - beta
IL-6	Interleukin 6
IL-10	Interleukin 10
iNOS	inducible Nitric Oxide Synthase
iTRAQ	isobaric Tagging for Relative and Absolute Quantification
LPS	Lipopolysaccaride
mTEC	marginal Thymic Epithelial Cells
NO	Nitric Oxides
NOS	Nitric Oxide Synthases

LIST OF ABBREVIATIONS (continued)

nNOS	neuronal Nitric Oxide Synthase
NuMa	Nuclear Mitotic apparatus
PAMP	Pathogen-Associated Molecular Pattern
PARP	Poly-ADP Ribose Polymerase
PB	Pacific Blue
PE	PhycoErythrin
PE/Cy7	a tandem PhycoErythrin and a cyanine dye
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKI	Protein kinase inhibitor peptide
PMA	Phorbol-12-myristate-13-acetate
TGF-β	Transforming Growth Factor – beta
TNF-α	Tumor Necrosis Factor – alpha
TLR	Toll-Like Receptor
TPI	Triosephosphate isomerase
R848	Resiquimod (Imidazoquinoline) compound
siRNA	small interfering Ribonucleic Acid
SLE	Systemic Lupus Erythematosis
STS:	Staurosporine
SUPER	Surface-exposed [during apoptotic cell death], Ubiquitously expressed, Protease-sensitive, Evolutionarily conserved, and Resident normally in viable cells
UTR	Un-Translated Region
VEGF	Vascular Endothelial Growth Factor

SUMMARY

About 10^{11} cells die daily by apoptosis (programmed physiological cell death) in the human body. Cells dying by apoptosis are rapidly engulfed and digested by their neighbors or by professional phagocytes in a process of phagocytosis, such that we are barely able to detect apoptotic cells *in vivo* normally. During the process of apoptosis, cells acquire a distinct "gain-of-function" trait which triggers a milieu of signaling events in the neighboring interacting cells, leading to an immunosuppressive (especially anti-inflammatory) state. While the mechanisms of apoptosis and those of clearance of effete cells have been studied extensively, those related to this gain-of-function accompanying apoptosis are much less understood. Perhaps the ultimate objective of the apoptotic process, along with the efficient elimination of inappropriate and non-functional cells, is to restore a physiological (non-inflammatory, "calm") setting. Understanding this immunosuppressive activity of apoptotic cells, which we have termed "Innate Apoptotic Immunity (IAI), is the focus of this thesis.

Apoptotic cells attenuate proinflammatory cytokine production in professional phagocytes, such as macrophages, as well as in other cell types. Our laboratory and others have determined that this attenuation occurs primarily on the level of transcription initiation. In addition to proinflammatory cytokines, key mediators of inflammation include Nitric Oxide (NO) species. We investigated whether NO production also is altered upon interaction with apoptotic cells, and verified that apoptotic cells are able to attenuate NO production in macrophages. We determined that this attenuation of NO production occurs through the complex transcriptional regulation of two genes, iNOS and Arginase II, which encode proteins controlling NO biosynthesis. Our data showed that these effects on Arginase II and iNOS transcription were mediated by apoptotic cell-cell contact, dependent on recognition, rather than apoptotic cell secreted factors. Further, while these studies indicated that the regulation of the Arginase II gene occurs at the level of transcriptional initiation, apoptotic cell-mediated regulation of iNOS occurs additionally on a post-transcriptional level. Upon further investigation of the Arginase II promoter, we identified a region around the *cAMP Response Element-Binding* Protein (CREB) binding site that may be responsible for apoptotic cell (recognition dependent) mediated Arginase II gene regulation. The

apoptotic cell-mediated regulation of Arginase II gene through the CREB binding site suggests cyclic Adenosine mono-phosphate (cAMP) signaling events triggered upon apoptotic cell recognition by responder cells.

The triggering of IAI depends upon the recognition of the apoptotic cell, but is not dependent upon its engulfment. Our laboratory has demonstrated that the apoptotic determinants responsible for the triggering of IAI are protease-sensitive cell surface molecules. In addition, we have found that the apoptotic determinants are resident normally in viable cells, ubiquitously expressed in all tissue types, and evolutionarily conserved among [at least] metazoans. We have used these criteria (for brevity, "SUPER": surface-exposed [during apoptotic cell death], ubiquitously expressed, protease-sensitive, evolutionarily conserved, and resident normally in viable cells) in our efforts to identify to apoptotic determinants for IAI molecularly. A variety of studies have revealed that numerous protein species, normally resident intracellularly, become externalized during the process of apoptosis. In an effort to characterize this externalization process more comprehensively, and to identify essential determinants for IAI, we took unbiased and quantitative approaches to analyzing the proteome of the apoptotic cell surface. We utilized two independent methodologies: two-dimensional gel electrophoresis as well as iTRAQ (Isobaric tagging for relative and absolute quantification) analysis, to identify novel surface-enriched proteins from apoptotic cell membranes. We identified numerous unique protein species that fit the SUPER criteria and which, therefore, could potentially be associated with IAI. Remarkably, among the most abundant of these, we found almost all of the enzymes of the glycolytic pathway. We found that the surface-exposed forms of these were enzymatically inactive and appeared to harbor post-translational modifications. I verified these findings using a cytofluorimetric approach, and established that the externalization of glycolytic enzyme molecules is a common and early aspect of apoptotic cell death in different cell types triggered to die with distinct suicidal stimuli. The externalization of the glycolytic enzyme molecules during apoptosis is extensive, but not quantitative; substantial pools of the molecules remain intracellular in apoptotic cells. While we have excluded a role for some of the externalized protein determinants in

IAI, we currently are evaluating whether the externalized glycolytic enzyme molecules serve an essential role as determinants for IAI.

Aging-associated immunosenescence involves a paradoxical dysregulation of the immune system, typically characterized by chronic elevations of the levels of proinflammatory cytokines and inflammatory mediators in the absence of an overt physiological stress, a diminution of protective responsiveness to exogenous and infectious immunogens, as well as elevated levels of autoimmune antibodies. It is striking that inflammatory responsiveness and tolerance are precisely the attributes of immunity that are known to be targets of active apoptotic immune modulation. Macrophages, in particular, have been implicated as the leukocyte population whose function is altered in an aging-associated manner. This prompted us to speculate that an alteration of IAI in macrophages might underlie the phenomenon of aging-associated immunosenescence. We investigated this hypothesis by examining IAI responsiveness of murine macrophages as a function of animal age. Our results were striking. We found that macrophages do not lose innate apoptotic immune responsiveness in an aging-associated manner. Importantly, we found that apoptotic cells generated from older animals were at least as effective as were apoptotic cells generated from younger animals in eliciting IAI responses in primary macrophages from younger or older animals. Surprisingly, we also detected no aging-associated alteration in macrophages responsiveness to TLR agonists. The older mouse population did exhibit well-characterized manifestations of aging, including a skewing of the T lymphocyte population toward a previously activated ("memory") phenotype, and altered susceptibility of bacterial pathogens. These results call into question previous work attributing marked aging-associated defects to macrophages. More significantly, however, we did find that in macrophages, the lifetime of the anti-inflammatory state triggered by encounter with apoptotic cells is prolonged with age, suggesting that macrophage "plasticity" is constrained in an aging-associated manner. We speculate that this subtle alteration in the nimbleness with which macrophages are able to alter their responsiveness may pertain to a reduced ability to dampen a proinflammatory response, and may be of causal significance to the hyper inflammatory state associated

with immunosenescence. We also observed modest differences in basal levels of proinflammatory cytokine expression, concordant with the reported aging-associated elevation in circulating cytokine levels. These results, highlighting nuanced alterations in cellular behaviors, prompt a reconsideration of expectations for changes that may underlie immunosenescence. Further studies are needed to examine these behaviors in greater detail, as well as to examine IAI responsiveness in other phagocytic populations in an aging-associated manner, including the characterization of antigen-presenting function and adaptive immune responsiveness to apoptotic cell-derived antigens.

CHAPTER I

BACKGROUND

"…One who has taken his birth is sure to die, and after death one is sure to take birth again."

The Bhagavad-Gita 2.27

A. Historical Overview

It is irrefutable that all cells die. The first observation of the process of cell death could have been made as early as when philosophers started asking questions about life and death. In fact, it was only after 1665, following the work of two scientific pioneers, Robert Hooke, who first identified and observed "cells" – a term he used because they reminded him of rooms in which monks lived, and Anton van Leeuwenhoek, who advanced the ability to microscopically examine microorganisms and other cells, that we started to make significant advances in exploring and understanding cells and their fates. However, it was not until 1842 that Carl Vogt, studying development and metamorphosis of tadpoles into toads, observed and reported the process of cells dying physiologically (1). This observation was followed by many others that described similar cases of cell death associated with embryogenesis as well as morphogenesis, including work from Walther Flemming in 1885. Most of the work that followed at the end of the 19th century and the beginning of the 20th century looked at phagocytosis. It was during that time Élie Metchnikoff, who later (1908) won the Nobel Prize in Physiology or Medicine for his work, described phagocytosis as a process that evolved both to clear foreign particles, identifying the relevant phagocytic cells as "neutrophils / microphages", and to eliminate effete self cells, characterizing the distinct phagocytic involved as "scavengers / macrophages" (reviewed in 2, 3).

In the first half of the 20th century, the phenomenon of physiological cell death was limited to its role development, morphogenesis, and metamorphosis (as reviewed in 4). Around the late 1960's, it became of known that the spontaneous loss of cells occurred in multi-cellular organisms, which was characterized by distinct morphological traits like shrinkage of cells,

nuclear condensation and fragmentation of the cell. In 1972, Kerr, Wyllie and Currie (5), coined the term “apoptosis” to describe this physiological cell death process, relating it to the process of "dropping off" or "falling off" seen during the shedding of petals from flowers, or leaves from trees. They also noted that the debris generated during the process of apoptosis is disposed rapidly by nearby intact cells and that apoptosis, in contrast to other cases of cell death (such as necrosis) is not associated with inflammation. While this pivotal work recognized apoptosis as a controlled process, mechanistic insights into the process, including elements of its execution and signaling required for its initiation, remained mysterious.

B. Molecular mechanisms of apoptosis

That a process of cell death (cell suicide) appeared to be imbedded within the cells of living organisms was a provocative conclusion, and it prompted substantial interest. In addition, the recognition that apoptosis might be important - and even underlie - serious disease states, including pathologies that involved excessive (neurodegenerative diseases such as Parkinsonism and Alzheimer’s diseases) or deficient (cancer; reviewed in 6) cell deaths encouraged greater interest. Processes of programmed cell death were observed to be conserved throughout metazoan evolution, including in the worm *Caenorhabditis elegans*, which gave rise to seminal insights (6-8).

The breakthrough in understanding the genetic regulation and mechanism of apoptosis came from genetic studies in *C. elegans*. *C. elegans*, a small self fertilizing hermaphrodite worm, is unusual in its invariant somatic cell lineages. Strikingly, among the “predetermined” fates of its 1030 somatic cells, 131 are committed to die apoptotically. Combined with tractable genetics, this became an important model system for mechanistic studies of apoptosis.

Studies from Robert Horvitz and John Sulston, who were later awarded the Nobel Prize in Physiology or Medicine for their work, led to the discovery of “cell death” genes, including *ced-*

3 and *ced-4*, which were required for apoptosis of all the 131 somatic cells. Further mutational studies led to identification of other genes involved in cell death: *ced-9*, the product of which inhibits the products of *ced-3* and *ced-4*, *egl-1*, which determines whether a cell will die or survive, and *nuc-1*, which encodes a nuclease responsible for death-associated DNA degradation (9, 10).

The expansion of the view of cell death developed from genetic studies of *C. elegans* into our current understanding of mammalian apoptosis dependent on a cascade of caspase activities developed from penetrating integrative biological analyses. Significant interest in IL-1β, an important proinflammatory mediator, led many groups to study the processing of this cytokine. Proteolytic processing from inactive to active IL-1β was found to be triggered by a cysteine protease, Interleukin-1β converting enzyme (ICE). Yuan *et al.* (12) reported a high homology between the gene for mammalian ICE and the *C. elegans* apoptosis gene ced-3 (11, 12). The identification of ICE led to the discovery of a whole family of proteins characterized as cysteine proteases (cysteine at the active site) that cleave targeted proteins after an aspartate residue, and subsequently termed "caspases" (ICE was renamed caspase-1). Molecular and genetic studies to elucidate the progression of apoptosis were rapid. The expanded family of death-specific caspases in mammals (note that other caspases, such as caspase 1, are involved in inflammation, rather than cell death) reflects the variety of cell death stimuli to which mammalian cells respond; this has led to the categorization of cell deaths triggered via cell surface "death receptors" (the "extrinsic" cell death pathway, which involve an initiator role for caspase 8) and cell deaths occurring as a result of the targeting of intracellular (especially mitochondrial) molecules (leading to caspase 9 activation; the "intrinsic" pathway of cell death). Key proteins involved in apoptosis, which are highly conserved evolutionarily, are listed in **Table I** (with the *C. elegans* and mammalian orthologues indicated).

The discovery of caspases coincided with the identification of the Bcl-2 family of proteins and the recognition that they orchestrate the execution of apoptosis. Bcl-2 had been identified and cloned previously as a gene involved lymphoblastic leukemia (13). The Bcl-2 family of proteins consists of both "anti-apoptotic members" (Bcl-2, Bcl-X_L), which are orthologous to the Ced-9 protein of *C. elegans*, as well as "pro-apoptotic members" (Bax, Bak, [multiple Bcl domain members] as well as Bid, Bad, Bim [BH3-only members] similar to *C. elegans* Egl-1). Proteins of the Bcl-2 family are associated typically with the mitochondrial membrane, and are thought to function through control of mitochondrial membrane potential. Upon treatment with [intrinsic] apoptotic stimuli, pro-apoptotic Bcl-2 family members become activated, leading to cytochrome *c* release from mitochondria. Cytochrome *c* release is essential for the activation of Apaf-1, encoded by the mammalian orthologue of *C. elegans* ced-4, which provides a platform for the activation of Caspase 3 and the execution phase of apoptosis. In addition to Caspase 3, the closely related Caspases 6 and 7 are associated with the execution phase of apoptosis, which involves the degradation of the chromosome (and the activation of an array of associated proteins, including caspase activated DNase [CAD], poly-ADP Ribose Polymerase [PARP], nuclear mitotic apparatus [NuMa], and DNA-dependent protein kinase [DNA-PK]), and the morphological and biochemical changes characteristic of apoptosis (1, 2, 14, 15). A simplified mechanistic overview of the core elements of the apoptotic process is shown in **Figure I**.

Table I: Conservation of key players in the apoptotic pathway

Players of apoptosis	***C. elegans***	**Mammalian**	
		Intrinsic pathway	**Extrinsic pathway**
Apoptosis promoter	EGL-1	Bax BH3-only proteins	Fas / CD95 TNFR1 DR4/5 TRAIL
Adaptor proteins	CED-4	Apaf-1	FADD TRADD
Inhibitor proteins	CED-9	Bcl-2 Bcl-X_L	FLIP
Initiator caspases	CED-3	Caspase 9	Caspase 8
Effector caspases	*none*	Caspase 3, Caspase 6, Caspase 7	Caspase 3, Caspase 6, Caspase 7

In contrast to physiological cell death (apoptosis), cell death in pathological settings (necrosis) typically is associated with cell swelling and relatively rapid cell lysis. In all cases, the cell death trigger and the context of cell death is responsible for the dictating the form of cell death (14-16). Importantly, the form of cell death also determines its physiological consequences: necrosis, again in contrast to apoptosis, is associated with pathology and triggers an inflammatory response.

Recently, additional forms of genetically "programmed" cell death have been identified. One of these, "autophagy", is primarily a cell survival mechanism. Like apoptosis, autophagy has important roles in developmental processes, human diseases and cellular responses to nutrient deprivation (17). Typically this program, at times of starvation, triggers active "cannibalism" of its own cellular contents by sequestration of cytoplasm and organelles in double-membrane vesicles and delivery to the cells own lysosomes for subsequent degradation. The immunological consequences of this mode of cell death are yet to be studied (18).

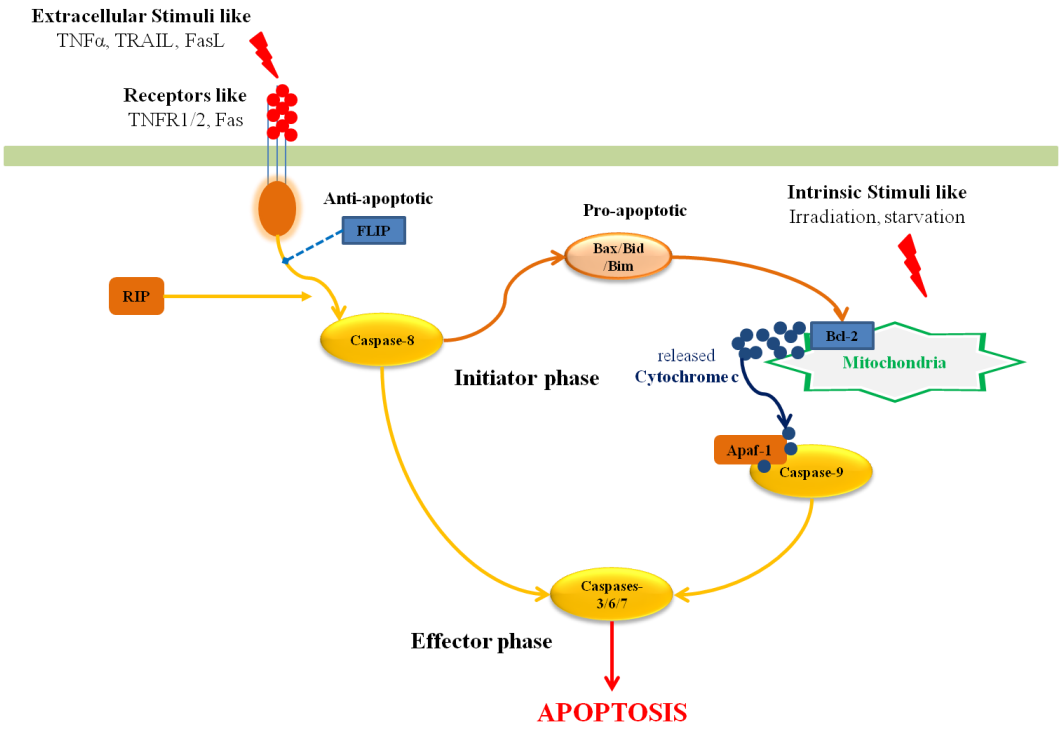


Figure I: Major components of the core apoptotic cascade. The two major apoptotic pathways - the extrinsic pathway, initiated via cell surface death receptors (such as the TNFR super-family), and the intrinsic pathway, initiated by death stimuli (especially cellular stressors) acting [especially] on mitochondrial targets - are depicted. In addition to mitochondrial targets, some death stimuli activate the intrinsic pathway through the endoplasmic reticulum. There is cross-talk between the two pathways.

C. Regulation exerted by apoptotic cells on viable cell function

The original observations demonstrating a lack of inflammation associated with infrequent and dispersed apoptotic cells supported the view that apoptotic cells themselves did not stimulate inflammation in neighboring cells or phagocytes. More recent evidence demonstrates that apoptotic cells play an active role in suppressing inflammatory responses. Apoptotic cells trigger a milieu of recognition-dependent signaling events in the neighboring cells. Early signaling events triggered by apoptotic cells are summarized in **Table II**.

Even more remarkably, apoptotic cells appear to play a role in wound healing and tissue remodeling. The interaction (phagocytosis) of apoptotic cells with neighboring cells also leads to the secretion of growth and survival factors, such as vascular endothelial growth factors (VEGF), and hepatocyte growth factor (HGF), both of which are important in promoting proliferation and growth of endothelial and epithelial cells (32, 26, 31). HGF also is a critical factor important for tissue healing in the lungs and it has been demonstrated that interaction with apoptotic neutrophils induces alveolar macrophages to produce HGF during the course of bacterial pneumonia infection, thereby helping in tissue recovery and repair mechanism (33). Elegant studies in the Cnidarian *Hydra* demonstrate that apoptosis increases canonical Wnt signaling in neighboring cells, leading to active cell proliferation and tissue regeneration (25, 34). It is interesting to note that neutrophils, which have a high turnover, are phagocytes that engulf apoptotic cells and are themselves subject to apoptosis and clearance by macrophages.

Table II: Key signaling events triggered in neighboring viable cells in response to recognition of apoptotic cells

Signaling event	Recognition- Dependent Apoptotic regulation
PI3K / Akt signaling	Activation of Rho-A triggering downstream signaling leading to apoptotic cell-induced stabilization of translation of many proteins, including TGF-β, through Akt / mTOR / eIF4E (19, 20, 21, 22, 23).
ERK1/2 (MAPK) signaling	Activation and phosphorylation of p38 MAPK and JNK pathway leading to transcriptional regulation of genes. In contrast, ERK1/2 activation is inhibited (20, 21, 24).
Wnt signaling	Up-regulation of Wnt expression upon interaction with apoptotic cells, leading to rapid remodeling and cell proliferation (25-27).
β-catenin signaling	Increased sustenance of phosphorylation of β-catenin, leading to regulation of β-catenin-dependent transcriptional activation (28).
Erk5/CREB signaling	Up-regulation of Erk5 phosphorylation, in a sphingosine-1-phosphate receptor mediated manner leading to CREB activation and downstream transcriptional regulation (29).
PPAR-γ signaling	Apoptotic cell induced PPAR-γ sumoylation to attenuate the removal of NCoR, a trans-repressor, thereby blocking trans-activation of NF-κB and subsequent transcriptional regulation (30).

Apoptosis is commonly seen at sites of inflammation. While it was previously considered that the apoptosis process was only a consequence of the massive inflammation and served no other purpose; we now have begin to understand that the apoptosis also serves for another purpose, that is to dampen the inflammation, This dampening of inflammation therefore acts as means of resolution ("calming nature") of inflammation. There is now a growing awareness of the importance of this phase during a robust inflammation against infections as well as other diseased states (40-44).

Apart from the "calming" nature of apoptotic cell clearance exerted on responding cells, apoptotic cells trigger a broader systemic immunomodulatory effect in organisms. A number of reports suggest that dead corpses, apoptotic and necrotic, are sources of antigens for presentation. An instance where apoptotic cell derived antigens play an important role is during that of thymic selection. It has been observed that massive apoptosis of the marginal thymic epithelial cells (mTEC's) in the thymus, is a means of presenting tolerogenic self-antigens for generation of central tolerance (45). And indeed, groups have also reported that, professional phagocytes such as dendritic cells (DCs) which process self tissues are able to reduce the development of autoimmune conditions by the tolerogenic education of T cells in the periphery (46). Other reports also address direct *in vitro* as well as *in vivo* interactions of dendritic cells with apoptotic cells. Apoptotic cells are able to suppress the ability of dendritic cells to process and present their antigens by regulating the expression of co-stimulatory molecules such as and CD86 on dendritic cell surface, resulting in diminished antigen specific activation of T-cells to the apoptotic cell-derived antigen (47). The uptake of apoptotic cells has also been shown to influence immature dendritic cells to preferentially secrete cytokines such as IL-10 and TGF- β which has been shown to skew T-helper cell populations from a Th_{17} phenotype to a $FoxP3^{+}$ T regulatory phenotype (48-50). Another report also observed that apoptotic cells exert a profound

immune tolerance against autoimmune conditions through the generation of $CD19^+$ regulatory B cells, which are able to influence the cytokine profile of antigen-specific T cells, which in turn are able to suppress autoimmune phenotype (51).

Our growing recognition of the multifaceted regulations by apoptotic cells, prompts us to study the complex mechanisms involved in apoptotic-cell mediated signaling.

D. Thesis aims

One of the most intriguing aspects of the apoptotic cell death process is the characteristic non-inflammatory nature of its clearance. The immunomodulatory activity of apoptotic cells was first recognized in studies *in vitro* with apoptotic neutrophils (39). The efficiency of the very rapid removal of apoptotic cells by professional phagocytes and neighboring cells in the absence of inflammation is made evident by the technical difficulty of finding apoptotic cells *in vivo* (36-38)! Apoptotic cells trigger an active signaling response in responder cells, leading to inflammatory suppression (20, 52). This is profoundly interesting in scenarios of abundance of cell death during development, thymic development and most interestingly for the resolution of inflammation post infection (33, 44, 54, and 55). This immunomodulation by apoptotic cells predominantly occurs at the level of transcription for both pro- and anti-inflammatory cytokines in the macrophages with which they interact (20, 21, 29 and56).

Still, while the molecular mechanisms and the signaling cascades involved in the induction of apoptosis have been well elucidated, those pertaining to apoptotic immunomodulation remain incompletely described. Previous work from our laboratory has demonstrated that this apoptotic-cell-mediated immune suppression is a conserved trait and that the specific recognition of apoptotic cells and the responses elicited occur independent of the origin (species, tissue type, or suicidal stimulus) of the apoptotic cell (57). Moreover, this profound innate immune discrimination of apoptotic from viable cells is manifest by professional and non-professional

phagocytes, as well as by non-phagocytic cell types. Together, these observations suggest that innate apoptotic immunity is ubiquitous and conserved evolutionarily.

I explored three distinct issues of innate apoptotic immunity in an effort to advance our understanding of this process:

1) the regulation of genes encoding proteins involved in the production of Nitric Oxide (NO) species, important inflammatory mediators,
2) the identification and characterization of conserved molecular determinants on the surface of apoptotic cells that trigger innate apoptotic immune responses, and
3) the involvement of innate apoptotic immunity in aging-associated immune dysregulation (immunosenescence).

Cited Literature for Chapter I

1. Zakeri Z, Lockshin RA. 2008. Cell death: history and future. *Adv Exp Med Biol.* **615**:1-11.

2. Lockshin RA, Zakeri Z. 2001. Programmed cell death and apoptosis: origins of the theory. *Nat Rev Mol Cell Biol.* **2(7)**:545-50.

3. Clarke PG, Clarke S. 1996. Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol (Berl).* **193(2)**:81-99.

4. Glucksmann A. 1951. Cell deaths in normal vertebrate ontogeny. *Biological Reviews.* **26(1)**:59–86.

5. Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* **26(4)**:239-57.

6. Vaux DL. 2002. Apoptosis timeline. *Cell Death Differ.* **9(4)**:349-54.

7. Hedgecock EM, Sulston JE, Thomson JN. 198. Mutations affecting programmed cell deaths in the nematode Caenorhabditis elegans. *Science.* **220(4603)**:1277-9.

8. Lockshin RA, Williams CM. 1965. Programmed cell death. IV. The influence of drugs on the breakdown of the intersegmental muscles of silkmoths. *J Insect Physiol.* **11(6)**:803-9.

9. Sulston JE. 1976. Post-embryonic development in the ventral cord of Caenorhabditis elegans. *Philos Trans R Soc Lond B Biol Sci.* **275(938)**:287-97.

10. Ellis HM, Horvitz HR. 1986. Genetic control of programmed cell death in the nematode C. elegans. *Cell.* **44(6)**:817-29.

11. Yuan J, Horvitz HR. 2004. A first insight into the molecular mechanisms of apoptosis. *Cell.* **116(2 Suppl):**S53-6, 1 p following S59.

12. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. 1993. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell.* **75(4)**:641-52.

13. Cleary ML, Smith SD, Sklar J. 1986. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell.* **47(1)**:19-28.

14. Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* **35(4)**:495-516.

15. Lawen A. 2003. Apoptosis-an introduction. *Bioessays*. **25(9)**:888-96.

16. Zeiss CJ. 2003. The apoptosis-necrosis continuum: insights from genetically altered mice. *Vet Pathol.* **40(5)**:481-95.

17. Debnath J, Baehrecke EH, Kroemer G. 2005. Does autophagy contribute to cell death? *Autophagy.* **1(2)**:66-74.

18. Petrovski G, Zahuczky G, Májai G, Fésüs L. 2007. Phagocytosis of cells dying through autophagy evokes a pro-inflammatory response in macrophages. *Autophagy*. **3(5)**:509-11

19. Reddy SM, Hsiao KH, Abernethy VE, Fan H, Longacre A, Lieberthal W, Rauch J, Koh JS, Levine JS. 2002. Phagocytosis of apoptotic cells by macrophages induces novel signaling events leading to cytokine-independent survival and inhibition of proliferation: activation of Akt and inhibition of extracellular signal-regulated kinases 1 and 2. *J Immunol.* **169(2)**:702-13.

20. Cvetanovic M, Ucker DS. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol.* **172(2)**:880-9.

21. Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM. 2008. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *J Immunol.* **181(5)**:3575-85.

22. Tosello-Trampont AC, Nakada-Tsukui K, Ravichandran KS. 2003. Engulfment of apoptotic cells is negatively regulated by Rho-mediated signaling. *J Biol Chem.* **278(50)**:49911-9.

23. Park HJ, Choi YH, Cho YJ, Henson PM, Kang JL. 2011. RhoA-mediated signaling up-regulates hepatocyte growth factor gene and protein expression in response to apoptotic cells. *J Leukoc Biol.* **89(3)**:399-411

24. Patel VA, Lee DJ, Feng L, Antoni A, Lieberthal W, Schwartz JH, Rauch J, Ucker DS, Levine JS. 2010. Recognition of apoptotic cells by epithelial cells: conserved versus tissue-specific signaling responses. *J Biol Chem.* **285(3)**:1829-40.

25. Galliot B, Chera S. 2010. The Hydra model: disclosing an apoptosis-driven generator of Wnt-based regeneration. *Trends Cell Biol.* **20(9)**:514-23

26. Li F, Huang Q, Chen J, Peng Y, Roop DR, Bedford JS, Li CY. 2010. Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. *Sci Signal.* **3(110)**:ra13

27. Chera S, Ghila L, Dobretz K, Wenger Y, Bauer C, Buzgariu W, Martinou JC, Galliot B. 2009. Apoptotic cells provide an unexpected source of Wnt3 signaling to drive hydra head regeneration. *Dev Cell.* **17(2)**:279-89.

28. Patel VA, Feng L, Lee DJ, Massenburg D, Pattabiraman G, Antoni A, Schwartz JH, Lieberthal W, Rauch J, Ucker DS, Levine JS. 2012. Recognition-dependent signaling events in response to apoptotic targets inhibit epithelial cell viability by multiple mechanisms: implications for non-immune tissue homeostasis. *J Biol Chem.* **287(17)**:13761-77.

29. Barra V, Kuhn AM, von Knethen A, Weigert A, Brüne B. 2011. Apoptotic cell-derived factors induce arginase II expression in murine macrophages by activating ERK5/CREB. *Cell Mol Life Sci.* **68(10)**:1815-27.

30. Jennewein C, Kuhn AM, Schmidt MV, Meilladec-Jullig V, von Knethen A, Gonzalez FJ, Brüne B. 2008. Sumoylation of peroxisome proliferator-activated receptor gamma by apoptotic cells prevents lipopolysaccharide-induced NCoR removal from kappaB binding sites mediating transrepression of proinflammatory cytokines. *J Immunol.* **181(8)**:5646-52.

31. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest.* **83(3)**:865-75.

32. Golpon HA, Fadok VA, Taraseviciene-Stewart L, Scerbavicius R, Sauer C, Welte T, Henson PM, Voelkel NF. 2004. Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. *FASEB J.* **18(14)**:1716-8.

33. Morimoto K, Amano H, Sonoda F, Baba M, Senba M, Yoshimine H, Yamamoto H, Ii T, Oishi K, Nagatake T. 2001. Alveolar macrophages that phagocytose apoptotic neutrophils produce hepatocyte growth factor during bacterial pneumonia in mice. *Am J Respir Cell Mol Biol.* **24(5)**:608-15.

34. King RS, Newmark PA. 2012. The cell biology of regeneration. *J Cell Biol.* **196(5)**:553-62.

35. Lang RA, Bishop JM. 1993. Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell.* **74(3)**:453-62.

36. Ravichandran KS. 2010. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med.* **207(9)**:1807-17.

37. Surh CD, Sprent J. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature.* **372(6501)**:100-3.

38. Gardai SJ, Bratton DL, Ogden CA, Henson PM. 2006. Recognition ligands on apoptotic cells: a perspective. *J Leukoc Biol.* **79(5)**:896-903.

39. Meagher LC, Savill JS, Baker A, Fuller RW, Haslett C. 1992. Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B2. *J Leukoc Biol.* **52(3)**:269-73.

40. Devitt A, Marshall LJ. 2011. The innate immune system and the clearance of apoptotic cells. *J Leukoc Biol.* **90(3)**:447-57.

41. Birge RB, Ucker DS. 2008. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ.* **15(7)**:1096-102.

42. Maderna P, Godson C. 2003. Phagocytosis of apoptotic cells and the resolution of inflammation. *Biochim Biophys Acta.* **1639(3)**:141-51.

43. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. 2004. Macrophages and apoptotic cell clearance during pregnancy. *Am J Reprod Immunol.* **51(4)**:275-82.

44. Elliott MR, Ravichandran KS. 2010. Clearance of apoptotic cells: implications in health and disease. *J Cell Biol.* **189(7)**:1059-70.

45. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol.* **9(12)**:833-44.

46. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM. 2002. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J Exp Med.* **196(8)**:1091-7.

47. Stuart LM, Lucas M, Simpson C, Lamb J, Savill J, Lacy-Hulbert A. 2002. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J Immunol.* **168(4)**:1627-35.

48. Zhou F, Lauretti E, di Meco A, Ciric B, Gonnella P, Zhang GX, Rostami A. 2013. Intravenous transfer of apoptotic cell-treated dendritic cells leads to immune tolerance by blocking Th17 cell activity. *Immunobiology.* **218(8)**:1069-76.

49. Kushwah R, Wu J, Oliver JR, Jiang G, Zhang J, Siminovitch KA, Hu J. 2010. Uptake of apoptotic DC converts immature DC into tolerogenic DC that induce differentiation of Foxp3+ Treg. *Eur J Immunol.* **40(4)**:1022-35.

50. Brereton CF, Blander JM. 2010. Responding to infection and apoptosis--a task for TH17 cells. *Ann N Y Acad Sci.* **1209**:56-67.

51. Gray M, Miles K, Salter D, Gray D, Savill J. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci* **104(35)**:14080-5.

52. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. 1997. Immunosuppressive effects of apoptotic cells. *Nature.* **390(6658)**:350-1.

53. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest.* **101(4)**:890-8.

54. Carrero JA, Unanue ER. 2012. Mechanisms and immunological effects of apoptosis caused by Listeria monocytogenes. *Adv Immunol.***113**:157-74.

55. Krysko DV, D'Herde K, Vandenabeele P. 2006. Clearance of apoptotic and necrotic cells and its immunological consequences. *Apoptosis.* **11(10)**:1709-26.

56. Kim S, Elkon KB, Ma X. 2004. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity.* **21(5)**:643-53.

57. Cvetanovic M, Mitchell JE, Patel V, Avner BS, Su Y, van der Saag PT, Witte PL, Fiore S, Levine JS, Ucker DS. 2006. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. *J Biol Chem.* **281(29)**:20055-67.

CHAPTER II

METHODS AND MATERIALS

A. Materials and Reagents

Antibodies: Affinity purified polyclonal rabbit antibodies specific for Triosephosphate isomerase (TPI) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Abcam Inc. (Cambridge, MA). Affinity purified polyclonal goat antibody reactive with the carboxyl terminus of α-enolase was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Fluorescein isothiocyanate (FITC) - conjugated Plasminogen was obtained from BioMac (Leipzig, German). Affinity purified monoclonal mouse antibody against Calreticulin was obtained from Enzo Life Sciences International, Inc. (Farmingdale, NY). Affinity purified polyclonal rabbit antibody detecting Arginase II was obtained from Pierce Thermo Fisher Scientific (Rockford, IL). Affinity purified polyclonal rabbit antibody specific for β-actin was obtained from Sigma-Aldrich, Inc. (St. Louis, MO). FITC - conjugated monoclonal antibody against Annexin-II, FITC - conjugated Annexin V, PhycoErythrin (PE) - conjugated Annexin-V were obtained from BD Biosciences (San Jose, CA). Affinity purified polyclonal rabbit antibody against iNOS, affinity purified monoclonal rabbit antibodies against total CREB, and affinity purified monoclonal rabbit antibodies against phosphorylated CREB at Serine 133 were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Pacific Blue (PB) – conjugated monoclonal rat antibody against F4/80 was obtained from Invitrogen Corporation (Frederick, MD). AlloPhycoCyanin (APC) – conjugated rat antibody against CD3, APC - conjugated rat antibody against IL-6, FITC - conjugated rat antibody against CD44, FITC - conjugated mouse antibody against TLR2, PE - conjugated rat antibody against CD4, PE - conjugated rat antibody against TNF-α, tandem PhycoErythrin / cyanine dye (PE/Cy7) – conjugated rat antibody against TLR4/MD2 complex, PE/Cy7 - conjugated rat antibody against IL-10, FITC – conjugated mouse IgG1, κ isotype control antibody, and PE/Cy7 - conjugated rat IgG2a, κ isotype control antibody

were obtained from BioLegend Inc. (San Diego, CA). FITC - conjugated rat monoclonal antibody against Interferon-α (IFN-α) was obtained from PBL InterferonSource (Piscataway, NJ).

Secondary antibodies were obtained from several vendors. FITC - conjugated rat monoclonal antibody against mouse IgG2b and PE - conjugated rat monoclonal antibody against mouse IgG1 were obtained from BD Biosciences. FITC - conjugated goat polyclonal antibody against rabbit IgG and FITC - conjugated goat polyclonal antibody against mouse IgG were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-linked donkey anti-rabbit $F(ab')_2$ from GE Healthcare (Amersham, United Kingdom) was used as a secondary antibody for detection of western blots by enhanced chemiluminescence.

Reagents, Inhibitors, and drugs: All primers were purchased from Integrated DNA Technologies, Inc (Coralville, IA). DNA polymerases (except PfuUltra High-fidelity DNA polymerase), restriction endonucleases and modifying enzymes were purchased from Fermentas (Thermo Fisher Scientific Inc.); PfuUltra High-fidelity DNA polymerase was purchased from Stratagene (Agilent Technologies, Inc., Santa Clara, CA). Cell permeable cAMP-dependent protein kinase (PKA) inhibitor (protein kinase inhibitor, PKI) was purchased from Invitrogen. The pan-caspase inhibitor quinolylvalyl-aspartyl-difluorophenoxy methyl ketone (Q-VD-OPh) was purchased from R&D Systems, Inc. (Minneapolis, MN). Actinomycin D and Staurosporine (STS); from *Streptomyces sp.,* 7-Aminoactinomycin D (7-AAD), Propidium Iodide (PI), Lipopolysaccaride (LPS; from *E.coli* 011:B4), 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE), Brefeldin A (from *Penicillium brefeldianum*), and Trypsin (from bovine pancreas) were purchased from Sigma-Aldrich. Phorbol-12-myristate-13-acetate (PMA) and LY294002 (a potent, cell-permeable, and reversible specific phosphatidylinositol 3-kinase inhibitor) were

purchased from Calbiochem (EMD Millipore Corporation, Billerica, MA). TLR agonists Zymosan, Heat killed *Listeria monocytogenes* (HKLM), Imidazoquinoline compound – R848, synthetic analog of dsRNA – Poly(I:C)-Low Molecular Weight (LMW), purified Flagellin from *S. typhimurium*, and synthetic bacterial lipoproteins Pam2CSK4 and Pam3CSK4, were purchased from InvivoGen (San Diego, CA).

B. Cells and Death Induction

Primary murine splenocytes, thymocytes, and macrophages (from C57BL/6 mice; see below), S49 murine thymoma cells, DO11.10 murine T cell hybridomas, RAW 264.7 murine macrophages, Jurkat human T leukemia cells, and U937 human monocytic (histiocytic) leukemia cells were cultured at 37°C in a humidified 5% (v/v) CO2 atmosphere in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with heat-inactivated 10% (v/v) FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. HeLa human cervical carcinoma cells and B2 cells, a stable NFκB-luciferase transfectant reporter clone of 293T human transformed kidney epithelial cells (1), and 293T cells were grown in DMEM with 4.5 g / liter glucose (Mediatech) supplemented with 10% (v/v) FBS and 2 mM L-glutamine (Mediatech).

Physiological cell death (apoptosis) was induced by treatment of cells with the macromolecular synthesis inhibitor actinomycin D (200 ng / ml, 12 hr), with the protein kinase inhibitor staurosporine (1 μM in serum-free medium, 3 hr), by irradiation with UVC light (254 nm, 20 mJ / cm2), or by γ-irradiation (2500 rad). Autophagy was induced by serum starvation with L-canavanine (1 mM) in the presence of the pan-caspase inhibitor Q-VD-OPh (10 μM) and was confirmed by the development of LC3-GFP puncta in transfected cells (2). Pathological cell death (necrosis) was triggered by incubation of cells at 56°C for 20 min. (until trypan blue

uptake indicated compromise of membrane integrity). In some experiments, apoptotic and viable target cells were fixed by incubation with formaldehyde (125 mM in PBS [unless otherwise noted, "PBS" signifies "1× PBS"], 25°C, 20 min; Polysciences, Inc., Warrington, PA). In all cases, target cells (fixed or unfixed viable, apoptotic, and necrotic cells) were washed four times in complete media and resuspended in the medium of the responder cells to be tested.

C. Vesicle Preparation and Quantification

Plasma membrane vesicles were prepared from HeLa cells as described (1). Briefly, mono-layers of cells, either untreated or induced to die for 4 h with actinomycin D (and still adherent), were stimulated to vesiculate by incubation at 37°C in vesiculation buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$, 2 mM DTT, and 25 mM formaldehyde). Supernatants were collected after 2.5 hr. (when abundant small membrane vesicles were apparent in the culture fluid). Non-adherent whole cells were removed by centrifugation at 1000 × g for 10 min. at 4°C, and vesicles were enriched from the cleared supernatant by centrifugation at 30,000 × g for 60 min. at 4°C. Vesicles were quantitated using cytofluorimetric method and analysis indicated that vesicles were ~0.8 μm in diameter and that the level of contaminating intact cells was less than one cell per 100 vesicles. Viable vesicles were comparable in size.

D. Mice

Adult C57BL/6 mice (male and female) of specific ages (2 - 3 months old, hereafter referred to as "Young"; 14 - 15 months old, "Middle-Aged"; and 24 - 25 months old, referred to as "Old"; see Chapter IV) were purchased from the National Institute of Aging (Bethesda, MD). Young (2 - 3 months old) female C57BL/6 mice for other studies (Chapter VII) were purchased from Charles River Laboratories (Wilmington, MA). All mice were maintained in-house in the environmentally controlled Biological Resources Laboratory (BRL). Animal procedures were

approved by the UIC Animal Care Committee and performed in the BRL in accordance with Protocol ACC 12-172.

E. Isolation of Macrophages

Splenic Macrophages: Spleens were removed aseptically and disrupted to yield a cell suspension in complete RPMI medium. Red blood cells were lysed by treating the cells briefly with TAC buffer (Tris-Ammonium Chloride) @ 37˚C for 5 min. Cells were washed twice in complete RPMI medium and plated (1×10^7 cells / 2 ml / well) in wells of 6-well plates. Splenic macrophages were isolated by means of adherence to plastic. After 2 hr. incubation, non-adherent cells were removed; the remaining (adherent) cells were washed twice with warm complete RPMI medium.

Resident Peritoneal Macrophages: Peritoneal cells were collected from unmanipulated mice by flushing the peritoneal cavity with 5 ml complete RPMI medium; recovered cells were washed twice in complete RPMI medium and plated (0.5×10^6 cells / ml / well) in wells of 24-well plates. After 2 hr. incubation, non-adherent cells were removed and the remaining (adherent) cells were washed twice with warm complete RPMI medium.

Thioglycollate-Elicited Peritoneal Macrophages: Thioglycollate (2 ml of 4% thioglycollate; Difco; Irvine, CA) was injected intraperitoneally. Three days later, elicited cells were collected by flushing the peritoneal cavity with in 5 ml complete RPMI medium. Cells were washed twice with complete medium and plated (0.5×10^6 cells / ml / well) in wells of 24-well plates. After 2 hr. incubation, non-adherent cells were removed and the remaining (adherent) cells were washed twice with warm complete RPMI medium.

For analysis, cells were removed from the wells washed with PBS, followed by a wash with PBS supplemented with 0.4 mM Na_2EDTA, followed by a wash with 0.05% trypsin-EDTA (Mediatech) to remove any bound targets. Cells then were gently lifted by scarping.

F. Bacterial Strains, Plasmids, and Media

E. coli DH5α strain used for generation and preparations of plasmids used for transfection was grown in Luria broth (LB) (Difco Laboratories, Detroit, MI). ***Needs clarification*****L. monocytogenes* strain 10403S, derived from the 1/2a serotype, a streptomycin resistant derivative of strain 10403; was grown in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) and supplemented with the appropriate antibiotic. Antibiotics were used at the following concentrations unless otherwise noted: ampicillin (100 μg / ml), kanamycin (30 μg / ml) and streptomycin (200 μg / ml). All strains of bacteria used were grown overnight at 37°C without agitation prior to *in vitro* and *in vivo* assays.

The rat iNOS promoter (-2.2 kbp) – luciferase reporter plasmid (rpiNOS[-2.2 kbp]-Luc) was kindly provided by Dr. Douglas Feinstein (6). The rat iNOS promoter (-1.1 kbp) – luciferase (rpiNOS[-1.1 kbp]-Luc-SVpA) and rat iNOS promoter (-1.1 kb) – luciferase with the 3'UTR (rpiNOS[-1.1 kbp]-Luc-3'UTR) constructs were kindly provided by Dr. Tadayoshi Okumura (3). The murine Arginase II promoter (-1.8 kbp) – luciferase reporter plasmid (mpArgII[-1.8 kbp]-Luc) was kindly provided by Dr. Bernard Brüne (4). The murine Arginase II promoter (-878 bp) – luciferase reporter (mpArgII[-878 bp]-Luc) and Arginase II (-852 bp) – luciferase reporter (mpArgII[-852]-Luc) plasmids were kindly provided by Dr. Antonio Castrillo (5). The pRL-TK, pNFκB-luc, and pGL3-basic vectors were purchased from Promega Corporation (Madison, WI).

Experiments involving recombinant DNA followed procedures approved by the UIC Institutional Biosafety Committee (Protocol IBC 13-040).

G. Intravenous Mouse Infections

Infections of C57BL/6 mice were performed as previously described (7). Overnight cultures of *L. monocytogenes* (strain 10403S) were diluted 1:20 into fresh medium and grown to an OD_{600} of approximately 0.6 (corresponding to 6×10^8 colony forming units [CFU] / ml). Bacteria were washed twice in PBS and resuspended in PBS for intravenous (tail vein) injection. Apoptotic splenocytes (induced by γ-irradiation) were washed twice in PBS, and resuspended in PBS for injection. The volume of injections was 200 μl in all cases. Seventy-two hr. after infection with *L. monocytogenes*, livers and spleens were collected from sacrificed mice. Organs were homogenized with a Tissue Master 125 homogenizer (Omni, Marietta, GA) and 10-fold serial dilutions were plated onto BHI plates containing 200 μg / ml streptomycin.

H. Construction of Mutant Arginase II Promoter – Luciferase Constructs

Truncated Arginase II promoter constructs were derived from the mpArgII(-878 bp)-Luc plasmid by restriction digestion, generating specific promoter fragments (the staggered ends of which were "filled-in" by the Klenow fragment of DNA polymerase I) , which then were inserted into the pGL3-basic reporter vector. Double digestion with NheI in combination with ApaI, PstI or AatII produced the -814 bp to +58 bp (mpArgII(-814bp)-Luc), the -420 bp to +58 bp (mpArgII(-420 bp)-Luc)., and the -55 bp to +58 bp (mpArgII(-55bp)-Luc) Arginase II promoter fragment.

Mutant Arginase II promoter constructs were generated by PCR-dependent site-directed muragenesis, and reinserted into pGL3r. The CREB binding-site mutant (TGACGTCA to TGGCGGCA) Arginase II promoter luciferase construct (mpArgII(-878 bp ΔCREB)–Luc) was generated using the following primer pairs:

Fwd: 5' GGAGGCGTGTCCGAGAGATGCTGGCGGCACAGGGCGGTGCGCTCGCCTAG 3'

Rev: 5' CTAGGCGAGCGCACCGCCCTGTGCCGCCAGCATCTCTCGGACACGCCTCC 3'

All promoter mutations and constructs were confirmed by sequencing.

I. Transfection and Luciferase Assay

Apoptotic modulation of specific transcription (e.g. iNOS promoter) was assessed with appropriate transcriptional reporter constructs, using a dual luciferase strategy, as described previously (1). Cells were co-transfected with a plasmid containing the firefly (*Photinus pyralis*) luciferase gene (in the pGL3-basic reporter system), the expression of which is driven by a gene-specific transcriptional promoter, together with pRL-TK, a Renilla (sea pansy; *R. reniformis*) luciferase control vector, the constant expression of which is dependent on the thymidine kinase enhancer / promoter region (Promega, Madison, WI).

RAW 264.7 macrophages (3×10^6 cells / 60 mm diameter dish) were transfected using JetPEI Transfection Reagent (Polyplus transfection, Bioparc, Illkirch-Graffenstaden, France). The next day, the cells were re-plated in 48-well plates (at 1×10^5 cells / well), allowed to rest for 8 hr. prior to incubation without or with the indicated target cells and / or a proinflammatory stimulus (LPS [100 ng / ml] unless indicated).

Cell extracts were prepared after further incubation as indicated, and luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) in an FB12 luminometer (Zylux; Oak Ridge, TN). Each condition was repeated in triplicate wells, and the luciferase activities in cells from each well were determined independently. Within any experiment, Renilla luciferase activities among samples varied less than 6%. The firefly luciferase activity in each sample was normalized with respect to the internal Renilla luciferase activity. The relative level of normalized firefly luciferase activity in an experimental sample,

compared with the normalized firefly luciferase activity in an untreated population, was expressed as the fold-induction in specific transcriptional activity.

siRNA transfections: CREB-specific predesigned siRNA - SignalSilence® CREB siRNA II (Mouse Specific) (Cell Signaling Technology) and Control siRNA (Cell Signaling Technology) were transfected into RAW264.7 cells using INTERFERin (Polyplus transfection, Bioparc, Illkirch-Graffenstaden, France) according to the manufacturer's instructions. After 4 hr. the medium was changed and cells were incubated for another 36 hr. in complete medium before use. CREB knockdown was confirmed by western blot analysis.

J. RNA Extraction, Reverse Transcription and Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase to remove any contaminating DNA using TURBO DNA-free (Ambion, Life Technologies Corporation, Grand Island, NY) (All procedures involving commercial products followed the manufacturers' protocols). cDNA synthesis (starting with 2 μg of total RNA) was performed with random hexamers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Corporation; Grand Island, NY). qRT-PCR reactions were performed using Fast SYBR green (Applied Biosystems) and run on the ViiA™ 7 Real-Time PCR System (Invitrogen). The primers used are listed in **Table III**. Primer pairs were designed to hybridize with murine-specific sequences exclusively, and to prime cDNA synthesis spanning exons, thereby minimizing the background of cDNA synthesis from contaminating genomic DNA. Standard curves were generated using a cDNA template prepared from cells stimulated with LPS alone for GAPDH, TNF-α, IL-6, iNOS and Endothelin-I; and a cDNA template prepared from cells treated with apoptotic cells (of human origin) and LPS for IL-10,

Arginase I, and Arginase II. Amplifications for each qRT-PCR primer / probe set were calibrated by linear regression according to the formula:

$$CN = 10^{-[(TC - b) / m]}$$

where CN is the relative template copy number and TC is the observed threshold crossing point. Transcript concentrations were calculated assuming an efficiency of cDNA synthesis of 1.0; samples were normalized relative to GAPDH mRNA content, determined in the same qRT-PCR reaction. Experimental cDNA samples were diluted as necessary to yield qRT-PCR results within the linear range of relevant standard curve. For the normalization of two samples (treated [t] and untreated [u], with the untreated sample serving as the normalization control),

$$CN_t / CN_u = \{10^{[-(TCt - b) / m]}\} / \{10^{[-(TCu - b) / m]}\}, \text{ and}$$

$$\log (CN_t - CN_u) = (TC_u - TC_t) / m$$

K. Cytofluorimetric (extra-cellular and intracellular) Analyses

For extra-cellular staining with primary antibodies conjugated to FITC (Ex_λ = 488 nm; Em_λ = 520 nm), phycoerythrin (PE; Ex_λ = 561 nm; Em_λ = 578 nm), allophycocyanin (APC; Ex_λ = 640 nm; Em_λ = 660 nm), Pacific Blue (PB; Ex_λ = 405 nm; Em_λ = 452 nm), or a tandem phycoerythrin / cyanine dye (PE-Cy7; Ex_λ = 561 nm; Em_λ = 785 nm), or for staining with FITC - conjugated plasminogen (BioMac), cells were washed twice with cold PBS containing FBS (1%) before resuspension and staining in the same buffer for 25 min. at 4°C in the dark prior to washing and cytofluorimetric analysis. Staining involving unconjugated primary antibodies followed the same procedure and was followed by a second incubation with an appropriate conjugated secondary antibody.

Table III: Primer pairs used in qRT-PCR

Each primer pair is designed to hybridize with murine-specific sequences exclusively, and to prime cDNA synthesis spanning exons, thereby minimizing the background of cDNA synthesis from contaminating genomic DNA.

Gene	Primer Orientation	Sequence
GAPDH	Forward	GGCATTGCTCTCAATGACAA
	Reverse	ATGTAGGCCATGAGGTCCAC
TNF-α	Forward	CAAATGGCCTCCCTCTCA
	Reverse	CTCCTCCACTTGGTGGTTTG
IL-10	Forward	TGAATTCCCTGGGTGAGAAG
	Reverse	GCTCCACTGCCTTGCTCTTA
iNOS	Forward	AGCTGAACTTGAGCGAGGAG
	Reverse	TGCCCCATAGGAAAAGACTG
Arginase II	Forward	ATATGGTCCAGCTGCCATTC
	Reverse	ACCACTTCAGCCAGTTCCTG
Arginase I	Forward	GGAACCCAGAGAGAGCATGA
	Reverse	TTTTCCAGCAGACCAGCTTT
Endothelin-I	Forward	AGTGCGCTCACCAAAAAGAC
	Reverse	CCAACTTGGAACAGGGTTTT

The accessibility of phosphatidylserine was revealed by the binding of FITC - or PE - conjugated annexin V (BD Biosciences). Cells that had been washed twice with PBS were re-suspended in 100 µl of annexin V binding buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, and 2.5 mM $CaCl_2$) and incubated with 5 µl of the conjugated annexin V for 25 min. in the dark at 25°C. Propidium iodide (PI; Ex_λ = 561 nm, Em_λ = 610 nm) and 7-aminoactinomycin D (7-AAD; Ex_λ = 561 nm, Em_λ = 650 nm) were employed to assess plasma membrane integrity. PI or 7-AAD were added to cells (at final concentrations of 1 or 4 µg / ml, respectively) immediately before cytofluorimetric analysis.

For intracellular staining, cells were washed twice with cold PBS and fixed and permeabilized in a solution of 4% formaldehyde and 0.1% saponin in PBS for 20 min. at 4°C in the dark. After fixation, cells were washed twice with PBS buffer containing 0.1% saponin and 1% FBS and stained in this same buffer with appropriate antibodies. In the case of intracellular cytokine detection, the normal process of secretion was reversed by treatment of cells with Brefeldin A (5 µg / ml), an inhibitor of endoplasmic reticulum to Golgi transport, for 3 hr. prior to fixation and permeabilization of cells for staining.

Cells were analyzed cytofluorimetrically on a FACSCalibur instrument (BD Biosciences) or the BD LSRFortessa (BD Biosciences). Cytofluorometric data were processed with WinMDI software (Joe Trotter, The Scripps Research Institute, La Jolla, CA) or Summit version 4.3 software (Dako, Carpentaria, CA). Where appropriate, fluorescence data are expressed as mean fluorescence intensity (MFI). Attributes of cell death, including changes in forward-angle and side-angle light scatter, also were evaluated, as described previously (8).

L. Assessment of Phagocytosis

Phagocytosis was assessed as previously described (1). Target cells were labeled green with (5,6)-carboxyfluorescein diacetate succinimidyl ester (CFDA; 0.2 μM; Molecular Probes; Eugene, OR) and then were induced to undergo apoptotic cell death, killed pathologically by heat treatment, or left untreated. Macrophages were co-cultured with apoptotic, necrotic, or viable target cells for 2 hr. at 37°C. Macrophages then were washed with PBS, followed by a wash with PBS supplemented with 0.4 mM Na_2EDTA, followed by a wash with 0.05% trypsin-EDTA (Mediatech) to remove any bound targets. Cells then were gently lifted by scarping, stained with PB - conjugated F4/80 antibody (to identify macrophages), and analyzed cytofluorimetrically on the BD LSRFortessa (BD Biosciences). Cells with macrophage-like scatter properties that stained positively for F4/80 and also were CFDA-positive (Ex_λ = 488 nm; Em_λ = 530 ± 15 nm), represented macrophages that had engulfed targets. Phagocytosis is represented as the fraction of $F4/80^+$ macrophages that are $CFDA^+$. Most targets that are bound but not engulfed are disrupted and do not remain adherent during the analysis (1).

M. Nitrate and Nitrite Measurements using Nitric Oxide Analyzer

Nitrite and nitrates were measured by chemiluminescence (Sievers Nitric Oxide Analyzer, NOA 280i) according to the manufacturer's protocols. Briefly, aliquots of culture media from experimental samples were injected into the reaction chamber containing either vanadium (III) chloride / HCl for the determinataion of total nitric oxides (nitrite, nitrate, and nitrosothiols), or I_3^- / HCl for the determination of nitrite content exclusively.

N. Griess Assay

Nitrite was determined in cell supernatants. Cells (e.g. primary macrophages. RAW264.7) were seeded in 6-well plates at a density of 1×10^6 cells / well using 1 ml medium. After

incubation for 24 hr., cells were co-incubated with appropriate stimuli for an additional 15 hr. Nitrite formation was determined by the Griess assay according to the manufacturer's instructions (Promega, Heidelberg, Germany). The absoute nitrite concentrations in supernatants were determined based on a standard concentration curve of $NaNO_2$ dissolved in culture medium.

O. Preparation of Cell Extracts and Immunoblot (Western Blot) Analysis

Briefly, after experimental incubations, cells were lysed in 1× RIPA buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% Sodium deoxycholate) supplemented with 50 µg / ml PMSF, 10 µg / ml pepstatin-A, Protease inhibitor cocktail (cOmplete mini; Roche *) and phosphatase inhibitor (Sigma-Aldrich). The lysate was cleared by centrifugation (15,000 × g, 15 min.), and soluble proteins (~100-150 µg / sample; determination by a detergent compatible protein assay; DC Protein Assay, Bio-Rad Laboratories, Hercules, CA) were resolved on 4–20% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories). Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) using a semi-dry transfer apparatus. Membranes were blocked by incubation in TBST (150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.1% Tween-20) + 5% dehydrated skim milk (30 min., 25°C), incubated with primary rabbit antibodies specific for proteins of interest (16 hr., 4°C), and developed with a horse radish peroxidase-labeled goat anti-rabbit secondary antibody (1:20,000; 1 hr., 25°C) and ECL detection (Pierce, Thermo Fisher Scientific). Membranes were washed four times for 15 min. each with TBST between antibody incubations.

P. Protein Identification by Two-Dimensional Gel Electrophoresis (2DE)

Vesicles were lysed by sonication in isoelectric focusing rehydration buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% Biolytes (pH 5–8), 0.01% Bromophenol Blue and

protease inhibitor). Seventy-five µg of protein in a total of 185 µl of rehydration buffer was applied to 11 cm BioRad ReadyStrip IPG Strips (pH 5–8) for overnight rehydration. First-dimension isoelectric focusing was carried out on a Bio-Rad PROTEAN IEF System for a total focusing time of 75000 VH. After focusing, strips were equilibrated with equilibration buffer I (6 M Urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% [w/v] DTT) for 15 min. The strips were further equilibrated with equilibration buffer II (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% [w/v] iodoacetamide) for 15 min. and directly applied to a 12.5% isocratic SDS-PAGE gel for second dimension. The resulting gel was then fixed (10% acetic acid and 40% ethanol) for 30 min. and stained overnight with SYPRO Ruby. Gels were destained (10% methanol, 7.5% acetic acid) for 60 min. After washing with water, gels were scanned on a 9400 Typhoon Variable Mode Imager (GE Healthcare, Inc.; Piscataway, NJ) using a Green (532 nm) Laser and 610BP30 emission filter. Quantitative analysis of spots on gels was performed by PDQuest (Bio-Rad) and visually confirmed.

Protein spots from SYPRO Ruby- stained gels were picked for protein identification. The gel spots were diced into 1 mm^3 pieces and washed with 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate prior to DTT reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37ºC overnight. The resulting peptides were extracted with 30 µl of 1% trifluoracetic acid followed by desalting with C18 Ziptip (Millipore; Bedford, MA). For the mass spectrometry (MS) analysis, the peptides were mixed with 7 mg / ml α-cyano-4-hydroxy-cinnamic acid matrix (in 60% ACN) in a 1:1 ratio and spotted onto a matrix assisted laser desorption / ionization (MALDI) plate. The peptides were analyzed on a 4800 MALDI-TOF-TOF analyzer (Applied Biosystems). Mass spectra (m / z 880-3,200) were acquired in positive ion reflector mode. The 15 most intense ions were selected for subsequent tandem mass

spectrometry (MS / MS) sequencing analysis in 1 keV mode. Protein identification was performed by searching the combined MS and MS / MS spectra against the human NCBI database using a local MASCOT search engine (V. 1.9) on a GPS (V. 3.5, Applied Biosystems) server. Proteins containing at least two peptides with Confidence Interval values no less than 95% were considered being identified.

Q. Protein Quantification by iTRAQ Analysis

For iTRAQ labeling, proteins from vesicles were extracted in an iTRAQ lysis buffer (1% NP40, 1% Triton X-100, 10 mM HEPES, 500 mM triethylammonium bicarbonate buffer [TEAB]) using probe sonication at 50% duty for 3 cycles of 15 sec. with a 60 sec. incubation in ice cold water between cycles. The lysate was cleared by centrifugation at 16,100 × g for 15 min. The pH of samples was adjusted to 8.0 with 1.0 M TEAB. The iTRAQ labeling procedures were performed according to the manufacturer's instructions as further described (9). Briefly, after reduction with Tris (2-carboxyethyl) phosphine hydrochloride and alkylation with methyl methanethiosulfonate, tryptic digestion of each sample (100 μg) was initiated by the addition of 10 μg of trypsin (Promega, Madison, WI), and each sample was incubated at 37°C overnight. Peptides derived from viable samples were labeled with iTRAQ tags 114 and 115 while the peptides from apoptotic samples were labeled with iTRAQ tags 116 and 117. The labeled samples then were mixed together and fractionated via strong cation exchange and reverse phase chromatography according to a procedure described previously (10). The HPLC eluate was mixed with a matrix solution (7 mg / ml α-cyano-4-hydroxycinnamic acid in 60% ACN, 5 mM of ammonium monobasic phosphate, and internal mass calibrants [50 fmol / μl each of Glu-Fib and ACTH]) through a 30 nl mixing tee and directly spotted onto the MALDI plates. The peptides were analyzed on a 4800 Proteomics Analyzer MALDI-TOF-TOF tandem mass

spectrometer (Applied Biosystems) in a data-dependent fashion using job-wide interpretation. MS spectra (m/z 800–3,600) were acquired in positive ion reflectron mode with internal mass calibration. A maximum of the fifteen most intense ions (S / N >50) per spot were selected for subsequent MS / MS analysis in 1.0 keV mode. Each spectrum was averaged over 2,000 laser shots.

R. Bioinformatic Analysis

TS2Mascot (Matrix Science Inc.; Boston, MA, USA) was used to generate peak lists in mascot generic file (MGF) format from the tandem MS spectra, using the following parameters (mass range from 20 to 60 Dalton below precursor, S/N ratio ≥10). The peak lists were submitted for automated search using a local Mascot server (version 2.2) against 20,244 proteins in the SwissProt human protein sequence database downloaded from ftp://ftp.ebi.ac.uk/pub/databases/uniprot/knowledgebase. The following parameters were used for the search; iTRAQ 4plex (K), iTRAQ 4plex (N-terminal) and methylthio (C) as fixed modifications; iTRAQ 4plex (Y) and Oxidation (M) as variable modifications; trypsin as the digestive enzyme with up to two missed cleavages allowed; monoisotopic mass with peptide precursor mass tolerance of 50 ppm; MS/MS ion mass tolerance of 0.3 Da. Scaffold (version 3_03_01, Proteome Software Inc., Portland, OR) was used to filter MS / MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at or greater than 95.0% probability and ≤ 1.0% false discovery rate (FDR) as specified by the Peptide Prophet algorithm (11). Protein identifications were accepted if they could be established at or greater than 95.0% probability and contained at least 1 identified peptide at 95% confidence. Protein probabilities were assigned by the Protein Prophet algorithm (12). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were

grouped to satisfy the principles of parsimony. Peptides were quantified using the centroided iTRAQ reporter ion peak intensity. Protein quantitative values were derived from only uniquely assigned peptides. Protein quantitative ratios were calculated as the median of all relevant peptide ratios for each protein. Log 2 fold ratios for each protein reported by Scaffold were transformed to normal relative protein abundance fold ratios in Excel (Microsoft; Redmond, WA, USA). A two-tailed t-test was performed using Excel for final statistical evaluation. In addition, only proteins altered in abundance by at least 20% from the viable vesicles were considered significant. For each identified protein, associated gene ontology terms were automatically fetched from European Bioinformatics Institute EBI by Scaffold software and plotted with respect to enrichment.

S. Enzymatic Assays

Enolase activity was assessed as the fluoride-inhibitable (13) conversion of 2-phosphoglycerate to phosphoenolpyruvate, by a modification of an assay described by Pancholi and Fischetti (14). Intact viable and apoptotic HeLa cells were washed once with PBS. Graded numbers of cells (ranging from 1×10^6 to 3×10^4) were added to a 200 µl reaction in enolase buffer (10 mM $MgCl_2$ in PBS). Reactions were started with the addition of 2-phosphoglycerate (to a final concentration of 3 mM). After 4 min. of incubation at 25°C, reactions were terminated by the addition of 800 µl of enolase stop buffer (10 mM $MgCl_2$ and 3 mM NaF in PBS). Cells were removed by centrifugation (600 × g for 5 min) and phosphoenolpyruvate in supernatants was quantified spectrophotometrically (λ = 240 nm). The molar extinction coefficient of phosphoenolpyruvate at 240 nm is 1.164×10^3. Enolase activity in cell extracts (comparably graded cell equivalents) was assessed similarly. Extracts were prepared from viable and apoptotic HeLa cells by sonication (6 × 10 sec at 60 watts; VibraCell sonicator, Sonics and

Materials, Danbury, CT) after allowing the cells to swell on ice in 0.1× PBS for 30 min. Enolase activity in cell supernatants was assessed after incubating graded numbers of intact cells (as described above) in mock reactions in enolase buffer without 2-phosphoglycerate. After 4 min, cells were removed by centrifugation. 2-Phosphoglycerate was then added to the supernatant, and the 2-phosphoglycerate-dependent production of phosphoenolpyruvate was assessed after 4 min. as described above.

The determination of GAPDH activity followed a similar set of procedures and was assessed as the conversion of NAD to NADH dependent on glyceraldehyde 3-phosphate, by a modification of an assay described by Pancholi and Fischetti (15). In particular, the reaction buffer was adjusted to iso-osmolarity. Graded numbers of intact, washed, apoptotic, and viable HeLa cells (ranging from 3×10^5 to 1×10^4) were added to a 200 µl reaction in GAPDH buffer (60 mM NaCl, 50 mM Na_2HPO_4, 40 mM triethanolamine, and 1 mM NAD [pH 8.6]). Reactions were started by the addition of glyceraldehyde 3-phosphate to a final concentration of 2 mM. After 4 min. of incubation at 25°C, reactions were terminated by the addition of 800 µl of GAPDH stop buffer (60 mM NaCl, 50 mM Na_2HPO_4, and 40 mM triethanolamine [pH 10.0]). Cells were removed by centrifugation, and NADH in supernatants was quantified spectrophotometrically (λ = 340 nm). The molar extinction coefficient of NADH at 340 nm is 6.22×10^3.

T. Statistical Analyses

Statistical analysis was applied using the 2-tailed Student's t test. In some figures, representative data are presented for example. In these experiments, error bars represent Standard Error of Mean [SEM]. All data points are the means (± SEM) of a minimum of

triplicate determinations, and each of the experiments presented is representative of multiple (typically > 4) repetitions.

In chapter VI, statistical analysis of line graph analyses between age groups of mice, where two different variables (age gourps, and dose response) were involved, were applied using 2-way Analysis of variance (ANOVA). In these experiments, error bars represent Standard Error of Mean [SEM]. All data points are the means (± SEM) of multiple (typically > 6) different experiments (mice of each age group).

Cited Literature for Chapter II

1. Cvetanovic M, Mitchell JE, Patel V, Avner BS, Su Y, van der Saag PT, Witte PL, Fiore S, Levine JS, Ucker DS. 2006. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. *J Biol Chem.* **281(29)**:20055-67.

2. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19(21)**:5720-8.

3. Ozaki T, Habara K, Matsui K, Kaibori M, Kwon AH, Ito S, Nishizawa M, Okumura T. 2010. Dexamethasone inhibits the induction of iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. *Shock.* **33(1):**64-9.

4. Johann AM, Barra V, Kuhn AM, Weigert A, von Knethen A, Brüne B. 2007. Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production. *FASEB J.* **21(11)**:2704-12.

5. Marathe C, Bradley MN, Hong C, Lopez F, Ruiz de Galarreta CM, Tontonoz P, Castrillo A. 2006. The arginase II gene is an anti-inflammatory target of liver X receptor in macrophages. *J Biol Chem.* **281(43): 32197-206**.

6. Bhat NR, Feinstein DL, Shen Q, Bhat AN. 2002. p38 MAPK-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells. Roles of nuclear factors, nuclear factor kappa B, cAMP response element-binding protein, CCAAT/enhancer-binding protein-beta, and activating transcription factor-2. *J Biol Chem.* **277(33)**: 29584-92.

7. Alonzo F 3rd, Port GC, Cao M, Freitag NE. 2009. The posttranslocation chaperone PrsA2 contributes to multiple facets of Listeria monocytogenes pathogenesis. *Infect Immun.* **77(7)**:2612-23.

8. Cocco RE, Ucker DS. 2001. Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol Biol Cell.* **12(4)**:919-30.

9. Jain MR, Bian S, Liu T, Hu J, Elkabes S, Li H. 2009. Altered proteolytic events in experimental autoimmune encephalomyelitis discovered by iTRAQ shotgun proteomics analysis of spinal cord. *Proteome Sci.* **7:25**.

10. Jain MR, Liu T, Hu J, Darfler M, Fitzhugh V, Rinaggio J, Li H. 2008. Quantitative Proteomic Analysis of Formalin Fixed Paraffin Embedded Oral HPV Lesions from HIV Patients. *Open Proteomics J.* **1**:40-45.

11. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem.* **74(20)**:5383-92.

12. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* **75(17)**:4646-58.

13. Kanapka JA, Hamilton IR. 1971. Fluoride inhibition of enolase activity in vivo and its relationship to the inhibition of glucose-6-P formation in Streptococcus salivarius. *Arch Biochem Biophys.* **146(1)**:167-74.

14. Pancholi V, Fischetti VA. 1988. alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *J Biol Chem.* **273(23)**:14503-15.

15. Pancholi V., Fischetti V. A. (1992). A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. *J. Exp. Med.* **176**:415–426.

CHAPTER III

Apoptotic Cell-Mediated Attenuation Of The Production Of Nitric Oxide Species

A. INTRODUCTION

Cell deaths occur throughout life and play a critical role in the development, maintenance and homeostasis in multi-cellular organisms (1, 2). Cells that die, either by the process of apoptosis (physiological cell death) or by necrosis (pathological cell death), are cleared rapidly and efficiently by professionally phagocytic cells such as dendritic cells and macrophages (3, 4). The rare instances where apoptotic cells are detected in tissues are in the thymus during the thymic selection or in tissues upon an active infection, and even in those tissues they are usually immediately engulfed by phagocytes (32, 33). The clearance of dying cells represents the final step - perhaps the ultimate objective - of the cell death program. While the clearance of pathogens, infected cells and necrotic cells are typically associated with inflammatory responses (5), the clearance of apoptotic cells, at early as well as late stages of cell death (6, 7), is associated with a repertoire of distinct signaling events that ultimately leads to clearance without any associated inflammation and pathology (8-10). The absence of inflammation results from an active suppression of proinflammatory cytokine secretion, such as TNF-α and IL-6, as well as by an increase in anti-inflammatory mediators (11). The anti-inflammatory effect on phagocytes occurs in a process independent of phagocytosis like IL-10 and TGF-β (12, and 34). Moreover, the nature of recognition and the subsequent establishment of the non-inflammatory state in the responding cells is not species specific, suggesting that the molecular determinants involved in the recognition of apoptotic cells is evolutionarily conserved (11, 13).

Previous studies have reported that the recognition of apoptotic cells by macrophages attenuates the production of reactive oxygen species (14) and nitric oxides (15), another free radical species, macrophages. Nitric oxides (NO), apart from being important signaling molecules involved in many different processes including vasodilation, also are important

inflammatory mediators and play a critical role in the elimination of intracellular pathogens (16). NO acts on pathogens either directly (for example, by inducing DNA damage, inhibiting DNA repair, destroying iron-sulfur clusters, and inactivating proteins via nitrosylation; ref. 17) or indirectly (by sequestering endogenous arginine, which is necessary for pathogen protein synthesis and replication; ref. 18). Many pathogens have developed mechanisms to detoxify NO and / or evade its effects. For example, *M. tuberculosis* and *S. typhimurium* are equipped with peroxiredoxins that detoxify $ONOO^-$ to nitrite (19). Some pathogens might even utilize mechanisms like apoptotic mimicry, or the induction of apoptosis as a strategy for immune evasion (see Discussion).

The production of nitric oxides is stringently regulated in cells. It is generated by the action of nitric oxide synthases (NOS) on L-Arginine, converting it into citrulline and NO via the intermediate product N-hydroxyl-Arginine (20). There are three isoforms of nitric oxide synthases: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). As its name implies, only the levels of iNOS, the predominant form in macrophages, are subject to very dynamic regulation; eNOS and nNOS are considered generally to be constitutively expressed (20). Expression of iNOS, in macrophages, is a critical step in the regulation of nitric oxide formation in response to microbial compounds or cytokines. NO production also is regulated by the action of another group of enzymes, arginases, that compete with the NO synthases for their common substrate, degrading L-arginine to L-ornithine and urea (see **Figure II**; refs. 21, 22). Arginases are expressed in two different isoforms: Arginase I, which is expressed in the liver and is commonly referred to as the hepatic arginase, and Arginase II, which is expressed in several other tissues and cell types, including macrophages (23).

Since apoptotic cells have been shown to play a critical role in regulating and actively suppressing inflammation, we investigated the molecular mechanism by which apoptotic cells might attenuate NO production by looking at the regulation of the genes involved in its production.

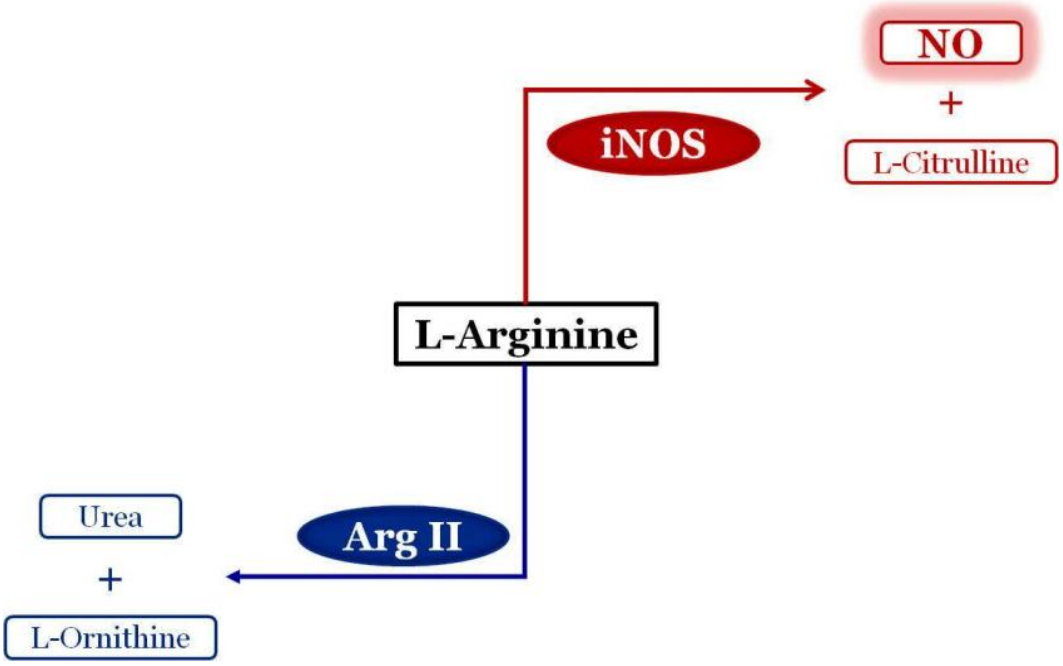


Figure II: Schematic representation of the regulation of NO production by iNOS and Arginase II. NO is generated in cells by the action of iNOS on L-Arginine; Arginase II also catabolizes L-Arginine and converts it into Urea and L-Ornithine.

B. RESULTS

i. Apoptotic cells suppress Nitric Oxide production in stimulated macrophages.

Apoptotic cells have been shown to modulate inflammatory responses in viable cells (including macrophages) with which they interact, establishing an anti-inflammatory state in those macrophages (9, 11-13, 15). This inherent ability of apoptotic cells to induce immunosuppression in neighboring interacting cells is termed as "innate apoptotic immunity" (IAI). Because of the ability of apoptotic cells ("targets") to suppress actively the expression of proinflammatory cytokines in responding cells following their inflammatory (e.g. Toll-like receptor [TLR]–dependent) stimulation, as well as to up-regulate the expression of anti-inflammatory cytokines, we asked whether apoptotic cells also can regulate the production of reactive nitrogen oxides species.

We challenged the murine macrophage like cell-line RAW264.7 cells with apoptotic or necrotic targets in the presence or absence of LPS, and then quantified the levels of two of the predominant nitric oxide intermediates, nitrite and nitrate, in cell supernatants. As see in **Figure III – A** & **B**, we observed a robust time-dependent increase in nitrite and nitrate levels upon LPS stimulation. In the presence of apoptotic cells, the LPS response was attenuated significantly. This activity of apoptotic cells was evident over a wide dose of LPS (**Figure III - C**). Necrotic targets, in contrast, did alter the production nitric oxide species.

These data suggest that the specific anti-inflammatory activity of apoptotic cells extends to the production of nitric oxide species. In contrast to RAW264.7 cells, we were unable to detect significant levels of nitrites or nitrates in the supernatants of LPS-stimulated primary elicited peritoneal macrophages (data not shown), precluding the evaluation of NO modulation by apoptotic targets in those cells.

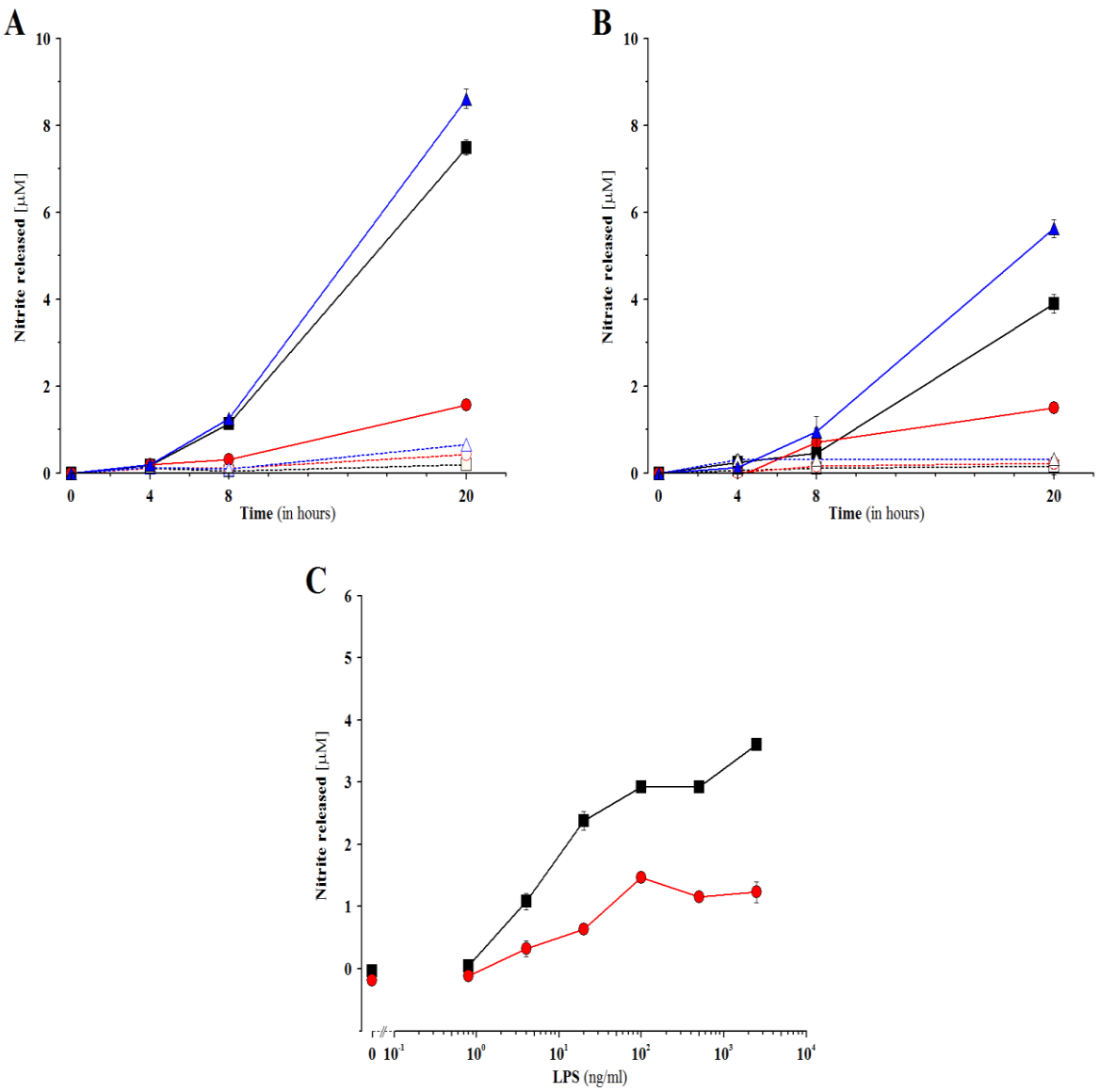


Figure III: **Attenuation of NO production by apoptotic cells.** RAW264.7 macrophages were incubated without (○△□) or with (●▲■) LPS in the presence of apoptotic (○●) or necrotic (△▲) targets (derived from Jurkat T cells; target to macrophage ratio of 6:1), or without added targets (□■). Culture supernatants were collected at the time points indicated (**A**, **B**) or at 20 hr. (**C**) after target addition, and levels of secreted nitrite (**A**, **C**) and nitrate (**B**) were quantified. In these experiments, target cells themselves produced no detectable levels of nitrites or nitrates.

ii. Macrophages that interact with apoptotic cells have lowered iNOS levels.

Since the anti-inflammatory effect of apoptotic cells, at least with regard to cytokines, is exerted primarily on the level of transcription initiation, we wondered whether the action of apoptotic cells in controlling the production of nitric oxides involves transcriptional regulation of genes involved in nitric oxide biosynthesis. Nitric oxides in stimulated macrophages are predominantly generated by the action of the inducible isoform of nitric oxide synthases; iNOS, on L-Arginine (**Figure II;** ref 20). Consequently, we began by investigating whether apoptotic cells exert an effect on iNOS transcription.

Just as iNOS protein levels increase with LPS treatment (**Figure IV - A**) in macrophages, iNOS transcript levels are induced by LPS (**Figure IV - B**). As we imagined, treatment with apoptotic cells resulted in a significant decrease in iNOS protein (**Figure IV - A**) and transcript (**Figure IV - B**) levels in LPS-stimulated macrophages. Notably, the effect of apoptotic cells on iNOS transcript levels is evident already after 2 hr. of treatment; iNOS protein levels are barely detectable at this time. These data suggest that apoptotic cell-mediated regulation of iNOS transcripts contributes significantly to apoptotic regulation of the production of nitric oxides.

iii. Apoptotic cells also up-regulate Arginase II levels in macrophages to further regulate NO production.

While the production of NO in macrophages is dependent on the action of iNOS, the availability of the substrate L-Arginine also is determinative. L-Arginine is destructively converted to L-ornithine and urea by arginase enzymes (**Figure II**). We wondered whether apoptotic cells might also regulate arginase levels in macrophages in order to attenuate NO production.

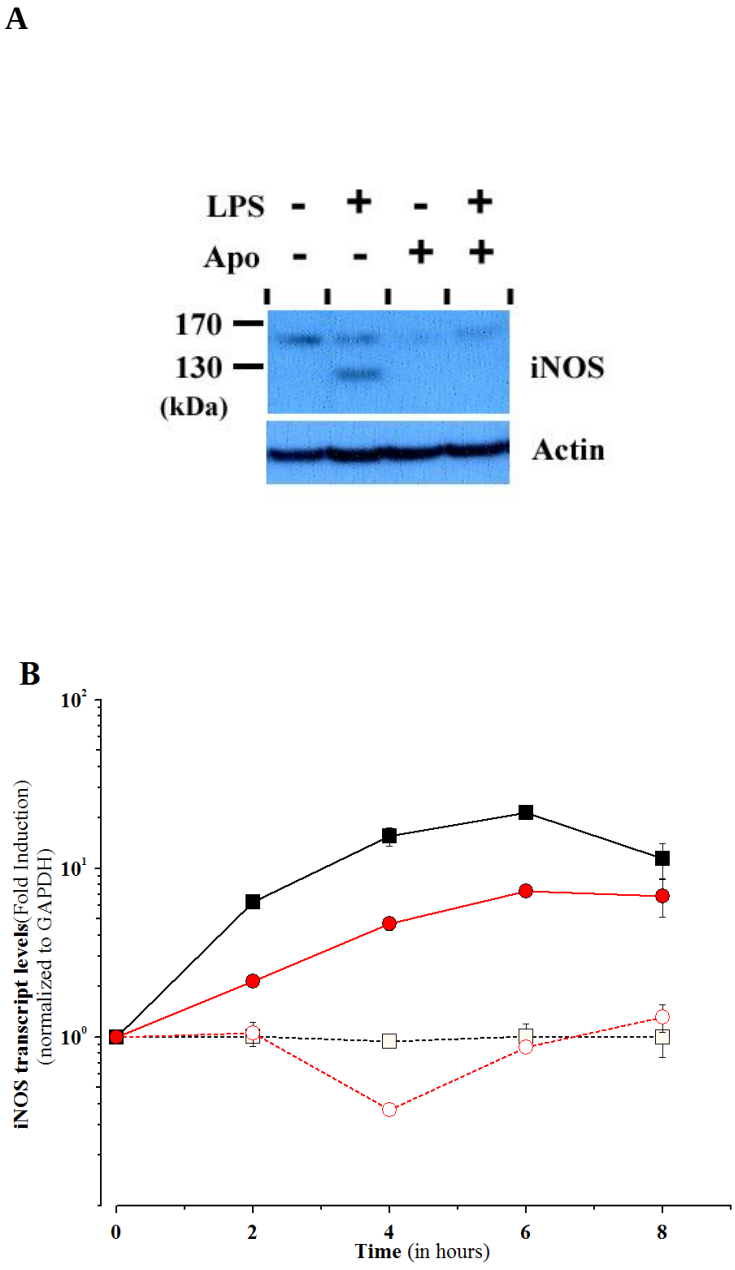
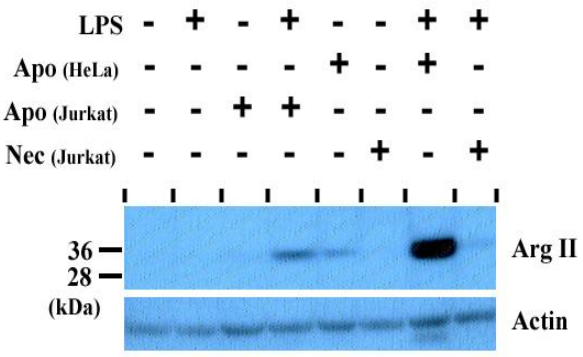


Figure IV: **Apoptotic cells down-regulate iNOS levels in macrophages.** **A**) RAW264.7 macrophages were incubated without or with LPS in the presence or absence of apoptotic targets (derived from Jurkat T cells; target to macrophage ratio of 6:1). Cells were lysed after 18 hr. and cell extracts were immunoblotted for iNOS and β-actin (as a loading control). **B**) RAW264.7 macrophages were incubated without (○□) or with (●■) LPS in the presence (○●) or absence (□■) of apoptotic targets (target to macrophage ratio of 6:1). Cells were lysed at indicated time points, RNA was prepared, and RAW264.7-specific iNOS transcripts were quantified by qRT-PCR analysis.

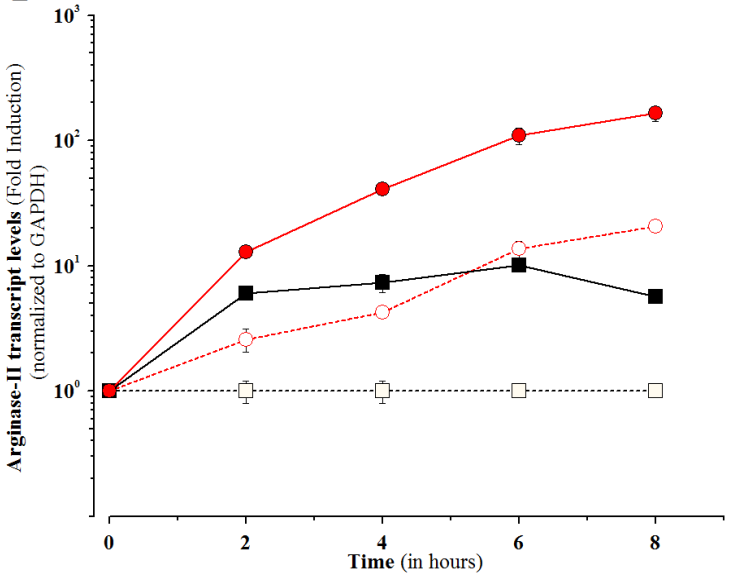
We confirmed that Arginase II is the predominantly expressed arginase form in macrophages (**Figure V - A**), and we focused on its expression on the protein (**Figure V - A**) and transcript levels (**Figure V - B**). Arginase II protein levels are significantly increased in macrophages upon treatment with apoptotic cells, even in the absence of LPS stimulation (**Figure V - A**). LPS and apoptotic cells act cooperatively to further elevate Arginase II levels (**Figure V - A**). Similarly, Arginase II transcript levels are enhanced by treatment with apoptotic cells alone or cooperatively by apoptotic cells and LPS (**Figure V - B**), and this apoptotic effect is rapid (**Figure V - B**).

Interestingly, while the expression of Arginase I in macrophages is considered generally to be constitutive, we found that Arginase I transcripts, although of much lower abundance than those of Arginase II (by qRT-PCR analysis, the relative abundance of Arginase II transcripts is approximately 100-fold greater than that of Arginase I), were similarly up-regulated in response to apoptotic cells (**Figure V - C**). Importantly, apoptotic cell interactions do not cause a global change in macrophage transcription; this fact is further documented here by an analysis of transcript levels of Endothelin-I, which encodes an important molecule involved in vascular constriction (**Figure V - D**). These data suggest that apoptotic cells exert a complex pattern of transcriptional regulation of genes affecting NO production. Although apoptotic cells regulate both iNOS and Arginase II expression, it may be that the profound regulation of Arginase II is of greater import. The work of Johann *et al.* (24), demonstrating that Arginase II ablation alone abrogates apoptotic cell-mediated attenuation of NO production significantly, is consistent with this view.

A



B



(**Figure V** cont...)

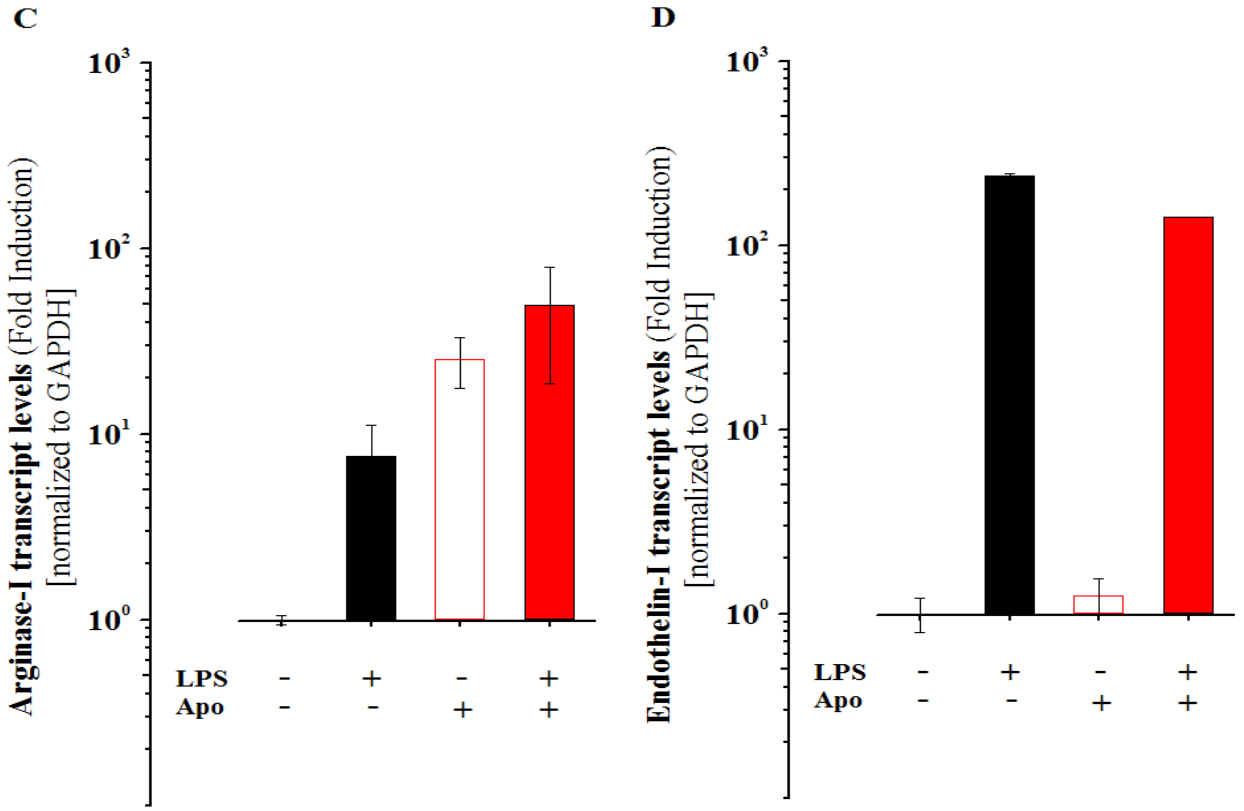


Figure V: **Apoptotic cells up-regulate Arginase II levels in macrophages.** **A**) RAW264.7 macrophages were incubated without or with LPS in the presence or absence of apoptotic or necrotic targets (derived from Jurkat T cells; target to macrophage ratio of 6:1). Cells were lysed after 18 hr. and cell extracts were immunoblotted for Arginase II and β-actin (as a loading control). **B**, **C**, **D**) RAW264.7 macrophages were incubated without (○□) or with (●■) LPS in the presence (○●) or absence (□■) of apoptotic targets (derived from Jurkat T cells; target to macrophage ratio of 6:1). Cells were lysed at the indicated time points (**B**) or at 4 hr. (**C**, **D**), RNA was prepared, and RAW264.7-specific Arginase II (**B**), Arginase I (**C**) and Endothelin-I (**D**) transcripts were quantified by qRT-PCR analysis.

iv. Transcriptional regulation of Arginase II and iNOS by apoptotic cells is dependent on cell-cell contact and is independent of apoptotic secreted factors.

The regulation of iNOS and Arginase II transcript levels by apoptotic cells could result either from direct cell to cell interaction between the apoptotic cell and the macrophage, or from the action of factors secreted by the apoptotic cells. We cultured macrophages with apoptotic cells, fixed apoptotic cells (eliminating factor secretion), or the culture media supernatants from apoptotic or control cells, in the presence and absence of LPS, and determined iNOS and Arginase II transcript levels.

Both iNOS and Arginase II transcript levels were regulated only in the presence of apoptotic cells or fixed apoptotic cells, while conditioned media from apoptotic cells did not alter transcript levels (**Figures VI - A** & **B**). We independently evaluated the requirement for cell – cell contact by separating macrophages from the apoptotic targets by a trans-well membrane. Alteration in iNOS and Arginase II transcript levels in response to apoptotic cells occur only when target cells and macrophages are allowed to interact in the same chamber (**Figures VI - C** & **D**).

No significant changes in iNOS and Arginase II transcript levels were induced by necrotic cells as well as fixed viable target cells (data not shown). These data indicate that apoptotic cells specifically, and upon direct cell-cell interaction with macrophages, exert a transcriptional regulation on iNOS and Arginase II genes, leading to the attenuation of the production of nitric oxide species. This model suggests that the observed transcriptional regulation exerted by apoptotic cells depends on determinant(s) on the surface of apoptotic cells, which are recognized by and engage responder cell molecules (receptors). This model underlies our search for apoptotic cell-surface determinant(s) involved in immunomodulation (see Chapter V).

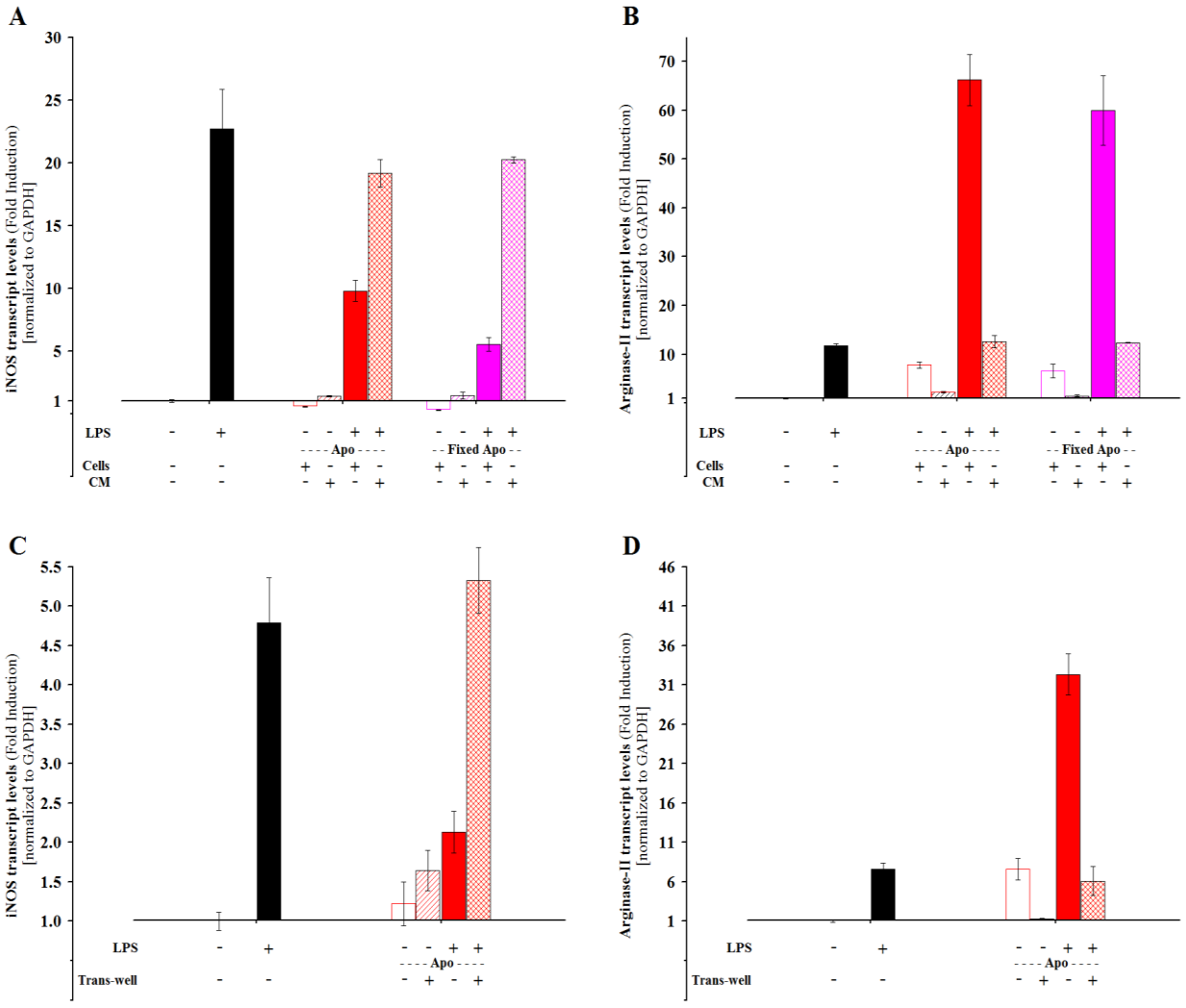


Figure VI: **Apoptotic regulation of iNOS and Arginase II transcription is dependent on cell-cell contact.** RAW264.7 cells were incubated for 3 hr. without or with LPS in the absence or presence of **(A**, **B**) apoptotic target cells (derived from Jurkat T cells; "Apo"), targets that had been fixed ("Fixed Apo"), or the culture medium (CM) prepared from those targets (at a ratio of targets [or target equivalents] to macrophages of 6:1), or **(C**, **D**) apoptotic target cells (at a ratio of targets to macrophages of 6:1) together in the same chamber, or separated by a trans-well membrane. Cells then were lysed, RNA was prepared, and RAW264.7-specific iNOS (**A**, **C**) and Arginase II (**B**, **D**) transcripts were quantified by qRT-PCR analysis.

v. Apoptotic cell mediated regulation of iNOS and Arginase II transcripts is an immediate-early response, independent of new protein synthesis.

We asked whether this transcriptional regulation by apoptotic cells, like the apoptotic regulation of cytokine gene transcription (12), is an immediate-early response directly coupled to recognition (i.e. an effect that occurs independently of new protein synthesis). Macrophages were pre-treated with the translation inhibitor cycloheximide for 60 min., or left without pretreatment, before the addition of targets and / or LPS.

For both iNOS and Arginase II transcripts, the patterns of induction and repression are not altered by cycloheximide pretreatment (**Figures VII - B** & **D**). In both cases, cycloheximide causes an elevation in basal transcript levels, and it also results in a diminution in the induction of iNOS transcripts by LPS (**Figures VII - A** & **C**; these affects may reflect cycloheximide-dependent alterations in mRNA stability [data not shown]). Nonetheless, the extent of apoptotic suppression of the LPS-induced levels of iNOS transcripts and the magnitude of the apoptotic induction of Arginase II transcripts are unaltered by the inhibition of protein synthesis (**Figures VII - B** & **D**; note that these data are normalized to relevant basal transcript levels). These findings suggest direct links between the recognition of apoptotic cells and the regulation of iNOS and Arginase II transcription.

vi. Apoptotic cell mediated regulation of Arginase II, but not of iNOS, occurs at the level of transcription initiation.

We have shown previously that the regulation of cytokine gene transcription by apoptotic cells is exerted primarily on the level of transcription initiation (12). Transcriptional reporters, constructed by the fusion of cytokine gene promoter regions and the firefly luciferase gene, provide convenient readouts of this apoptotic activity (12, 13, 25, and 26).

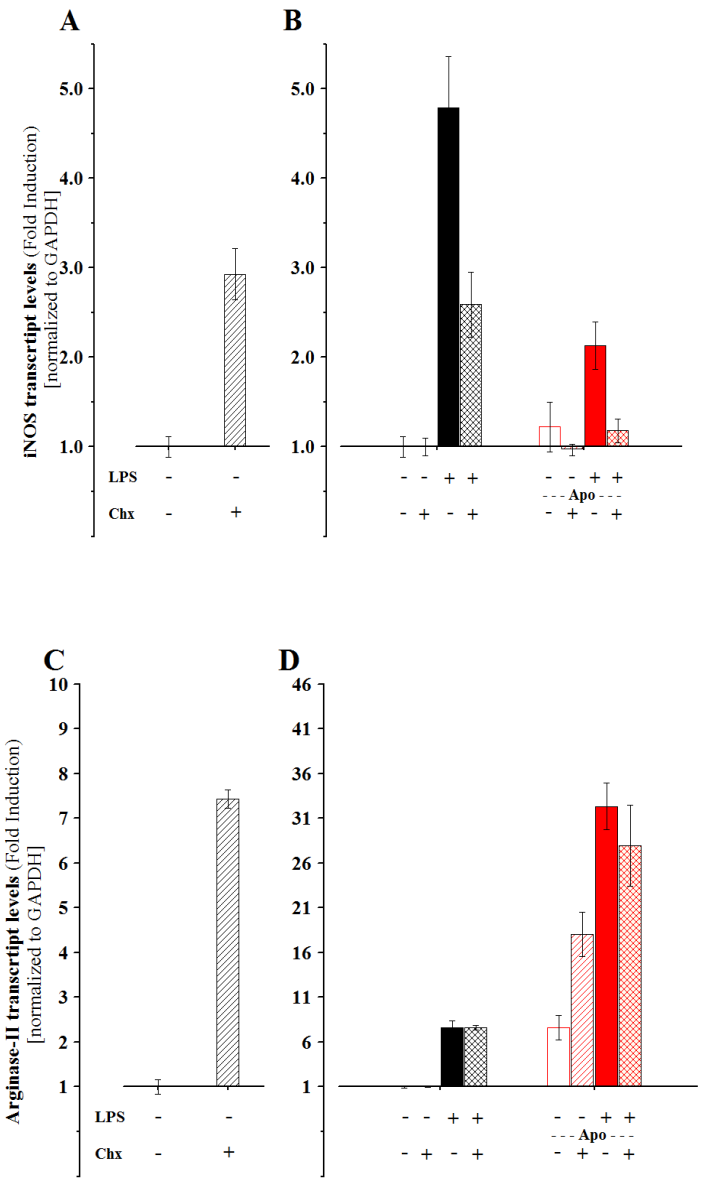


Figure VII: **Apoptotic regulation of iNOS and Arginase II transcription is a primary response, independent of new protein synthesis.** RAW264.7 macrophages were pre-incubated in the absence or in the presence of cycloheximide for 30 min. Basal levels of iNOS (**A**) and Arginase II (**C**) transcripts at that time were quantified by qRT-PCR analysis. Raw264.7 cells subsequently were incubated without or with LPS in the presence or absence of apoptotic targets (derived from Jurkat T cells; target to macrophage ratio of 6:1) for an additional 3 hr. RAW264.7-specific iNOS (**B**) and Arginase II (**D**) transcripts then were quantified by qRT-PCR analysis. Relative levels of iNOS and Arginase II transcripts ("fold induction") were calculated by normalization to the basal levels of pre-incubated cells (as determined in **A** and **C**, respectively).

We examined whether the apoptotic regulation of iNOS and Arginase II transcript levels is exerted similarly. We tested this using transcriptional reporters comprising luciferase fusions downstream of fragments of the iNOS promoter (a 2.2 kbp fragment from the 5' untranslated region [UTR] of the iNOS gene; ref 6) or the Arginase II 5'UTR (1.8 kbp; ref 4). Macrophages transiently transfected with the Arginase II promoter-luciferase plasmid (**Figure VIII - C**) or the iNOS promoter-luciferase plasmid (**Figure VIII - A**) were treated with apoptotic cells in the presence or absence of LPS for 18 hr. and luciferase activities were measured.

The ability of apoptotic cells to regulate Arginase II transcript levels was recapitulated with the Arginase II reporter construct (**Figure VIII - C**). In these transient transfection experiments, Arginase II promoter-dependent luciferase activity was induced about 4-6 fold by apoptotic cells specifically (**Figure VIII - C**); viable and necrotic cells had no effects (data not shown). These data indicate that apoptotic cells specifically regulate Arginase II transcription on the level of transcriptional initiation.

While LPS stimulation triggers iNOS promoter-dependent luciferase activity, the effect of apoptotic cells assessed with this reporter was discordant with the apoptotic effect we observed on iNOS transcript levels (**Figure VIII – A**). These data suggest that transcriptional initiation may not be the target of apoptotic regulation of iNOS expression. Previous work by Ozaki *et al*. (27) implicated a role for the 3'UTR of the iNOS transcript in regulating mRNA stability. Our data, involving an iNOS reporter construct containing a 3'UTR fragment, indicates that the apoptotic regulation of iNOS transcripts is not effected in this way **Figure VIII - B**). Other possible mechanisms of iNOS transcript regulation by apoptotic cells, including the involvement of antisense RNA (e.g. RNA complementary to the iNOS 3'UTR; ref. 28) have not been explored.

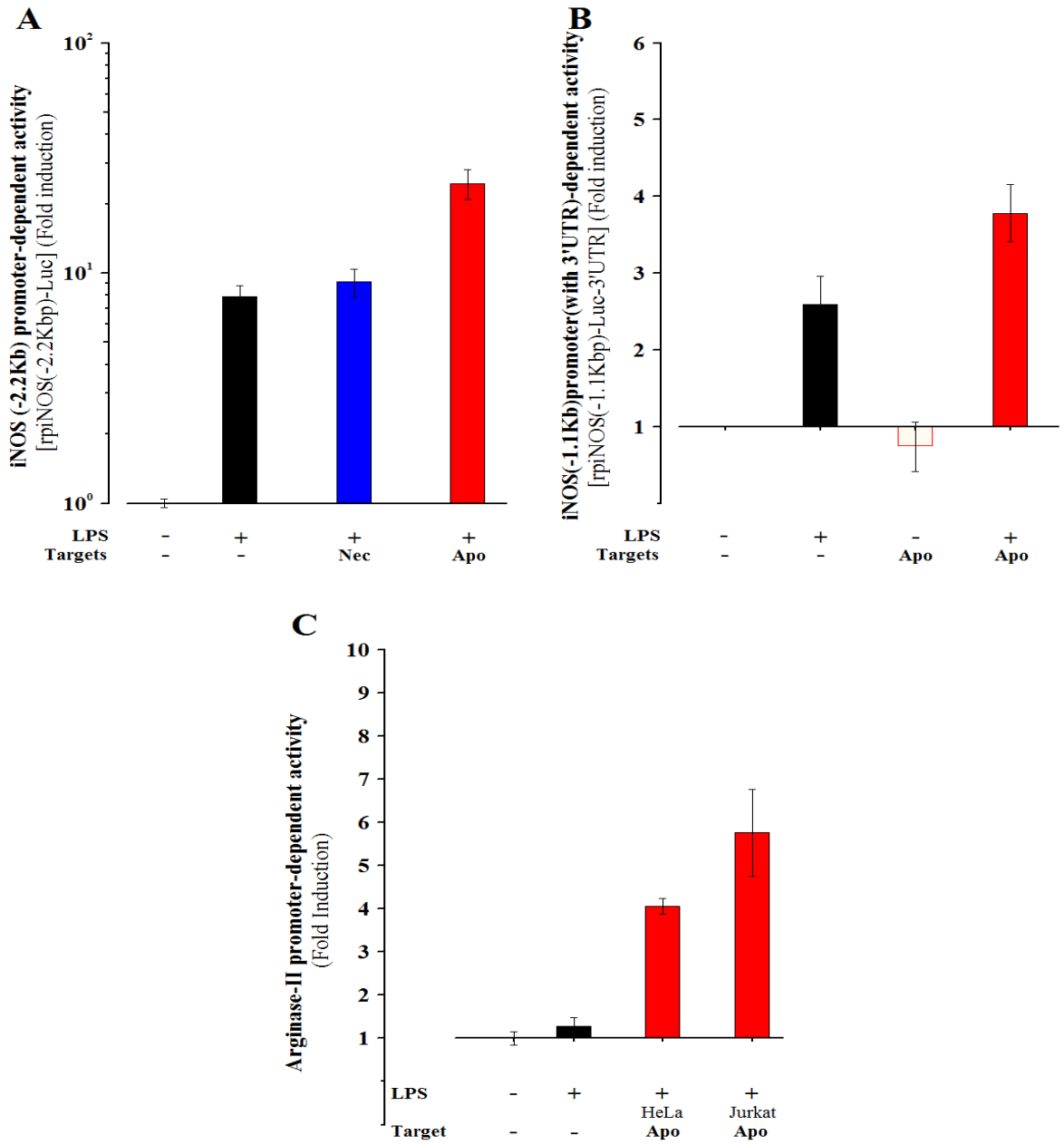


Figure VIII: **Apoptotic regulation of Arginase II, but not of iNOS, occurs at the level of transcription initiation.** RAW 264.7 macrophages were co-transfected transiently with rpiNOS(-2.2 kbp)-Luc (**A**), rpiNOS(-1.1 kbp)-Luc-3'UTR (**B**), or mpArgII(-1.8 kbp)-Luc (**C**) together with the Renilla luciferase normalization control vector (see Materials and Methods; Chapter III, section H), and split into multiple wells. After 24 hr., the transfected macrophages were incubated without or with apoptotic or necrotic targets (derived from Jurkat T cells; target to macrophage ratio of 6:1) and / or LPS (100 ng / ml) as indicated. Cell extracts were prepared after another 18 hr. and luciferase activities were measured. Data are presented as normalized luciferase activities in treated transfected macrophages relative to the uninduced transfected population ("fold-induction").

Together, these data implicate complex regulatory (including transcriptional and post-transcriptional) control exerted by apoptotic cells in their modulation of NO production in macrophages with which they interact.

vii. Analysis of CREB in apoptotic regulation of the Arginase II promoter.

The ability of apoptotic cells to positively regulate Arginase II promoter activity prompted us to identify putative regulatory sites within the Arginase II promoter targeted specifically by apoptotic cells (i.e. apoptotic cell regulatory elements [ACRE]). We generated luciferase reporter constructs with various Arginase II 5'UTR promoter fragments modified by truncation or site-directed mutagenesis, and tested them upon transient transfection into macrophages.

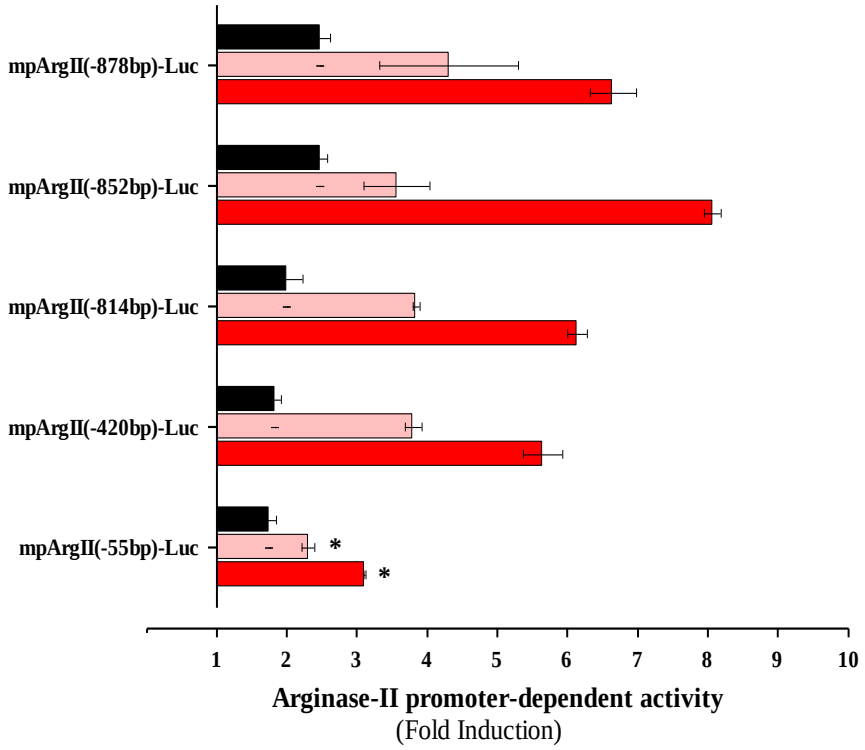
A truncated promoter construct consisting of only 420 bp of the 5' UTR (mpArgII[-420bp]-Luc) retained responsiveness to transcriptional up-regulation exerted by apoptotic cells (as measured by apoptotic induction of luciferase activity) comparable to that of the "full-length" (1.8 kbp of the 5' UTR) promoter construct (**Figure IX - A**). Responsiveness to LPS stimulation, and to the cooperative effects of apoptotic cells and LPS, also was retained. Further deletion (leaving only 55 bp of the 5' UTR) abrogated full promoter activity in response to apoptotic stimulation, as well as full responsiveness to LPS (**Figure IX - A**). These results implicate the Arginase II promoter region between -420 and -55 nucleotides as necessary for apoptotic responsiveness and, therefore, potentially harboring an ACRE.

A binding site for the cAMP Response Element-Binding Protein (CREB) has been identified previously in this vicinity (nucleotides TGACGTCA; ref.). We evaluated the role of that CREB site by site-directed mutagenesis. Functional loss of the CREB site from an otherwise fully functional promoter construct (mpArgII[-878bp]) resulted in loss of full apoptotic responsiveness, comparable to that observed in the very truncated (-55) construct (**Figure IX -**

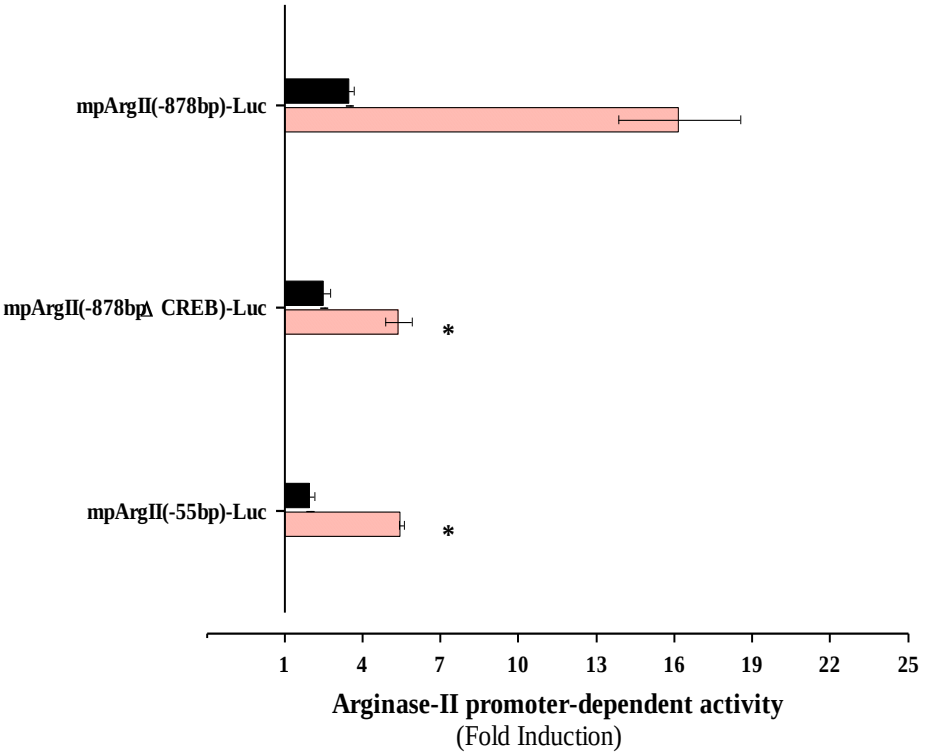
B). Complementarily, we addressed the possible involvement of CREB in the apoptotic regulation of the Arginase II promoter, by asking whether CREB is activated in response to apoptotic cells. Activation of CREB is marked by phosphorylation on Serine-133. Since an antibody specific for phospho-CREB (S-133) is not species-specific, we relied on multiparameter cytofluorimetric staining to evaluate CREB phosphorylation specifically in macrophages. No significant changes in the levels of macrophage CREB phosphorylation in the presence of apoptotic cells was observed (**Figure IX - C**).

Together, these data do not provide support for the view that CREB serves a specific role in the transcriptional regulation of Arginase II exerted by apoptotic cells. It is significant that the activation state of CREB is not altered significantly by apoptotic cells. It also is unclear that the defect in responsiveness associated with proximal deletion of the Arginase II promoter, or the specific CREB site mutation, represents a loss of a requisite ACRE as distinct from a general loss of promoter efficacy.

A


mpArgII(-878bp)-Luc
mpArgII(-852bp)-Luc
mpArgII(-814bp)-Luc
mpArgII(-420bp)-Luc
mpArgII(-55bp)-Luc
*
*
1
2
3
4
5
6
7
8
9
10
Arginase-II promoter-dependent activity
(Fold Induction)

B


mpArgII(-878bp)-Luc
mpArgII(-878bpΔ CREB)-Luc
mpArgII(-55bp)-Luc
*
*
1
4
7
10
13
16
19
22
25
Arginase-II promoter-dependent activity
(Fold Induction)

C

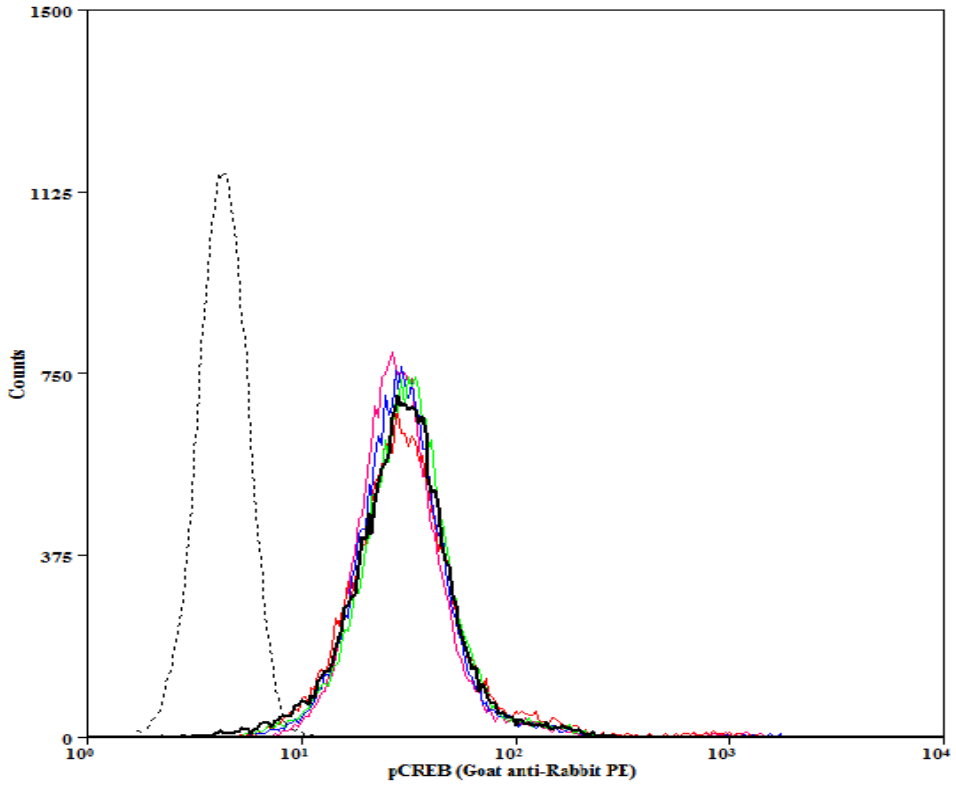


Figure IX: **CREB binding site of Arginase II promoter might act as an Apoptotic Cell Response Element (ACRE). A & B)** RAW 264.7 macrophages were co-transfected transiently with Arginase II mutant promoter constructs together with the Renilla luciferase normalization control vector, and split into multiple wells. After 24 hr., the transfected macrophages were incubated alone (normalization control), with LPS (100 ng / ml; black solid bars), with apoptotic targets only (derived from Jurkat T cells; target to macrophage ratio of 6:1; red hatched bars), or with apoptotic targets and LPS (red solid bars). Cell extracts were prepared after another 18 hr. and luciferase activities were measured. Data are presented as normalized luciferase activities in treated transfected macrophages relative to the uninduced transfected population ("fold-induction") [* - represents P-value < 0.05 (statistical significance); as measured by T-Test]. **C)** RAW 264.7 macrophages were incubated alone (black, solid line), or with apoptotic targets (@ 1 : 1; green - solid line, @ 2 : 1; blue - solid line, @ 4 : 1; red - solid line, @ 8 : 1; magenta - solid line, isotype control; black – dotted line) for 30 min. and analyzed cytofluorimetrically for intra-cellular levels of pCREB in RAW 264.7 macrophages alone (**C**).

C. DISCUSSION

Here, we have shown that apoptotic cells are able to attenuate LPS-induced NO production in macrophages. While this is consistent generally with the work of Brüne's group (14, 15, 24), our findings expand, and in part differ, in molecular details from their conclusions. We demonstrate that apoptotic cells attenuate NO production through complex transcriptional and post-transcriptional regulation of both iNOS and Arginase II. Significantly, we document that these regulatory events are mediated through direct cell-cell interactions between apoptotic targets cell and responding macrophages. While Barra *et al.* (24) identified the CREB binding site of the Arginase II promoter as crucial for transcriptional regulation mediated by soluble factors released from apoptotic cells, our data do not implicate CREB or the CREB site definitively in cell contact-dependent apoptotic regulation. This difference seen between the results from Barra *et al.* (24) and our experiment might predominantly be because of the timing of our assays. While we have assessed most of these experiments in a manner to address immediate-early signaling events by apoptotic cells, the experimental setup used by Brüne's group (14, 15, 24) has been an extended assay (>24 hr) looking at secondary events. Further analyses, including dissection of putative sites closely adjacent to the CREB site, are needed to clarify the relevant sites and transcription factors involved.

The well characterized phenomenon of "Innate Apoptotic Immunity" (IAI) involves apoptotic cell-mediated suppression of proinflammatory cytokine (e.g. IL-6, TNF-α) expression as well as up-regulation of anti-inflammatory cytokines (such as IL-10 and TGF-β). Particularly with respect to macrophages, apoptotic cells seem to trigger an M2-like (alternate activation) anti-inflammatory set of responses, which also has been described to ensue in the context of TH_2 cytokines (29-31). The regulation of nitric oxide species by apoptotic cells, leading to reduced

production of these inflammatory mediators, expands this view of apoptotic regulation and macrophage reprogramming.

Pathogens utilize a variety of mechanisms to subvert host immune responses. The critical role of NO species in the elimination of many pathogens likely makes this a particularly important target for pathogen subversion. The link between IAI and NO regulation adds to our understanding of the role of immune modulation effected by apoptotic cells, either host cells induced to undergo apoptosis directly by pathogens, or the mimicry of apoptotic cells by pathogens themselves, as a mechanism that can enhance pathogenesis (35-39).

Cited Literature for Chapter III

1. Surh CD, Sprent J. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature.* **372(6501)**:100-3.

2. Vaux DL. 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci U S A.* **90(3)**:786-9.

3. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest.* **83(3)**:865-75.

4. Duvall E, Wyllie AH, Morris RG. 1985. Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology.* **56(2)**:351-8.

5. Henson PM, Johnston RB Jr. 1987. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *J Clin Invest.* **79(3)**:669-74.

6. Cocco RE, Ucker DS. 2001. Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol Biol Cell.* **12(4)**:919-30.

7. Patel VA, Longacre A, Hsiao K, Fan H, Meng F, Mitchell JE, Rauch J, Ucker DS, Levine JS. 2006. Apoptotic cells, at all stages of the death process, trigger characteristic signaling events that are divergent from and dominant over those triggered by necrotic cells: Implications for the delayed clearance model of autoimmunity. *J Biol Chem.* **281(8)**:4663-70.

8. Gray M, Miles K, Salter D, Gray D, Savill J. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci* **104(35)**:14080-5.

9. Birge RB, Ucker DS. 2008. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ.* **15(7)**:1096-102.

10. Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, Tam PK, Savill J. 2008. Apoptotic cells protect mice against lipopolysaccharide-induced shock. *J Immunol.* **180(7)**:4978-85.

11. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. 1997. Immunosuppressive effects of apoptotic cells. *Nature.* **390(6658)**:350-1.

12. Cvetanovic M, Ucker DS. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol.* **172(2)**:880-9.

13. Cvetanovic M, Mitchell JE, Patel V, Avner BS, Su Y, van der Saag PT, Witte PL, Fiore S, Levine JS, Ucker DS. 2006. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. J Biol Chem. 281(29):20055-67.

14. Johann AM, von Knethen A, Lindemann D, Brüne B. 2006. Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-activated receptor-gamma and attenuates the oxidative burst. *Cell Death Differ.* **13(9)**:1533-40.

15. Johann AM, Barra V, Kuhn AM, Weigert A, von Knethen A, Brüne B. 2007. Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production. *FASEB J.* **21(11)**:2704-12.

16. Bogdan C. 2001. Nitric oxide and the immune response. *Nat Immunol.* **2(10)**:907-16.

17. Bogdan C, Röllinghoff M, Diefenbach A. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol*. **12(1)**:64-76.

18. Eckmann L, Laurent F, Langford TD, Hetsko ML, Smith JR, Kagnoff MF, Gillin FD. 2000. Nitric oxide production by human intestinal epithelial cells and competition for Arginine as potential determinants of host defense against the lumen-dwelling pathogen Giardia lamblia. *J Immunol.* **164(3)**:1478-87.

19. Bryk R, Griffin P, Nathan C. 2000. Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature.* **407(6801)**:211-5.

20. Stuehr DJ. 1999. Mammalian nitric oxide synthases. *Biochim Biophys Acta.* **1411(2-3)**:217-30.

21. Gotoh T, Mori M. 1999. Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J Cell Biol.* **144(3)**:427-34.

22. Gotoh T, Sonoki T, Nagasaki A, Terada K, Takiguchi M, Mori M. 1996. Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. *FEBS Lett.* **395(2-3)**:119-22.

23. Wu G, Morris SM Jr. 1998. Arginine metabolism: nitric oxide and beyond. *Biochem J.* **336 (Pt 1)**:1-17.

24. Barra V, Kuhn AM, von Knethen A, Weigert A, Brüne B. 2011. Apoptotic cell-derived factors induce arginase II expression in murine macrophages by activating ERK5/CREB. *Cell Mol Life Sci.* **68(10)**:1815-27.

25. Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM. 2008. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *J Immunol.* **181(5)**:3575-85.

26. Kim S, Elkon KB, Ma X. 2004. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity.* **21(5)**:643-53.

27. Ozaki T, Habara K, Matsui K, Kaibori M, Kwon AH, Ito S, Nishizawa M, Okumura T. 2010. Dexamethasone inhibits the induction of iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. *Shock.* **33(1)**:64-9.

28. Matsui K, Nishizawa M, Ozaki T, Kimura T, Hashimoto I, Yamada M, Kaibori M, Kamiyama Y, Ito S, Okumura T. 2008. Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes. *Hepatology.* **47(2)**:686-97.

29. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**:23-35.

30. Mantovani, A., Sozzani, S., Locati, M., Allavena, P., Sica, A. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. **23:**549-555.

31. Sica A, Mantovani A. 2012. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. **122(3)**:787-95.

32. Ravichandran KS. 2010. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med.* **207(9)**:1807-17.

33. Gardai SJ, Bratton DL, Ogden CA, Henson PM. 2006. Recognition ligands on apoptotic cells: a perspective. *J Leukoc Biol.* **79(5)**:896-903.

34. Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM. 2008. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *J Immunol.* **181(5)**:3575-85.

35. Gazzinelli RT, Oswald IP, Hieny S, James SL, Sher A. 1992. The microbicidal activity of interferon-gamma-treated macrophages against Trypanosoma cruzi involves an L-Arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. *Eur J Immunol.* **22(10)**:2501-6.

36. Zamboni DS, Rabinovitch M. 2003. Nitric oxide partially controls Coxiella burnetii phase II infection in mouse primary macrophages. *Infect Immun.* **71(3)**:1225-33.

37. Zamboni DS, Rabinovitch M. 2004. Phagocytosis of apoptotic cells increases the susceptibility of macrophages to infection with Coxiella burnetii phase II through down-modulation of nitric oxide production. *Infect Immun.* **72(4)**:2075-80.

38. Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, DosReis GA, Lopes MF. 2000. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature.* **403(6766)**:199-203.

39. Benoit M, Ghigo E, Capo C, Raoult D, Mege JL. 2008. The uptake of apoptotic cells drives Coxiella burnetii replication and macrophage polarization: a model for Q fever endocarditis. *PLoS Pathog.* **4(5)**.

CHAPTER IV

Apoptosis-Specific Externalization Of Glycolytic Enzymes

A. INTRODUCTION

Apoptosis is the primary mechanism by which cells die physiologically and is ongoing throughout life in multi-cellular organisms. During the process of apoptosis, cells externalize many determinants that mark them for efficient clearance by professional phagocytes like macrophages and dendritic cells. A surprising array of cellular components comprising autoantigens are also exposed on the surface of apoptotic cells (1). Apoptotic cells are cleared rapidly *in vivo*; that this phagocytic clearance occurs in the absence of inflammation has long been recognized (2). The clearance of apoptotic cells is a key homeostatic process, and represents a final step of the physiological cell death program (3, 4). The failure to promptly clear apoptotic cells even has been linked to chronic inflammation and autoimmunity characteristic of systemic lupus erythematosis (SLE), rheumatoid arthritis, and other pathologies including atherosclerosis (5-8). Independent of phagocytosis, specific apoptotic recognition elicits a profound repertoire of affirmative signaling and effector responses in macrophages and neighboring cells associated generally with the suppression of inflammation and immune responsiveness; we have termed this "innate apoptotic immunity" (IAI; refs. 4,9,10). It may be that the autoimmune pathologies observed in association with the persistence of apoptotic corpses are consequences of deficits in recognition-specific non-phagocytic responses (11).

The anti-inflammatory effects elicited upon the specific recognition of apoptotic cells (12) result primarily from the triggering of transcriptional responses (especially the repression of inflammatory cytokine gene expression) in cells (both professional and non-professional phagocytes) that interact with them (10,13). Subsequent responses, including the production of anti-inflammatory cytokines (e.g. TGF-β, IL-10), extend and may enhance the anti-inflammatory state (14). While numerous molecules have been implicated in the process of apoptotic cells

clearance (15), the critical determinants involved in the recognition of apoptotic cells and in the triggering of functional responses to them remain undefined. Our studies have demonstrated that these determinants are evolutionarily conserved and become membrane-exposed during the process of apoptotic cell death without a requirement for ensuing new gene expression (10, 13). Here, we add to this characterization and show that they are protease-sensitive. We note that determinants for apoptotic immune recognition and for the phagocytosis of apoptotic cells may not be identical; phosphatidylserine, for example, has been implicated functionally in engulfment (16) and not in innate apoptotic recognition (12, 13).

In an effort to understand the molecular basis for innate immune responses to apoptotic cells, we have taken a comprehensive approach toward the identification of the determinants of apoptotic recognition. We have employed two distinct proteomic approaches, based on two-dimensional electrophoretic separations and on "Isobaric tagging for relative and absolute quantification" (iTRAQ), and we have exploited apoptotic membrane vesicles as an enriched source of apoptotic recognition determinants. From our analyses, we identified a large number of over- and under-represented proteins in apoptotic vesicles. We categorized the identified molecules according to previously assigned molecular functions. Notably, these independent approaches both led to the novel observation that numerous components of the glycolysis pathway are enriched on the apoptotic cell surface. Through cytofluorimetric analyses, we have confirmed the apoptosis-associated surface exposure of glycolytic enzymes. Moreover, we have extended these findings to reveal that externalization of glycolytic enzymes is a common attribute of apoptotic cell death, occurring independently of the particular suicidal stimulus and in a variety of cells of different tissue types and species of origin.

Although we have not completed our evaluation of all externalized glycolytic enzyme molecules as determinants of innate apoptotic responses, it is clear that surface-exposed glycolytic enzyme molecules represent novel, early, and unambiguous markers (biomarkers) of the apoptotic cell death process. Surface exposure of glycolytic enzymes has been noted previously in a variety of enteric bacteria and pathogens, and is responsible for specific plasminogen binding (17-27). This striking commonality of glycolytic enzyme externalization raises the possibility that the exposure of glycolytic enzymes on microorganisms reflects a subversion of innate apoptotic immunity though apoptotic mimicry that facilitates commensalism or pathogenesis. In this light, it may be appropriate to reevaluate the significance of reported plasminogen binding activities of glycolytic enzymes.

B. RESULTS

i. Apoptotic suppressive determinants, enriched in membrane vesicles, are protease-sensitive.

We have demonstrated that apoptotic determinants for recognition and immune modulation are evolutionarily conserved and arise on the surface of cells during the process of apoptotic death without a requirement for ensuing new gene expression (10, 12). The ability of apoptotic cells to modulate inflammatory responses occurs primarily on the level of transcription (10) and can be assessed reliably with transcriptional reporters that disclose primary inflammatory responses (i.e. transcriptional promoters linked to the firefly luciferase gene and responsive to critical transcriptional activators involved in inflammatory responses, such as NFκB; refs. 10, 13). **Figure X** provides examples of the specific dose-dependent effects of apoptotic cells on a responsive cell line harboring such a transcriptional reporter. B2.1 is a highly responsive clone of stably transfected human HEK 293T reporter cells (13). The results presented show that apoptotic cells repress NFκB-dependent transcription whereas viable cells do not, and recapitulate cytokine responses of those cells (13). Importantly, apoptotic cells of distinct species and tissues of origin (S49 [in **Figure X-A**] is a murine T-lymphocyte cell line, and HeLa [in **Figure X-B**] is a human epithelial cell line) trigger equivalent responses.

Previously, we found that the phospholipid phosphatidylserine is neither a sufficient determinant (12) nor a necessary component (13) for specific apoptotic recognition and immune modulation. We wondered whether apoptotic immunosuppressive activity involves protein determinants and would therefore be susceptible to proteolytic digestion. Indeed, we found that when apoptotic cells were digested with trypsin, their immunomodulatory activity was lost (**Figure X-A**). Interestingly, we find that upon extended incubation following trypsin digestion,

apoptotic cells recover modulatory activity (data not shown). Fixation with formaldehyde after protease treatment precludes this recovery, although apoptotic immunomodulatory activity is stable to fixation with formaldehyde (13). We interpret these results to suggest that apoptotic immunomodulatory determinants are protease-sensitive molecules (or molecular complexes including essential protein components) that are resident in all cells prior to cell death, and that some fraction of the intracellular stores of the relevant molecules becomes surface-exposed (and susceptible to trypsin digestion) due to apoptosis-specific post-translational modification (PTM). For brevity, the acronym SUPER (surface-exposed during apoptotic cell death, ubiquitously-expressed, protease-sensitive, evolutionarily-conserved, and resident normally in viable cells) serves to emphasize these defining properties of apoptotic determinants for recognition and immune modulation.

We have shown that membrane vesicles prepared from apoptotic cells expose these determinants (13). In fact, assays for specific NFκB-dependent transcriptional suppression as a function of the dose of apoptotic vesicles reveal that apoptotic membrane vesicles are enriched in immunomodulatory determinants, relative to whole apoptotic cells and comparable vesicles prepared from viable cells. As shown in **Figure X-B**, titration of the immunomodulatory activity of intact apoptotic HeLa cells and apoptotic vesicles prepared from them demonstrate that vesicles have approximately 25% of the immunomodulatory activity of whole cells. (The low level at which whole cells contaminate vesicle preparations does not account for this activity.) Given that the surface area of whole cells is about 125-fold greater than that of these vesicles (we estimate the ratio of surface areas as $4 \pi r_c^2 / 4 \pi r_v^2 \cong 500$, where r_c, the radius of an intact HeLa cell, is approximately 9 μm and r_v, the average radius of a vesicle, is 0.8 μm) and that the membrane-associated protein content per nanomole of phospholipid of a vesicle is

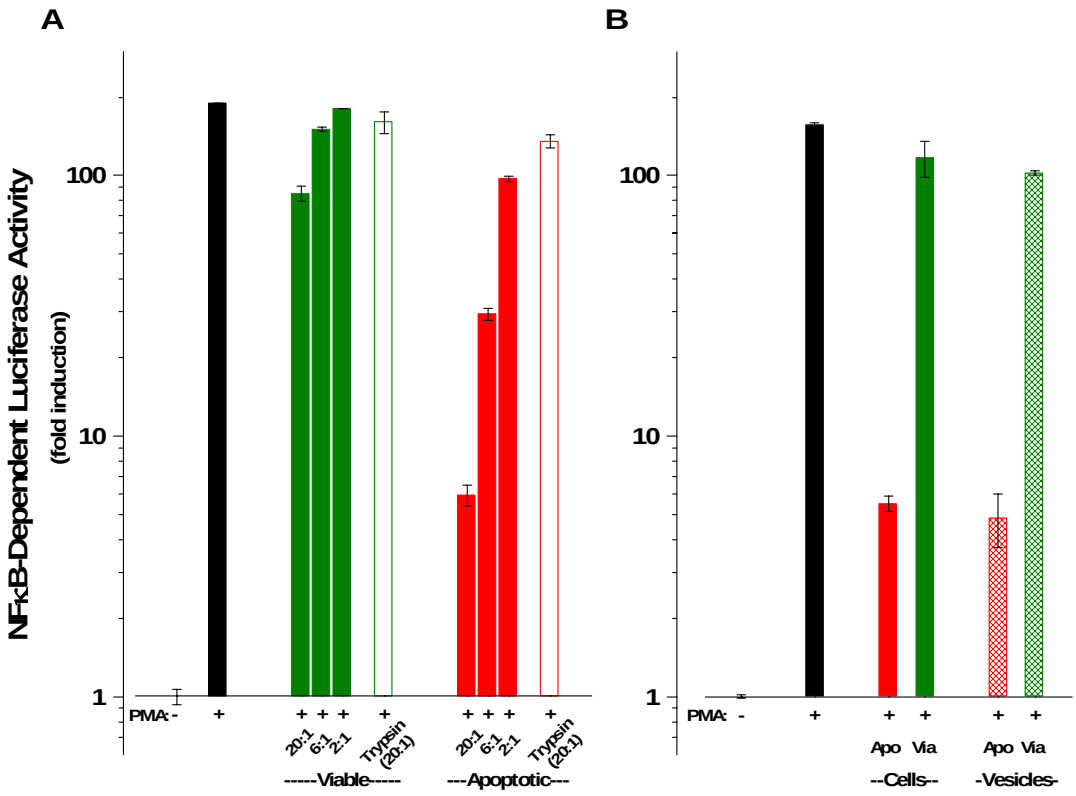


Figure X: Apoptotic suppressive determinants are protease-sensitive and enriched in membrane vesicles. Apoptotic suppression of NFκB-dependent transcription was assessed with respect to NFκB-dependent luciferase activity in B2.1 reporter cells (13). **A)** Suppression of phorbol myristate acetate (PMA; 1.25 ng / ml)-induced NFκB-dependent luciferase activity by graded numbers (indicated as the target : responder ratio) of viable (green) or apoptotic (actinomycin D, 200 ng / ml; red) S49 murine T cells (solid bars) and targets that had been digested with trypsin (0.1%, 15 min., 37°C; open bars) was assessed. Trypsin was removed from targets by extensive washing and residual activity was quenched by incubation in 10% serum-containing medium. **B)** Human epithelial (HeLa) cell targets were left untreated or treated with actinomycin D. The suppressive activity of cells (solid bars; target : responder ratio = 8:1) and vesicles (solid bars; target : responder ratio = 30:1), prepared by incubation of untreated and treated cells in Vesiculation Buffer (see Experimental Procedures), was assessed independently, as in **A**.

approximately 30-fold greater than that of a whole cell (4.70 μg protein / nmole phospholipid for intact cells versus 0.15 μg protein / nmole phospholipid for vesicles), we calculate the vesicle enrichment of immunomodulatory protein determinants to be approximately 1000-fold (i.e. 0.25 × 125 × 30 = 938).

ii. Proteomic analyses of apoptotic membrane vesicles reveal the membrane association of glycolytic enzyme molecules.

The implication that determinants of innate apoptotic immunity necessarily include essential protein components and that membrane vesicles provide an enriched source of those determinants led us to undertake a systematic analysis of the proteome of apoptotic plasma membrane vesicles. We performed a comparative analysis of membrane vesicle proteins prepared from apoptotic and viable cells by two-dimensional gel electrophoresis (2DE; **Figure XI**). After electrophoretic resolution, spots of proteins that were distinctly altered in abundance (over- or under-represented) were excised and subjected to tryptic digestion followed by nanoflow liquid chromatography (LC) and tandem mass spectrometry (MS / MS) analysis.

Interestingly, among the most prominent of the over-represented species we observed were proteins known to be involved as enzymes in the terminal stages of glucose metabolism, including pyruvate kinase (PK; highlighted in blue), α-enolase (EnoA; indicated in magenta), and Triosephosphate isomerase (TPI; marked in green). Each of these proteins was identified in multiple gel spots (with distinct pI's), and the abundance of each of the distinct spots was found to be increased among proteins from the apoptotic (relative to viable) membrane vesicles (**Figure XI**). The multiple spots may be indicative of isoforms of each of these proteins harboring PTM's. Notably, apoptosis-associated proteolysis does not appear generally to

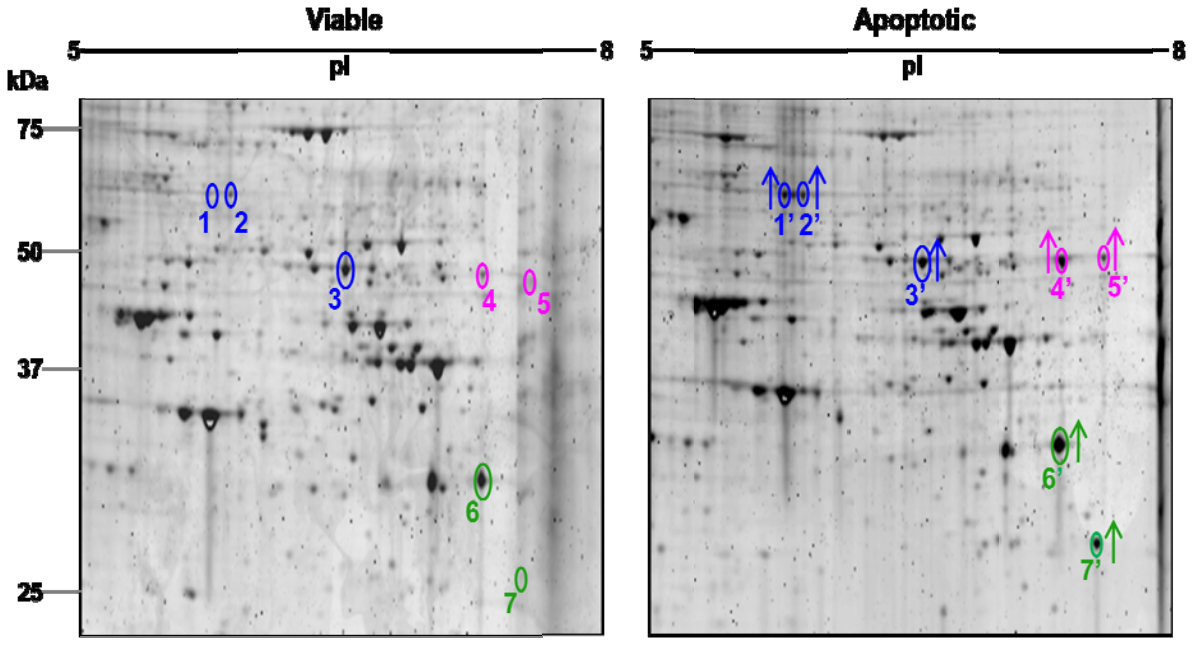


Figure XI: Analysis of membrane vesicle proteins by Two-Dimensional Gel Electrophoresis. Comparative analysis of membrane vesicle preparations from viable and apoptotic HeLa cells by 2-dimensional gel electrophoresis**.** Multiple gel spots corresponding to Pyruvate Kinase (blue), α-Enolase (magenta), and Triosephosphate Isomerase (green) are indicated. The increased abundance of protein spots among apoptotic (relative to viable) membrane vesicles is indicated by up-arrows. Details are listed below.

Pyruvate Kinase (predicted molecular weight = 58 kDa, pI = 7.96; ref. 28):

Spots	apparent M_r	apparent pI	ratio[1]
1, 1'	~58 kDa	~5.7	1.9
2, 2'	~58 kDa	~5.8	1.3
3, 3'	~48 kDa	~6.5	1.1

α-Enolase (predicted molecular weight = 47 kDa, pI = 7.01; ref. 29):

Spots	apparent M_r	apparent pI	ratio[1]
4, 4'	~48 kDa	~7.1	2.1
5, 5'	~48 kDa	~7.5	1.4

Triosephosphate Isomerase (predicted molecular weight = 31 kDa, pI = 5.65; ref. 30):

Spots	apparent M_r	apparent pI	ratio[1]
6, 6'	~31 kDa	~7.1	1.2
7, 7'	~26 kDa	~7.3	3.6

1. The ratio of normalized densitometric intensities of a spot in the apoptotic sample to the comparable viable spot was determined.

underlie these modifications; only in the case of TPI (spot 7') is an apoptosis-enriched isoform of distinctly lower apparent molecular weight (presumably due to proteolytic cleavage) evident.

Independently, we undertook a complete, quantitative proteomic characterization of apoptotic and viable membrane vesicles, employing iTRAQ technology, which permits the analysis of less abundant species. Apoptotic and viable vesicle extracts were denatured, alkylated, and labeled with isobaric iTRAQ reagent tags (see Experimental Procedures). Duplicate viable vesicle peptides were labeled with iTRAQ reagent tags 114 and 115 (differential tags of 114 and 115 Daltons, resp.); duplicate apoptotic membrane peptides were labeled with iTRAQ tags 116 and 117. Samples then were digested with trypsin, and the labeled peptides were mixed in even ratios and quantified by LC / MS / MS. The relative abundance (enrichment or depletion) of distinct peptides in apoptotic and viable samples was determined by replicate comparisons between the labeled samples. From these data, we identified a total of 564 proteins (graphic representation of this distribution, highlighting selected proteins, is shown in **Figure XII**). **Tables IV** and **V** list 56 over- represented and 105 under-represented proteins, respectively, that varied by at least 20% between apoptotic and viable membrane vesicle preparations at an FDR of less than 1%. We have previously established, with known standards, that the analytical coefficient of variance is less than 10%; therefore, changes between apoptotic and viable membrane proteins greater than 20% are reliably significant. Notably, the over-represented population included proteins from all intracellular locales (see below).

Table IV: Over-represented apoptotic membrane-associated proteins. Proteins identified by iTRAQ analysis and found to be over- represented among apoptotic membrane vesicles by at least 20% at a confidence level greater than 95% are listed.

	Identified Proteins	Accession ID	Ratio	T-test
1	Cytochrome c	CYC_HUMAN	2.71	0.01
2	Stathmin	STMN1_HUMAN	2.07	0.00
3	Macrophage migration inhibitory factor	MIF_HUMAN	2.00	0.01
4	Acyl-CoA-binding protein	ACBP_HUMAN	1.94	0.05
5	SUMO-conjugating enzyme UBC9	UBC9_HUMAN	1.81	0.02
6	Profilin-1	PROF1_HUMAN	1.80	0.01
7	T-complex protein 1 subunit epsilon	TCPE_HUMAN	1.74	0.01
8	Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	1.74	0.00
9	Calpastatin	ICAL_HUMAN	1.68	0.03
10	Glutathione S-transferase omega-1	GSTO1_HUMAN	1.68	0.00
11	Aldose reductase	ALDR_HUMAN	1.68	0.02
12	Heat shock protein HSP 90-alpha	HS90A_HUMAN	1.68	0.05
13	Transgelin-2	TAGL2_HUMAN	1.68	0.05
14	Plastin-3	PLST_HUMAN	1.62	0.01
15	Nucleoside diphosphate kinase B	NDKB_HUMAN	1.62	0.03
16	14-3-3 protein beta/alpha	1433B_HUMAN	1.57	0.02
17	Thioredoxin	THIO_HUMAN	1.56	0.03
18	Myristoylated alanine-rich C-kinase substrate	MARCS_HUMAN	1.52	0.05
19	Importin-7	IPO7_HUMAN	1.52	0.01
20	Glutathione S-transferase P	GSTP1_HUMAN	1.51	0.02
21	Alpha-actinin-1	ACTN1_HUMAN	1.46	0.01
22	Spectrin beta chain, brain 1	SPTB2_HUMAN	1.46	0.01
23	Cytosolic phospholipase A2	PA24A_HUMAN	1.46	0.01
24	ATP synthase subunit d, mitochondrial	ATP5H_HUMAN	1.46	0.01
25	Nuclear migration protein nudC	NUDC_HUMAN	1.46	0.01
26	Alpha-enolase	ENOA_HUMAN	1.46	0.03
27	Fructose-bisphosphate aldolase A	ALDOA_HUMAN	1.46	0.03
28	T-complex protein 1 subunit theta	TCPQ_HUMAN	1.46	0.03
29	Phosphoglycerate kinase 1	PGK1_HUMAN	1.46	0.03
30	Heat shock cognate 71 kDa protein	HSP7C_HUMAN	1.37	0.01
31	Actin-related protein 2/3 complex subunit 3	ARPC3_HUMAN	1.37	0.01
32	Nascent polypeptide-associated complex subunit alpha	NACA_HUMAN	1.37	0.01
33	Superoxide dismutase [Cu-Zn]	SODC_HUMAN	1.36	0.05
34	Proteasome subunit beta type-3	PSB3_HUMAN	1.36	0.05
35	Triosephosphate isomerase	TPIS_HUMAN	1.36	0.05
36	Heat shock protein HSP 90-beta	HS90B_HUMAN	1.36	0.05
37	14-3-3 protein epsilon	1433E_HUMAN	1.36	0.05
38	Tyrosyl-tRNA synthetase, cytoplasmic	SYYC_HUMAN	1.36	0.05
39	Ubiquitin-like modifier-activating enzyme 1	UBA1_HUMAN	1.36	0.05
40	Ezrin	EZRI_HUMAN	1.32	0.03

41	Cofilin-1	COF1_HUMAN	1.32	0.03
42	Transgelin	TAGL_HUMAN	1.32	0.03
43	Proteasome subunit alpha type-5	PSA5_HUMAN	1.32	0.00
44	T-complex protein 1 subunit gamma	TCPG_HUMAN	1.32	0.04
45	L-lactate dehydrogenase A chain	LDHA_HUMAN	1.32	0.04
46	Ras-related protein Rab-1B	RAB1B_HUMAN	1.28	0.02
47	Actin-related protein 2/3 complex subunit 2	ARPC2_HUMAN	1.27	0.02
48	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	ERF3A_HUMAN	1.27	0.02
49	Fatty acid synthase	FAS_HUMAN	1.23	0.00
50	Protein SET	SET_HUMAN	1.19	0.04
51	Phosphoglycerate mutase 1	PGAM1_HUMAN	1.19	0.04
52	Elongation factor 1-gamma	EF1G_HUMAN	1.19	0.04
53	Proliferation-associated protein 2G4	PA2G4_HUMAN	1.19	0.03
54	Elongation factor 2	EF2_HUMAN	1.19	0.03
55	Acidic leucine-rich nuclear phosphoprotein 32 family member A	AN32A_HUMAN	1.19	0.03
56	Importin subunit beta-1	IMB1_HUMAN	1.19	0.03

Table V: Under-represented apoptotic membrane-associated proteins. Proteins identified by iTRAQ analysis and found to be under- represented among apoptotic membrane vesicles by at least 20% at a confidence level greater than 95% are listed.

	Identified Proteins	Accession ID	Ratio	T-test
1	Histone H2A type 2-B	H2A2B_HUMAN	0.28	0.01
2	Large neutral amino acids transporter small subunit 1	LAT1_HUMAN	0.29	0.03
3	Dipeptidyl peptidase 1	CATC_HUMAN	0.31	0.02
4	Small nuclear ribonucleoprotein-associated proteins B and B'	RSMB_HUMAN	0.32	0.03
5	Alkaline phosphatase, tissue-nonspecific isozyme	PPBT_HUMAN	0.42	0.03
6	Asparagine synthetase [glutamine-hydrolyzing]	ASNS_HUMAN	0.42	0.02
7	60S ribosomal protein L19	RL19_HUMAN	0.45	0.03
8	Annexin A5	ANXA5_HUMAN	0.45	0.02
9	Annexin A6	ANXA6_HUMAN	0.47	0.02
10	60S ribosomal protein L24	RL24_HUMAN	0.50	0.02
11	Cytochrome b-c1 complex subunit 7	QCR7_HUMAN	0.50	0.02
12	40S ribosomal protein S17	RS17_HUMAN	0.52	0.00
13	Keratin, type I cytoskeletal 10	K1C10_HUMAN	0.54	0.04
14	60S ribosomal protein L34	RL34_HUMAN	0.56	0.04
15	40S ribosomal protein S2	RS2_HUMAN	0.57	0.03
16	Annexin A2	ANXA2_HUMAN	0.57	0.03
17	Heat shock protein beta-1	HSPB1_HUMAN	0.57	0.00
18	40S ribosomal protein S23	RS23_HUMAN	0.57	0.01
19	Protein S100-A4	S10A4_HUMAN	0.57	0.03
20	60S ribosomal protein L32	RL32_HUMAN	0.58	0.01
21	Programmed cell death protein 6	PDCD6_HUMAN	0.59	0.03
22	60S ribosomal protein L36a	RL36A_HUMAN	0.59	0.00
23	DNA replication licensing factor MCM5	MCM5_HUMAN	0.60	0.02
24	60S ribosomal protein L9	RL9_HUMAN	0.60	0.02
25	Protein transport protein Sec31A	SC31A_HUMAN	0.60	0.02
26	Tubulin beta-4 chain	TBB4_HUMAN	0.60	0.05
27	Protein S100-A10	S10AA_HUMAN	0.61	0.03
28	Small nuclear ribonucleoprotein Sm D3	SMD3_HUMAN	0.61	0.03
29	Polypeptide N-acetylgalactosaminyltransferase 2	GALT2_HUMAN	0.62	0.00
30	Non-POU domain-containing octamer-binding protein	NONO_HUMAN	0.62	0.01
31	60S ribosomal protein L14	RL14_HUMAN	0.62	0.04
32	Endothelin-converting enzyme 1	ECE1_HUMAN	0.62	0.04
33	Keratin, type II cytoskeletal 8	K2C8_HUMAN	0.64	0.04
34	Interleukin enhancer-binding factor 3	ILF3_HUMAN	0.64	0.04
35	Histone H2B type 1-J	H2B1J_HUMAN	0.64	0.01
36	DNA replication licensing factor MCM4	MCM4_HUMAN	0.64	0.02
37	Histone H1.5	H15_HUMAN	0.64	0.02
38	40S ribosomal protein S18	RS18_HUMAN	0.66	0.04
39	Annexin A1	ANXA1_HUMAN	0.66	0.04
40	60S ribosomal protein L26	RL26_HUMAN	0.66	0.00
41	Lysosome-associated membrane glycoprotein 1	LAMP1_HUMAN	0.66	0.01
42	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	ECH1_HUMAN	0.66	0.01

43	Splicing factor, Arginine/serine-rich 6	SFRS6_HUMAN	0.66	0.01
44	Mitochondrial carrier homolog 2	MTCH2_HUMAN	0.66	0.01
45	Probable ATP-dependent RNA helicase DDX17	DDX17_HUMAN	0.66	0.01
46	40S ribosomal protein S16	RS16_HUMAN	0.68	0.05
47	40S ribosomal protein S3a	RS3A_HUMAN	0.68	0.05
48	Clathrin light chain A	CLCA_HUMAN	0.68	0.05
49	EGF-like repeat and discoidin I-like domain-containing protein 3	EDIL3_HUMAN	0.68	0.05
50	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GBB1_HUMAN	0.68	0.05
51	40S ribosomal protein S9	RS9_HUMAN	0.68	0.01
52	N-acetylgalactosaminyltransferase 7	GALT7_HUMAN	0.68	0.01
53	40S ribosomal protein S15a	RS15A_HUMAN	0.68	0.01
54	Aspartyl-tRNA synthetase, cytoplasmic	SYDC_HUMAN	0.68	0.01
55	X-ray repair cross-complementing protein 5	XRCC5_HUMAN	0.68	0.03
56	Interleukin enhancer-binding factor 2	ILF2_HUMAN	0.68	0.03
57	Protein ERGIC-53	LMAN1_HUMAN	0.69	0.05
58	60S ribosomal protein L23	RL23_HUMAN	0.71	0.05
59	Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_HUMAN	0.71	0.00
60	Peroxiredoxin-2	PRDX2_HUMAN	0.71	0.00
61	60S ribosomal protein L13	RL13_HUMAN	0.71	0.02
62	Reticulon-4	RTN4_HUMAN	0.71	0.02
63	60S ribosomal protein L18	RL18_HUMAN	0.71	0.02
64	60S ribosomal protein L15	RL15_HUMAN	0.71	0.03
65	Myosin light polypeptide 6	MYL6_HUMAN	0.73	0.02
66	60S ribosomal protein L6	RL6_HUMAN	0.73	0.02
67	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRPC_HUMAN	0.73	0.02
68	60S ribosomal protein L35	RL35_HUMAN	0.73	0.01
69	Calumenin	CALU_HUMAN	0.73	0.01
70	DNA topoisomerase 2-alpha	TOP2A_HUMAN	0.73	0.01
71	Heterogeneous nuclear ribonucleoprotein G	HNRPG_HUMAN	0.73	0.01
72	60S ribosomal protein L3	RL3_HUMAN	0.73	0.05
73	ADP/ATP translocase 2	ADT2_HUMAN	0.73	0.05
74	Tubulin beta chain	TBB5_HUMAN	0.76	0.00
75	DNA-dependent protein kinase catalytic subunit	PRKDC_HUMAN	0.76	0.03
76	40S ribosomal protein S10	RS10_HUMAN	0.76	0.03
77	Vigilin	VIGLN_HUMAN	0.76	0.03
78	60S ribosomal protein L4	RL4_HUMAN	0.78	0.02
79	40S ribosomal protein S19	RS19_HUMAN	0.78	0.02
80	60S ribosomal protein L10	RL10_HUMAN	0.78	0.02
81	60S ribosomal protein L21	RL21_HUMAN	0.78	0.02
82	60S ribosomal protein L7a	RL7A_HUMAN	0.78	0.02
83	Heterogeneous nuclear ribonucleoprotein D0	HNRPD_HUMAN	0.78	0.02
84	Heterogeneous nuclear ribonucleoprotein R	HNRPR_HUMAN	0.78	0.02
85	40S ribosomal protein S4, X isoform	RS4X_HUMAN	0.81	0.05
86	60S ribosomal protein L13a	RL13A_HUMAN	0.81	0.05
87	60S ribosomal protein L23a	RL23A_HUMAN	0.81	0.05
88	60S ribosomal protein L18a	RL18A_HUMAN	0.81	0.05
89	Heterogeneous nuclear ribonucleoprotein L	HNRPL_HUMAN	0.81	0.05

90	Serpin H1	SERPH_HUMAN	0.81	0.05
91	Thymidylate kinase	KTHY_HUMAN	0.81	0.05
92	Trifunctional enzyme subunit alpha, mitochondrial	ECHA_HUMAN	0.81	0.05
93	ATP-dependent RNA helicase A	DHX9_HUMAN	0.84	0.04
94	26S protease regulatory subunit 4	PRS4_HUMAN	0.84	0.04
95	60S acidic ribosomal protein P0	RLA0_HUMAN	0.84	0.04
96	Actin-related protein 2	ARP2_HUMAN	0.84	0.04
97	Cytochrome b5	CYB5_HUMAN	0.84	0.04
98	Filamin-A	FLNA_HUMAN	0.84	0.04
99	Nucleolin	NUCL_HUMAN	0.84	0.04
100	Signal recognition particle 9 kDa protein	SRP09_HUMAN	0.84	0.04
101	60S ribosomal protein L7	RL7_HUMAN	0.84	0.03
102	40S ribosomal protein S8	RS8_HUMAN	0.84	0.03
103	60S ribosomal protein L35a	RL35A_HUMAN	0.84	0.03
104	Histone H2A.V	H2AV_HUMAN	0.84	0.03
105	Splicing factor, proline- and glutamine-rich	SFPQ_HUMAN	0.84	0.03

With the vesiculation protocol, as many as 8% of the cells in an otherwise untreated control ("viable") cell culture become apoptotic. Thus, we would expect that an idealized candidate SUPER molecule (i.e. a protein that is not associated with viable cell membranes and that is associated specifically with apoptotic cell membranes [and that actually is over-represented *x*-fold among apoptotic cell membrane proteins]) would appear to be enriched among apoptotic vesicle proteins no more than approximately 12-fold (i.e. x / .08 x). Although the membrane vesicles that we prepared have no ultrastructure and are depleted for non membrane-associated molecules, the non surface-exposed, intravesicular contents of viable vesicles may contribute further to a diminution of the apparent enrichment of apoptosis-specific membrane proteins in apoptotic vesicle preparations. Indeed, the maximum enrichment of a protein that we observed among apoptotic vesicles (**Figure XII**) was less than 3-fold.

iii. Inventory of apoptotic membrane proteins.

We categorized the identified molecules according to previous descriptions of their major molecular functions (**Figure XIII**; see below). Molecules characterized for their catalytic activities involved in metabolism constituted the largest group of over-represented proteins present in apoptotic cell vesicles (15/56). Among these, glycolytic enzymes (including aldolase, TPI, phosphoglycerate kinase [PGK], EnoA, and PK) were highly represented, consistent with our 2DE analysis. An example of this enrichment, observed by iTRAQ analysis, is presented for EnoA peptides (**Figure XIV**). Indeed, it is striking that almost all members of the glycolytic pathway are enriched among apoptotic cell membranes (see **Figure XII insert**). Other major classes among the over-represented proteins present in apoptotic vesicles are structural (including cytoskeletal) molecules (14/56), those (12/56) involved in macromolecular synthesis (especially translation) and processing (including proteases), and molecular chaperones (7/56).

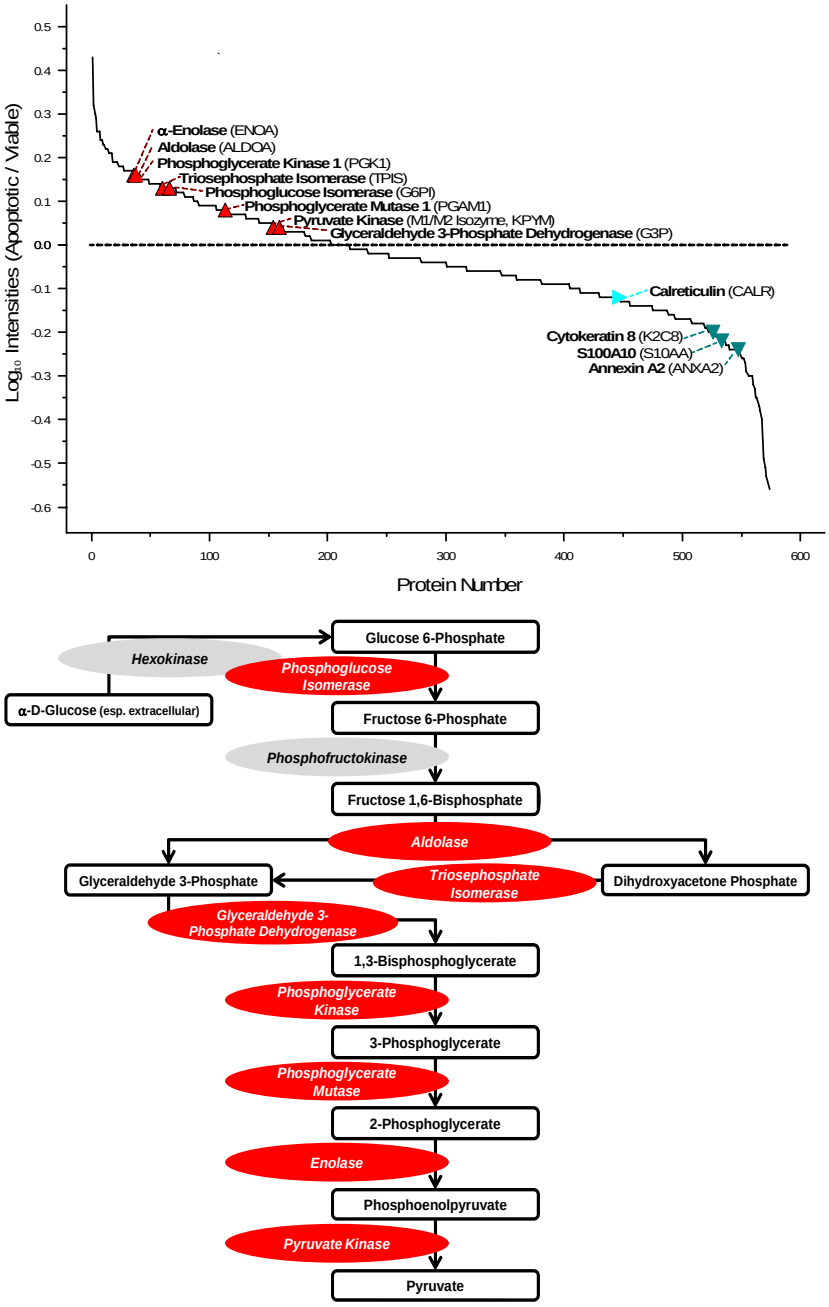


Figure XII: Relative abundance of apoptotic and viable membrane vesicle proteins determined by iTRAQ analysis. Compilation of the relative abundance of membrane vesicle proteins from apoptotic versus viable preparations ("Apoptotic /' Viable"), determined by iTRAQ analysis. The relative abundance of selected proteins is indicated. Proteins involved as enzymes in the glycolysis pathway (marked in red; see inset) are found to be over-represented. Other molecules identified as "plasminogen receptors" (see text; marked in green) are under-represented.

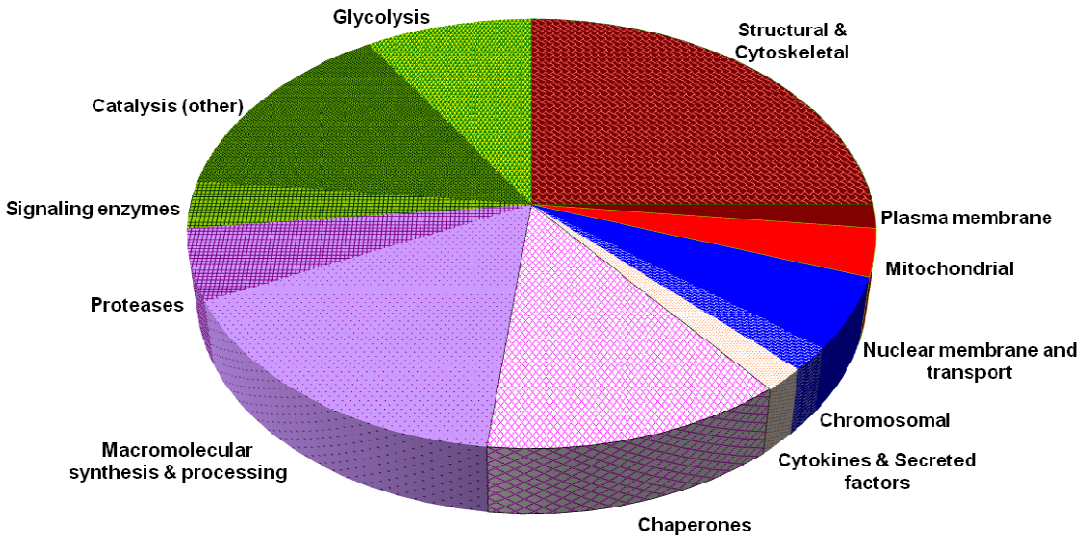


Figure XIII: Functional categorization of over-represented proteins from apoptotic membrane vesicles. Functional categorization (gene ontology) of proteins found to be enriched among apoptotic membrane vesicles by iTRAQ analysis (see Table IV). The two largest groups of proteins (26.8% each) are those characterized as membrane proteins and structural and cytoskeletal elements (marked in brown), and as catalytic proteins (marked in green). Proteins associated with the glycolytic pathway are the largest coherent cohort (9.0%) within either group. The next largest groups are proteins associated with macromolecular synthesis (especially translation) and processing and proteases (21.4%; shaded in violet), and molecular chaperones (12.5%; cross- hatched magenta area).

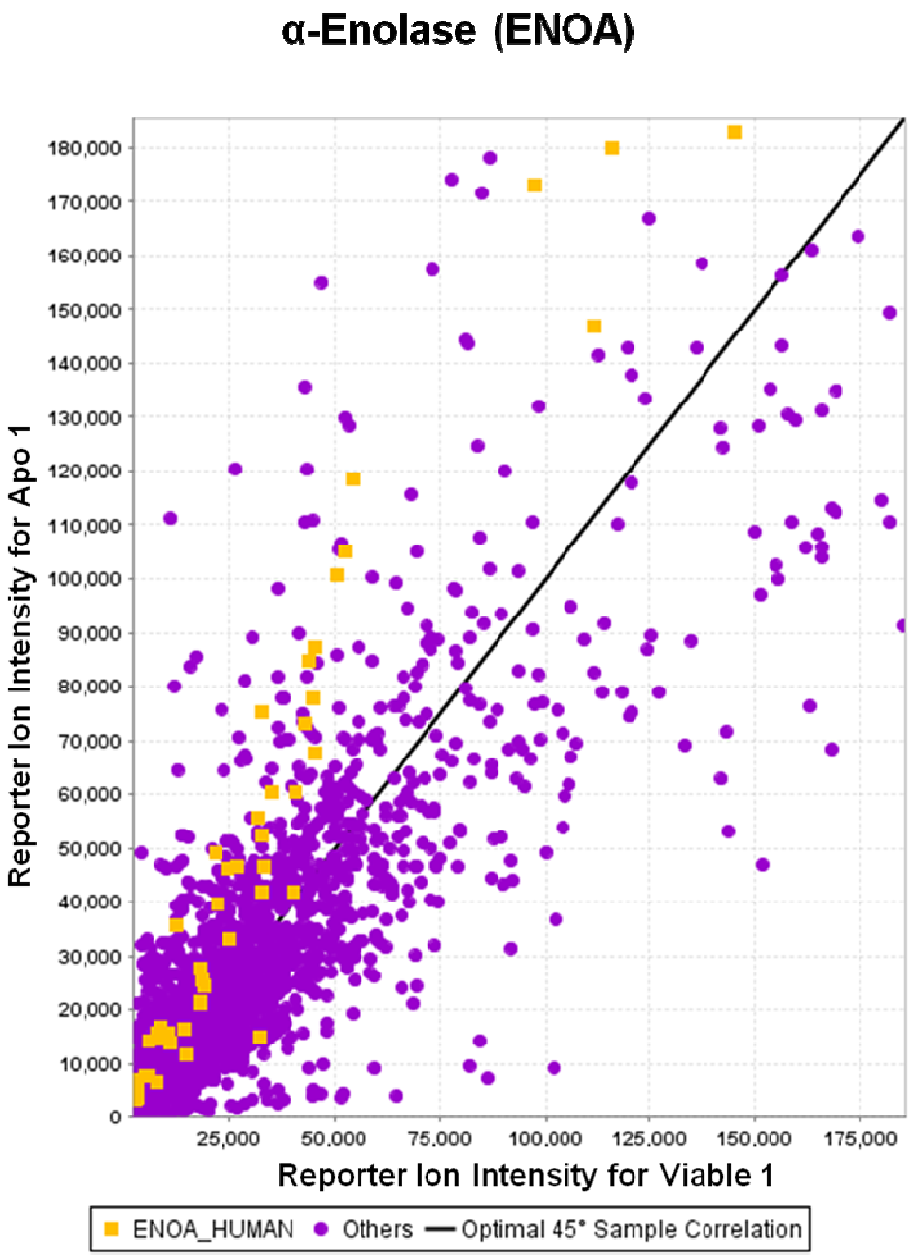


Figure XIV: Detail of EnoA data from iTRAQ analysis. Over-representation of α-enolase ascertained from the consistent iTRAQ ratios obtained from all related precursor ions. Plot of reporter ion 114 (Viable 1) versus 116 (Apoptotic 1) intensities for all peptides (purple dots) identified in iTRAQ experiment. Yellow dots represent the iTRAQ signals derived from spectra matched to α-enolase (A1).

Importantly, some of the proteins identified here as enriched among apoptotic vesicles (for example, the actin-associated proteins cofilin and ARP 2/3 [ref 31], numerous heat shock proteins and other chaperones associated with stress [refs 32-34]) fulfill expectations derived from other studies. Similarly, the mitochondrial protein cytochrome *c* is known to be released from mitochondria during apoptotic cell death (35, 36). It is significant that not all of the vesicle-enriched molecules are membrane-exposed; in particular, we do not detect surface-exposed cytochrome *c* (data not shown).

On the other hand, some proteins expected to be enriched in apoptotic vesicles were not identified in this array. Actin was expected to be among the apoptotic vesicle proteins (37-39), along with actin-associated and other structural molecules. Histones also were expected to be enriched among apoptotic vesicle proteins, based on previous studies documenting their unique presence on the apoptotic cell surface (1). The observation that vesicles have immunomodulatory activity - like intact apoptotic cells - but lack externalized histones - unlike intact apoptotic cells - allows us to exclude histones definitively as apoptotic recognition determinants. Annexin A1, which has been suggested previously to be involved in apoptotic recognition (40), was relatively depleted in our apoptotic vesicle preparation (only ~70% representation; **Table IV**) relative to the viable vesicle preparation. Other proteomic studies also have not found annexin A1 to be present generally among apoptotic membrane proteins (see, for example, ref. 41).

It is most striking that both of the independent proteomic analyses we employed identified the preferential membrane vesicle-association of glycolytic enzymes with apoptotic cell death. Since the issue of surface exposure is a defining criterion with regard to apoptotic determinants

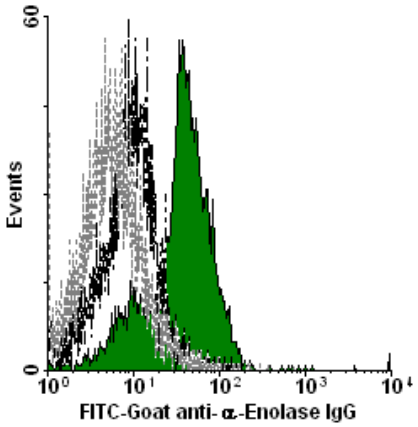
for recognition and immune modulation (SUPER) candidates, we sought to evaluate whether glycolytic enzyme molecules are present on the apoptotic cell surface.

iv. Cytofluorimetric confirmation of apoptotic externalization of glycolytic enzyme molecules.

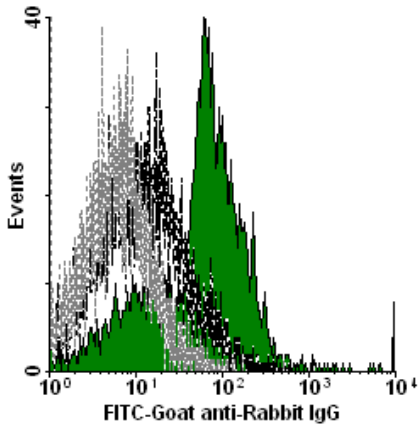
We examined independently the preferential enrichment of glycolytic enzyme molecules among apoptotic membrane proteins and tested specifically whether those molecules are exposed on the apoptotic cell surface. We analyzed apoptotic cells for the externalization of three glycolytic enzymes (EnoA, GAPDH, and TPI) by immunofluorescence and cytofluorimetric analyses. We examined a variety of cell types and cell lines (in addition to the HeLa cells used to prepare the membrane vesicles subjected to our protein analyses, we analyzed human and murine epithelial, lymphoid, and myeloid cell lines and primary cells induced to undergo apoptotic death with a variety of suicidal stimuli) for cell death-associated externalization of these proteins.

By immunofluorescence staining analysis, we found that EnoA is displayed generally on the surface of apoptotic, but not viable, cells. An example of these analyses (with primary murine splenocytes) is presented in **Figure XV-A**. Completely analogous staining patterns were observed for the two other glycolytic enzymes that were analyzed, GAPDH (**Figure XV-B**) and TPI (**Figure XV-C**). Thus, glycolytic enzyme molecules not only are enriched among apoptotic membrane proteins, but are exposed specifically on the apoptotic cell surface. Externalized glycolytic enzyme molecules fulfill the critical criteria for apoptotic determinants of recognition and immune modulation: they are evolutionarily conserved proteins that are resident in all cells ubiquitously, and, while unexposed on the surface of viable cells, are membrane-exposed on apoptotic cells.

A. α-Enolase



B. Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)



C. Triosephosphate Isomerase (TPI)

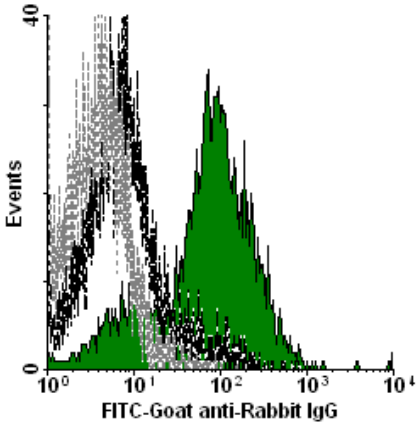
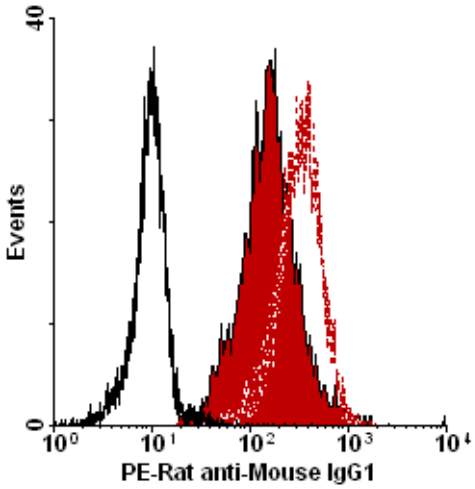


Figure XV: Cytofluorimetric analysis of externalization of glycolytic enzyme molecules. Murine splenocytes that had undergone apoptosis spontaneously in culture (12 hr.) and freshly isolated, viable splenocytes were analyzed cytofluorimetrically following staining with **(A)** FITC - conjugated polyclonal Goat anti- α-Enolase peptide IgG (Santa Cruz Biotechnology), **(B)** polyclonal Rabbit anti-GAPDH IgG (Abcam) and secondary FITC - conjugated Goat anti-Rabbit IgG (Santa Cruz Biotechnology), and **(C)** polyclonal Rabbit anti-Triosephosphate Isomerase IgG (Novus Biologicals) and secondary FITC - conjugated Goat anti-Rabbit IgG (Santa Cruz Biotechnology). Profiles shown are for apoptotic (solid green histogram) and viable (black, dashed line) cells stained with the specific FITC - conjugated reagents, and for apoptotic cells stained with the secondary antibody alone (gray, dotted line; the profile of viable cells is identical). Cells that had lost membrane integrity (PI^+, reduced forward- and expanded side-angle light scatter) were excluded from these analyses by electronic gating.

We evaluated the extent of glycolytic enzyme molecule externalization by comparing immunofluorescence staining of membrane-intact and permeabilized apoptotic cells. Examples of these analyses are presented in **Figure XVI**. Permeabilized viable and apoptotic cells stain identically and homogeneously for EnoA (**Figure XVI-A**), GAPDH (**Figure XVI-B**), and TPI (data not shown). The extent of externalization of these molecules ranged from 10 – 50% of the total cellular protein for each species during apoptotic cell death (by comparison of mean fluorescence intensities; see **Figure XVI** legend). Importantly, these data indicate that the observed apoptosis-specific externalization of glycolytic enzyme molecules is distinct from a general accessibility to intracellular molecules that would result from plasma membrane compromise. In contrast, the detection of glycolytic enzyme molecules on necrotic cells is not distinguishable from plasma membrane compromise (see next). Our data also demonstrate that there is no net elevation of cellular glycolytic enzyme concentration with apoptosis, but rather a preferential externalization of those molecules.

We explored the kinetics of the appearance of these glycolytic enzyme molecules by examining the earliest apoptotic cells (annexin V^+ 7-AAD^-) following brief treatment with staurosporine, a potent inducer of apoptosis (**Figure XVII**). Externalized EnoA, GAPDH, and TPI could be found already externalized on these early apoptotic cells. Interestingly, the extent of exposure ranged as high as the levels found on later apoptotic cells. By comparison, neither autophagic or necrotic cells expose glycolytic enzyme molecules, consistent with the notion that the externalization of glycolytic enzyme molecules is a specific attribute of the apoptotic cell death process (**Figure XX**). Furthermore, we have found that the process of glycolytic enzyme externalization, like apoptosis itself, is caspase-dependent (**Figure XXI**).

A. α-Enolase



B. GAPDH

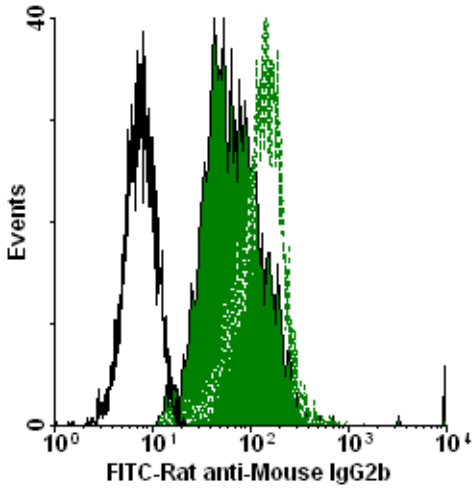
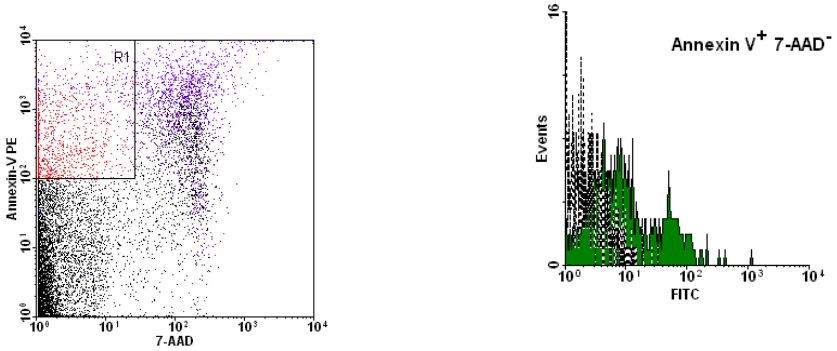
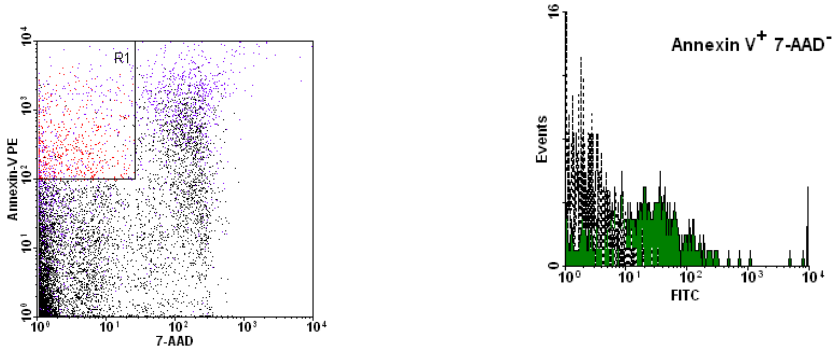


Figure XVI: Quantification of glycolytic enzyme molecules externalization. Apoptotic cells (induced with actinomycin D) were analyzed cytofluorimetrically without (solid histogram) or following permeabilization (colored dashed line). **A**) HeLa cells were stained with mouse monoclonal anti- α-Enolase (L-27; Santa Cruz Biotechnology) and secondary PE - conjugated Rat monoclonal anti-mouse IgG_1 (BD Biosciences). The mean fluorescence intensity (MFI) of staining for non-permeabilized cells is 156.5; MFI for permeabilized cells is 307.3; MFI for staining with secondary antibody alone (black, solid line) is 10.0. We calculate the fraction of total cellular EnoA that was externalized in this experiment as 49%. The profile of staining of permeabilized viable HeLa cells (MFI = 319.5) was very similar to that of permeabilized apoptotic HeLa cells. **B**) DO11.10 cells were stained with mouse monoclonal anti GAPDH (Abcam) and secondary FITC - conjugated Rat anti-mouse IgG_{2b} (BD Biosciences). MFI for non-permeabilized cells is 67.2; MFI for permeabilized cells is 142.0; MFI for staining with secondary antibody alone (black, solid line) is 15.2; the fraction of total cellular GAPDH externalized is 41%. The profile of staining of permeabilized viable DO11.10 cells (MFI = 138.5) was indistinguishable from that of permeabilized apoptotic DO11.10 cells.

A. α-Enolase



B. GAPDH



C. TPI

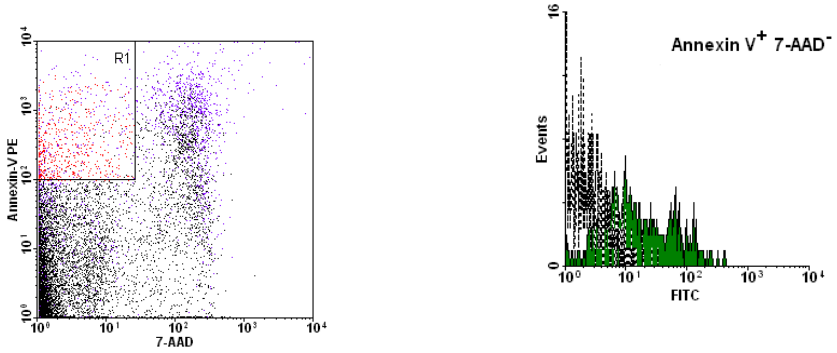


Figure XVII: Characterization of the appearance of exposed glycolytic enzyme molecules. Murine splenocytes that were induced to undergo apoptosis with staurosporine were analyzed cytofluorimetrically following staining with PE - conjugated annexin V, 7-AAD, and (**A**) FITC - conjugated polyclonal Goat anti- α-Enolase peptide IgG (Santa Cruz Biotechnology), (**B**) polyclonal Rabbit anti-GAPDH IgG (Abcam) and secondary FITC - conjugated Goat anti-Rabbit IgG (Santa Cruz Biotechnology), and (**C**) polyclonal Rabbit anti-Triosephosphate Isomerase IgG (Novus Biologicals) and secondary FITC - conjugated Goat anti-Rabbit IgG (Santa Cruz Biotechnology). Cells that met the criteria of staining positively with annexin V and negatively with 7-AAD (annexin V^+ 7-AAD^-; the "R1" region marked in red in the upper dot plots) were gated electronically, and the fluorescein signal of those cells was analyzed (shown as solid green histograms in the lower panels). The fluorescein signal of annexin V^+ 7-AAD^- cells stained with secondary antibody alone also is presented (gray, dotted lines).

These staining data involve a variety of mono- and polyclonal antibodies. Although all apoptotic cells react with polyclonal sera specific for the glycolytic enzymes, we have noted differences among apoptotic cell populations with regard to reactivity with glycolytic enzyme-specific monoclonal antibodies (data not shown). Some glycolytic enzyme epitopes appear not to be exposed in some cell lines (although those epitopes are immunologically detectable intracellularly). These data suggest that the process of apoptosis-specific externalization may be constrained conformationally (see next section).

In summary, our data from three independent approaches reveal that the externalization of glycolytic enzyme molecules is a dramatic event that occurs reliably and early during the process of apoptotic cell death. The surface-exposed, membrane-associated forms of glycolytic enzyme proteins represent novel and unambiguous apoptosis-specific biomarkers.

v. Externalized glycolytic enzyme molecules lack enzymatic activity.

We have assessed enolase and GAPDH activities in apoptotic and viable cells. While apoptotic cells have elevated levels of apparently externalized activity, accounting for 40 – 50% of the total cellular activity observed in whole cell extracts (**Table VI**), virtually all of that externalized activity can be attributed to activity leaking from broken cells. That is, we find equivalent activity in cell supernatants prepared by incubation of cells in reaction mixtures (that are of physiological osmolarity) without substrate. Thus, we have no evidence that the "externalized" activities we observe represent the enzymatic activity of enzyme molecules localized to the cell surface, as opposed to the activities of intracellular enzyme molecules released due to plasma membrane leakage. Indeed, we see similar absolute levels of enolase and GAPDH activities associated with undisrupted cells from untreated cultures (although total cellular activities in apoptotic cultures are 40 – 60% lower than in viable cultures; **Table VI**).

Table VI: Cell-associated enzymatic activities. Enolase and GAPDH activities were assessed as described in Experimental Procedures. Graded numbers of undisrupted viable or apoptotic HeLa cells, or the sonicated extracts of equivalent cell numbers (disrupted cells), were incubated in 200 µl reactions for 4 min. at 25ºC. Cells and cell debris then was removed by centrifugation, and reaction products were quantified spectrophotometrically. Activity in cell supernatants was assessed by incubating cells as above in mock reactions without substrate, removing cells by centrifugation, and then incubating supernatants with substrate for an additional 4 min. at 25ºC.

Enolase activity (HeLa cells) (conversion of 2 phosphoglycerate to phosphoenolpyruvate [PEP], nMol / 10^4 cells)		
	Cells from	
	Viable Populations	Apoptotic Populations
Disrupted cell activity	9.7 ± 1.1	3.7 ± 0.8
Undisrupted cell activity	1.9 ± 0.4	1.9 ± 0.5
Undisrupted cell supernatant activity	1.4 ± 0.2	1.2 ± 0.2

GAPDH activity (HeLa cells) (Glyceraldehyde 3-phosphate-dependent formation of NADH, nMol / 10^4 cells)		
	Cells from	
	Viable Populations	Apoptotic Populations
Disrupted cell activity	5.5 ± 0.8	3.4 ± 0.1
Undisrupted cell activity	0.8 ± 0.1	1.4 ± 0.4
Undisrupted cell supernatant activity	1.2 ± 0.3	1.2 ± 0.1

We conclude that apoptosis-specific externalized enzyme molecules are not enzymatically active. This conclusion is consistent with our suggestion that the process of apoptosis-specific externalization may be conformationally-constrained (above).

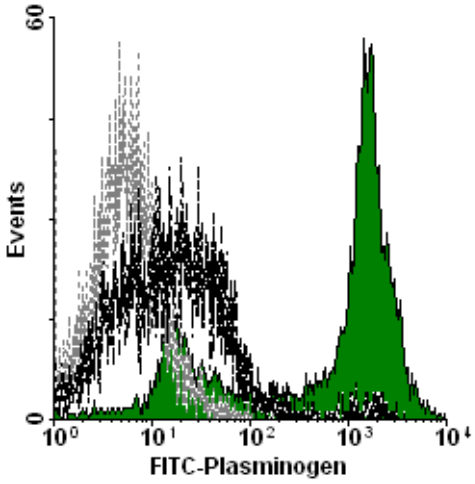
vi. Externalized glycolytic enzyme molecules bind plasminogen.

Several of the glycolytic enzyme molecules that we have shown to be externalized with apoptosis (especially EnoA, GAPDH, and PGK) have been implicated previously in the binding of plasminogen (42-47). Elevated plasminogen binding associated with apoptotic cell death also has been described (48). Consistent with these observations, we see robust plasminogen binding to apoptotic cells (**Figure XVIII-A**). Plasminogen binding, like glycolytic enzyme molecule externalization, is evident on the earliest apoptotic cells (**Figure XVIII-B**).

Pre-binding of plasminogen to apoptotic cells precludes the binding of α-enolase-specific antibodies, indicating that EnoA serves as a plasminogen-binding "receptor" (data not shown), although it appears that EnoA is not the only species responsible for plasminogen binding to apoptotic cells. Conversely, plasminogen binding to the apoptotic cell surface is inhibited competitively with the lysine analogue ε–aminocaproic acid (data not shown), consistent with the characterization of cell surface lysine residues as targets for plasminogen binding (42).

In contrast to the glycolytic enzyme molecules, annexin A2, which also has been implicated as a plasminogen-binding receptor (46), is not exposed preferentially during apoptotic cell death. There is concordance between iTRAQ and cytofluorimetric analyses in this regard (**Figure XII** and **Figure XIX-A**). By comparison, cytofluorimetric analysis revealed the apoptosis-specific externalization of calreticulin (**Figure XIX-B**), although our iTRAQ analysis did not identify calreticulin among apoptotic membrane vesicle-enriched molecules (**Figure XII**). The exposure of calreticulin in association with apoptotic cell death has been noted previously (49, 50).

A.



B.

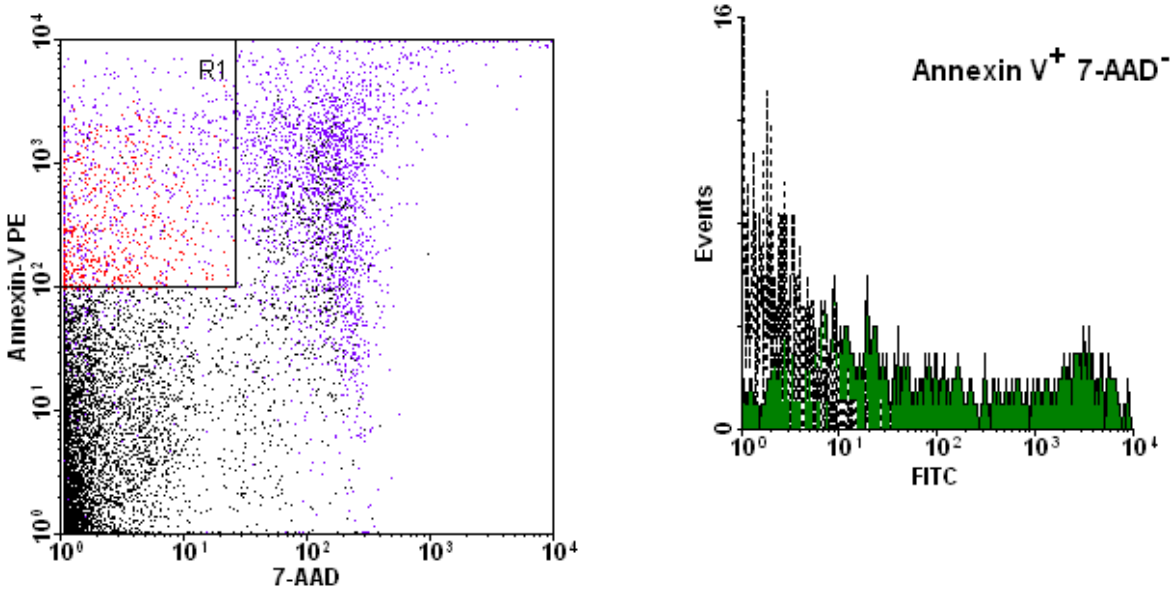


Figure XVIII: Cytofluorimetric analysis of plasminogen binding to apoptotic cells. A) Murine splenocytes that had undergone apoptosis spontaneously in culture (12 hr.) and freshly isolated, viable splenocytes were analyzed cytofluorimetrically as in Figure XV, following staining with FITC - conjugated plasminogen (BioMac). **B**) Murine splenocytes that were induced to undergo apoptosis with staurosporine were analyzed cytofluorimetrically following staining with PE - conjugated annexin V, 7-AAD, and FITC - conjugated plasminogen, and analyzed as in Figure XVII.

A. Annexin A2 **B. Calreticulin**

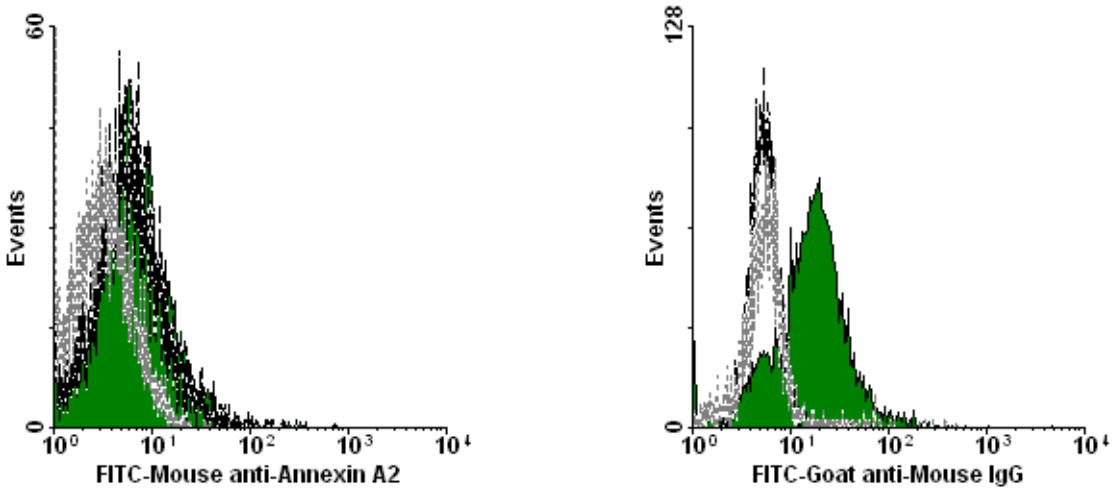


Figure XIX: Cytofluorimetric analysis of externalization of other molecules. Human transformed (Jurkat) T lymphocytes, that had been induced to undergo apoptosis by treatment with actinomycin D or had been left untreated, were analyzed cytofluorimetrically following staining with (**A**) FITC - conjugated mouse monoclonal anti-human Annexin-II (BD Biosciences) or (**B**) Mouse monoclonal anti-Calreticulin and secondary FITC - conjugated Goat anti-Rabbit IgG (Enzo Life Sciences). Apoptotic (solid green histogram) and viable (dashed line) cells were identified by scatter properties and gated electronically.

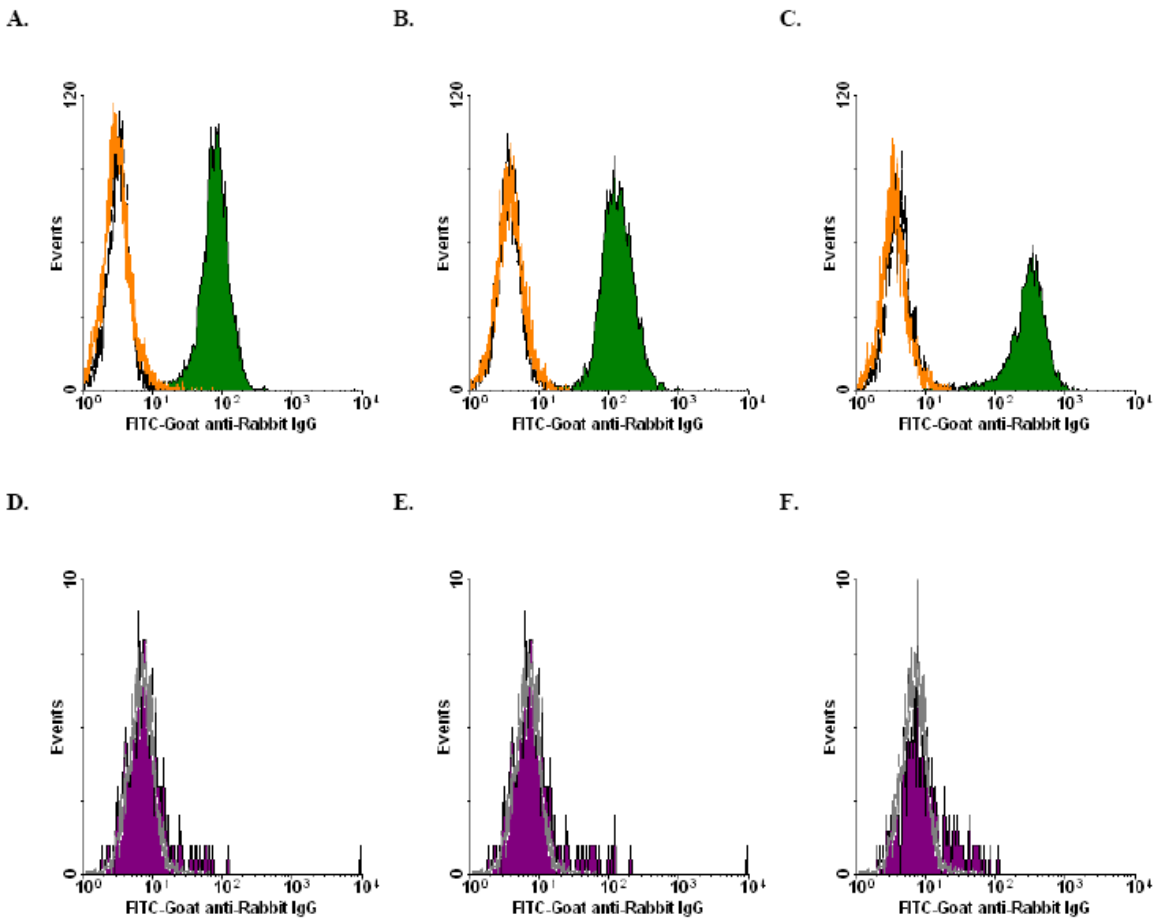


Figure XX: Glycolytic enzyme molecules are not externalized on autophagic or necrotic cells. HeLa cells that were induced to undergo autophagy by serum starvation with L-canavanine (1 mM) in the presence of the pan-caspase inhibitor Q-VD-OPh (10 μM), apoptotic HeLa cells (induced by serum starvation in the absence of QVD-OPh), and viable HeLa cells were analyzed cytofluorimetrically following staining for (**A**) α-Enolase (with polyclonal Rabbit anti- α-Enolase peptide IgG and secondary FITC-conjugated Goat ant-Rabbit IgG [Abcam]), (**B**) GAPDH (with polyclonal Rabbit anti-GAPDH IgG and secondary FITC-conjugated Goat anti-Rabbit IgG [Abcam]), and (**C**) Triosephosphate Isomerase (with polyclonal Rabbit anti-Triosephosphate Isomerase peptide IgG and secondary FITC-conjugated Goat anti-Rabbit IgG [Abcam]). Profiles shown (**A**-**C**) are for autophagic (orange, solid lines), apoptotic (solid green histograms), and viable (black, dashed lines) cells stained with the specific FITCconjugated reagents. Cells that had lost membrane integrity (PI^+, low forward- and side-angle light scatter) were excluded from these analyses by electronic gating. Cells triggered to die necrotically also were analyzed, as in **Figure XVII**, following staining with PE-conjugated annexin V, 7-AAD, and reagents specific (as above) for (**D**) α-Enolase, (**E**) GAPDH, and (**F**) Triosephosphate Isomerase. Cells that met the criteria of staining positively with annexin V and negatively with 7-AAD (annexin V^+ 7-AAD^-) were gated electronically, and the fluorescein signal of those cells was analyzed (shown as solid violet histograms in panels **D**-**F**). The fluorescein signal of annexin V^+ 7-AAD^- cells stained with secondary antibody alone also is presented (gray, dotted lines in panels **D**-**F**).

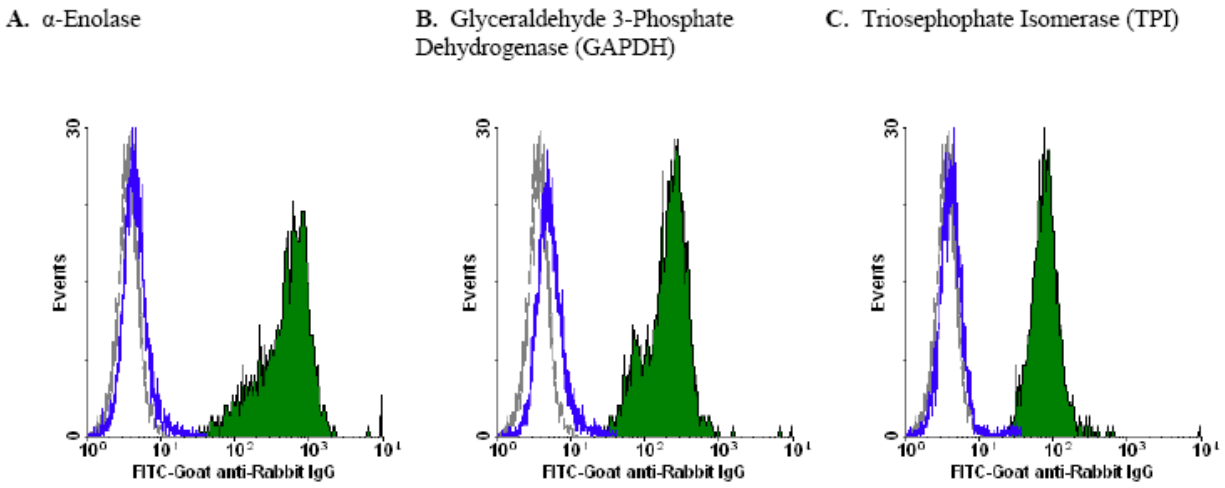


Figure XXI: Apoptotic externalization of glycolytic enzyme molecules is caspase-dependent. HeLa cells, subjected to irradiation with UV-C light in the presence of the pan-caspase inhibitor Q-VD-OPh (10 μM, including 90 min. pre-treatment) and in its absence, were analyzed cytofluorimetrically following staining, as in **Figure XX**, for (A) α-Enolase, (B) GAPDH, and (C) Triosephosphate Isomerase. Profiles shown are for cells irradiated in the presence of Q-VD-OPh (blue, solid lines) and in its absence (solid green histograms). The fluorescein signal of cells stained with secondary antibody alone also is presented (gray, dotted lines). Cells that had lost membrane integrity (PI^+, low forward- and side-angle light scatter) were excluded from these analyses by electronic gating.

C. DISCUSSION

Efficient recognition of the corpse, coupled to immunomodulation, is perhaps the ultimate functional consequence of apoptotic cell death, and the identification of the relevant molecules that determine those outcomes is of fundamental importance. Based on results from a variety of our studies indicating that apoptotic immunomodulatory determinants, which can be evaluated by the transcriptional responses they elicit, appear relatively early in the process of cell death, are enriched in plasma membrane vesicles from apoptotic cells, and are protease sensitive, we undertook distinct, independent, and unbiased proteomic approaches to characterize apoptosis-specific (as compared with viable) membrane vesicle-associated proteins. We elaborated criteria that represent critical features of the molecules that function as apoptotic determinants for recognition and immune response modulation (denoted "SUPER"): molecules that become surface-exposed specifically during apoptotic cell death process, that are expressed ubiquitously in cells of different cell types, that are protease-sensitive, evolutionarily-conserved, and that are resident normally in viable cells (albeit not on the cell surface of non-apoptotic cells).

There were intriguing and informative surprises in the array of proteins identified. For example, the absence of histones, which meet the SUPER criteria for apoptotic determinants for recognition and immune modulation, is striking. The absence of histones from apoptotic vesicles may reflect their loose association with the apoptotic cell membrane and loss as a consequence of the extensive washing and ultracentrifugation involved in vesicle preparation. The apparent absence of actin (as distinct from molecules with which it shares significant sequence identity [e.g. actinin, etc.]) may simply represent an artifact of the informatic paradigm by which protein assignments from identified peptides are made, although, by immunofluorescence staining, we have not detected actin associated with apoptotic vesicles (data not shown). Presumably, the

enrichment of cytochrome *c* among apoptotic vesicle proteins reflects the release of soluble cytochrome *c* from mitochondria during the apoptotic process, while the absence of cytochrome c from the cell surface underscores the selectivity of apoptosis-specific protein externalization.

Unexpectedly, we identified a group of glycolytic enzyme molecules that become redistributed and membrane-associated during apoptotic cell death, and we demonstrated cytofluorimetrically their externalization to the apoptotic cell surface. With the exception of two of the upstream members (hexokinase and phosphofructokinase), all of the enzymes of the aerobic glycolytic pathway (phosphoglucose isomerase, aldolase, TPI, GAPDH, PGK, EnoA, and PK) were identified as enriched in the membrane vesicle fraction from apoptotic cells. Previous work (51, 52) has documented that glycolytic enzymes are not [normally] associated with the plasma membrane. Still, our results are consistent with findings from other proteomic studies. For example, Gu *et al.* (53) observed that several glycolytic enzymes (including EnoA and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) were up-regulated at late times of death (triggered by activation of p53), and Jin *et al.* (39) found EnoA and GAPDH in microparticles recovered from human plasma, which include apoptotic blebs. Sunaga *et al.* (54) also noted that GAPDH is over-expressed during the apoptotic death of neuronal cells and that it is exposed in amyloid plaques. Interestingly, thesemolecules have been shown to form multimeric complexes intracellularly on cytoskeletal and membrane elements in viable cells (55-58); we do not know if they exist in an aggregated form when externalized.

Our data demonstrate that glycolytic enzyme molecule externalization is a common and early aspect of apoptotic cell death in different cell types triggered to die with distinct suicidal stimuli. Although all apoptotic cells expose glycolytic enzyme molecules, only a fraction of those molecules within a cell is externalized to the cell surface; the externalized molecules lack

enzymatic activity. Numerous metabolic processes and enzymes have been shown to be important for many aspects of apoptosis (59-62); however, the redistribution of a large subset of glycolytic enzymes to the apoptotic cell membrane has not been characterized previously. Our findings demonstrate that the externalization of glycolytic enzyme molecules is a unique feature and a definitive marker of apoptotic cell death.

GAPDH and EnoA externalization have been noted previously in particular cases. GAPDH, for example, has been reported to serve as a receptor for transferrin (63). Raje *et al.* found that GAPDH was externalized on cells of a macrophage cell line cultured in iron-depleted medium (63). We repeated those experiments (with the same macrophage cell lines and others) and found that the iron-deficient conditions employed trigger apoptotic cell death, leading to the exposure of GAPDH (and other glycolytic enzyme molecules; data not shown). Thus, those findings reiterate that externalized GAPDH is a definitive marker of the apoptotic cell, independent of transferrin-binding activity. The case of plasminogen binding to externalized EnoA is discussed below.

The cohort of externalized glycolytic enzyme molecules that we identified by several approaches fulfill the critical SUPER criteria. Others of the molecules enriched among apoptotic membrane vesicles and identified in our iTRAQ analysis also meet SUPER criteria. Those found not to be externalized (e.g. actin, cytochrome *c*, and histones; discussed above) likely can be excluded from further consideration. In addition, the group of chaperones likely does not function as apoptotic immunosuppressive determinants; heat shock proteins, in particular, have been implicated as immunostimulatory "danger signals" (64).

Further, since exposure of resident, intracellular molecules from necrotic cells does not confer apoptotic-like immunosuppressive activity (10, 12), protein externalization *per se* (in the

absence of apoptosis-specific PTM) likely is not sufficient for the appearance of apoptotic determinants for recognition and immune modulation. At least with regard to EnoA, our data demonstrating that the appearance of apoptotic determinants for recognition and immune response modulation is not dependent on *de novo* gene expression (10, 12) are consistent with the suggestion of Redlitz *et al.* (43) that the surface-exposed form of that molecule does not arise as the product of a specific or altered gene transcript.

It is not clear how apoptosis-specific protein externalization occurs, although our data indicate that it occurs in a caspase-dependent manner . The externalization of glycolytic enzymes has been observed in a wide variety of cells and among many species, independent of cell death (17-27, 42-44, 47). Among these examples are bacterial and protist pathogens, some of which have been shown to mimic apoptotic cell immune responses (65, 66). The export signals or mechanism by which those normally intracellular molecules become membrane-exposed have not been identified. The enzymatically inactive forms of externalized glycolytic enzyme molecules on apoptotic cells contrast with the enzymatically active glycolytic enzymes exposed on the bacterial surface (17, 18, 20), suggesting that different processes for membrane externalization may pertain.

As discussed above, we observe similar mobilities (both relative molecular weight and pI; **Figure XI**) for non-externalized glycolytic enzyme molecules that are derived from viable cells, and for the glycolytic enzyme molecules of apoptotic cells, at least a large fraction of which are externalized. With the exception of TPI, where there does appear to be apoptosis-enhanced proteolysis (**Figure XI**, spot 7'), it is clear from these data that apoptosis-specific post-translational modifications that give rise to glycolytic enzyme molecule externalization generally are not proteolytic. Our analysis of such modifications, including the possibility of oxidation-

dependent modification (67), is underway. Notably, apoptotic externalization appears to target proteins selectively and independently of protein abundance. The process of blebbing (68-71), which is characteristic of apoptosis, involves protein relocalization (1). We do not know whether blebbing *per se* is sufficient for the protein externalization that we have characterized.

Externalization of EnoA has been noted in the context of its ability to bind plasminogen (42-44). The nature of the cells exposing EnoA on the cell surface was not explored in those studies. Our data show that significant binding of plasminogen occurs specifically to apoptotic (and not viable) cells, and that EnoA is involved in [at least some of] this binding. These results confirm and extend previous observations (48). In particular, independent studies have implicated externalized glycolytic enzymes (especially EnoA and GAPDH) on mammalian and pathogen surfaces as sites for plasminogen binding and activation (18, 27, 72, 73), although no compelling physiological rationale for the presence of this activity on such disparate cells has been offered.

Plasminogen binding, leading to proteolytic cleavage and plasmin activation, has been suggested to be important for pathogen invasiveness (27, 74). With mammalian cells, several, but not all, species of plasminogen receptor molecules are enriched on the surface of apoptotic cells. It seems unlikely that migration and invasiveness (e.g. extracellular matrix degradation) are selected attributes for apoptotic cells, although plasmin-dependent proteolytic activation of latent, matrix-associated TGF-β may be significant (see ref. 75). Plasminogen also appears to be a component of serum that enhances phagocytosis of apoptotic cells (K. Lauber, personal communication). Although molecules identified as plasminogen receptors that are externalized in a non-cell death-related manner, such as annexin A2, may be physiologically relevant for plasminogen binding and its consequences (76, 77), plasminogen-binding molecules exposed in

an apoptosis-specific manner may function in an entirely distinct capacity on the apoptotic cell surface.

Several molecular species of "plasminogen receptors" other than glycolytic enzyme molecules have been identified. For example, Das and Plow (78, 79) have suggested that histone H2B is a plasminogen receptor, and cell surface actin has been implicated similarly (38, 46, 80). In this context, it is interesting that our iTRAQ analysis indicates that these and other molecules characterized as (or associated with) plasminogen receptors (S100A10, annexin A2, cytokeratin 8; refs. 77, 81, 82) are not preferentially enriched on the apoptotic cell surface (**Figure XII**).

We take our data to suggest that plasminogen binding *per se* may not represent the primary functional consequence of apoptosis-specific protein externalization; rather, the externalization of glycolytic enzyme molecules may be significant functionally in apoptotic cell recognition and immune modulation. In this context, the significance of the expression of orthologous molecules on bacterial (and other pathogenic) surfaces may relate to apoptotic mimicry, leading to immune suppression (attenuation of inflammatory and other immune responses), rather than to plasminogen binding. Our results, then, may serve to integrate diverse previous findings to suggest that the observed binding of plasminogen to glycolytic enzyme molecules exposed on the mammalian cell surface is a consequence of the apoptosis-specific externalization of determinants for recognition and immune modulation, and that apoptotic mimicry is the primary effect of exposed glycolytic molecules on commensal bacterial and pathogens.

Independently of their intracellular roles in metabolism (as well as more recently identified functions in transcription, cytoskeletal trafficking, and autophagy and cell death; refs. 83-87), our findings proffer glycolytic enzyme molecules, in an extracellular context, as candidate determinants of immunomodulation and suggest that they may fulfill an immunological

“moonlighting” (88) role. It is not surprising that in cases of autoimmune pathology, where normal innate apoptotic immunity and tolerance is broken, these molecules are recognized as autoantigens to which antibodies are generated. Indeed, glycolytic enzymes have been identified as potent autoantigens in several autoimmune syndromes (e.g. EnoA in Hashimoto's encephalopathy, Behçet's disease, SLE, inflammatory bowel disease, vasculitis, mixed cryoglobulinemia, and rheumatoid arthritis [refs. 89-95]; TPI in neuropsychiatric lupus, SLE, and osteoarthritis [refs. 96, 97]; PK in rheumatoid arthritis as well as SLE [ref. 98]). Further characterization of the apoptosis-specific mechanism by which glycolytic enzymes are post-translationally modified and externalized and their role in immune modulation holds promise for understanding and addressing causes of autoimmune and inflammatory pathology.

Cited Literature for Chapter IV

1. Cocca, B. A., Cline, A. M., and Radic, M. Z. 2002. Blebs and apoptotic bodies are B cell autoantigens. *J. Immunol.* **169**, 159-166.

2. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239-256.

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

4. Birge, R. B., and Ucker, D. S. 2008. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ.* **15**(7), 1096-1102.

5. Herrmann, M., Voll, R. E., Zoller, O. M., Hagenhofer, M., Ponner, B. B., and Kalden, J. R. 1998. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis Rheum.* **41**, 1241-1250.

6. Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A. S., Earp, H. S., Matsushima, G., and Reap, E. A. 2002. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J. Exp. Med.* **196**, 135-140.

7. Rothlin, C. V., Ghosh, S., Zuniga, E. I., Oldstone, M. B., and Lemke, G. 2007. TAM receptors are pleiotropic inhibitors of the innate immune response. *Cell* **131**(6), 1124-1136.

8. Thorp, E. B. 2010. Mechanisms of failed apoptotic cell clearance by phagocyte subsets in cardiovascular disease. *Apoptosis* **15**(9), 1124-1136.

9. Ucker, D. S. 2009. Innate apoptotic immunity: a potent immunosuppressive response repertoire elicited by specific apoptotic cell recognition. In: Krysko, D. V., and Vandenabeele, P. (eds). *Phagocytosis of dying cells: from molecular mechanisms to human diseases*, Springer, Berlin, New York.

10. Cvetanovic, M., and Ucker, D. S. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J. Immunol.* **172**, 880-889.

11. Patel, V. A., Longacre-Antoni, A., Cvetanovic, M., Lee, D. J., Feng, L., Fan, H., Rauch, J., Ucker, D. S., and Levine, J. S. 2007. The affirmative response of the innate immune system to apoptotic cells. *Autoimmunity* **40**(4), 274-280.

12. Cocco, R. E., and Ucker, D. S. 2001. Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol. Biol. Cell* **12**(4), 919-930.

13. Cvetanovic, M., Mitchell, J. E., Patel, V., Avner, B. S., Su, Y., van der Saag, P. T., Witte, P. L., Fiore, S., Levine, J. S., and Ucker, D. S. 2006. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. *J. Biol. Chem.* **281**, 20055-20067.

14. 14. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* **101**, 890-898

15. Savill, J. 1998. Apoptosis. Phagocytic docking without shocking. *Nature* **392**, 442-443.

16. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* **148**, 2207-2216.

17. Pancholi, V., and Fischetti, V. A. 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *J. Exp. Med.* **176**(2), 415-426.

18. Bergmann, S., Rohde, M., Chhatwal, G. S., and Hammerschmidt, S. 2001. alpha-Enolase of Streptococcus pneumoniae is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Mol. Microbiol.* **40**(6), 1273-1287.

19. Bergmann, S., Rohde, M., and Hammerschmidt, S. 2004. Glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pneumoniae is a surface-displayed plasminogen-binding protein. *Infect. Immun.* **72**(4), 2416-2419.

20. Pancholi, V., and Fischetti, V. A. 1998. alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *J. Biol. Chem.* **273**(23), 14503-14515.

21. Candela, M., Biagi, E., Centanni, M., Turroni, S., Vici, M., Musiani, F., Vitali, B., Bergmann, S., Hammerschmidt, S., and Brigidi, P. 2009. Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. *Microbiology* **155**(Pt 10), 3294-3303.

22. Vanegas, G., Quinones, W., Carrasco-Lopez, C., Concepcion, J. L., Albericio, F., and Avilan, L. 2007. Enolase as a plasminogen binding protein in Leishmania mexicana. *Parasitol. Res.* **101**(6), 1511-1516.

23. Lama, A., Kucknoor, A., Mundodi, V., and Alderete, J. F. 2009. Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated, fibronectin-binding protein of Trichomonas vaginalis. *Infect. Immun.* **77**(7), 2703-2711.

24. Bhowmick, I. P., Kumar, N., Sharma, S., Coppens, I., and Jarori, G. K. 2009. Plasmodium falciparum enolase: stage-specific expression and sub-cellular localization. *Malar. J.* **8**, 179.

25. Gil-Navarro, I., Gil, M. L., Casanova, M., O'Connor, J. E., Martinez, J. P., and Gozalbo, D. 1997. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of Candida albicans is a surface antigen. *J. Bacteriol.* **179**(16), 4992-4999.

26. Goudot-Crozel, V., Caillol, D., Djabali, M., and Dessein, A. J. 1989. The major parasite surface antigen associated with human resistance to schistosomiasis is a 37-kD glyceraldehyde-3P-dehydrogenase. *J. Exp. Med.* **170**(6), 2065-2080.

27. Jong, A. Y., Chen, S. H. M., Stins, M. F., Kim, K. S., Tuan, T.-L., and Huang, S.-H. 2003. Binding of Candida albicans enolase to plasmin(ogen) results in enhanced

invasion of human brain microvascular endothelial cells. *J. Med. Microbiol.* **52**(Pt 8), 615-622.

28. Kato, H., Fukuda, T., Parkison, C., McPhie, P., and Cheng, S. Y. 1989. Cytosolic thyroid hormone-binding protein is a monomer of pyruvate kinase. *Proc. Natl. Acad. Sci. USA* **86**(20), 7861-7865.

29. Giallongo, A., Feo, S., Moore, R., Croce, C. M., and Showe, L. C. 1986. Molecular cloning and nucleotide sequence of a full-length cDNA for human alpha enolase. *Proc. Natl. Acad. Sci. USA* **83**(18), 6741-6745.

30. Gracy, R. W. 1975. Triosephosphate isomerase from human erythrocytes. *Methods Enzymol.* **41**, 442-447.

31. Mannherz, H. G., Gonsior, S. M., Gremm, D., Wu, X., Pope, B. J., and Weeds, A. G. 2005. Activated cofilin colocalises with Arp2/3 complex in apoptotic blebs during programmed cell death. *Eur. J. Cell Biol.* **84**(4), 503-515.

32. Buttyan, R., Zakeri, Z., Lockshin, R., and Wolgemuth, D. 1988. Cascade induction of c-fos, c-myc, and heat shock 70K transcripts during regression of the rat ventral prostate gland. *Mol. Endocrinol.* **2**, 650-657.

33. Zinszner, H., Kuroda, M., Wang, X. Z., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Devel.* **12**, 982-995.

34. Huot, J., Houle, F., Rousseau, S., Deschesnes, R. G., Shah, G. M., and Landry, J. 1998. SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *J. Cell Biol.* **143**(5), 1361-1373.

35. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132-1136.

36. D'Herde, K., De Prest, B., Mussche, S., Schotte, P., Beyaert, R., Van Coster, R., and Roels, F. 2000. Ultrastructural localization of cytochrome c in apoptosis demonstrates mitochondrial heterogeneity. *Cell Death Differ.* **7**, 331-337.

37. Laster, S. M., and Mackenzie, J. M., Jr. 1996. Bleb formation and F-actin distribution during mitosis and tumor necrosis factor-induced apoptosis. *Microsc. Res. Tech.* **34**, 272-280.

38. Dudani, A. K., and Ganz, P. R. 1996. Endothelial cell surface actin serves as a binding site for plasminogen, tissue plasminogen activator and lipoprotein(a). *Br. J. Haematol.* **95**(1), 168-178.

39. Jin, M., Drwal, G., Bourgeois, T., Saltz, J., and Wu, H. M. 2005. Distinct proteome features of plasma microparticles. *Proteomics* **5**(7), 1940-1952.

40. Arur, S., Uche, U. E., Rezaul, K., Fong, M., Scranton, V., Cowan, A. E., Mohler, W., and Han, D. K. 2003. Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev. Cell* **4**, 587-598.

41. Miguet, L., Pacaud, K., Felden, C., Hugel, B., Martinez, M. C., Freyssinet, J. M., Herbrecht, R., Potier, N., van Dorsselaer, A., and Mauvieux, L. 2006. Proteomic analysis of malignant lymphocyte membrane microparticles using double ionization coverage optimization. *Proteomics* **6**(1), 153-171.

42. Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Plow, E. F. 1991. Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry* **30**, 1682-16891.

43. Redlitz, A., Fowler, B. J., Plow, E. F., and Miles, L. A. 1995. The role of an enolase-related molecule in plasminogen binding to cells. *Eur. J. Biochem.* **227**(1-2), 407-415.

44. Nakajima, K., Hamanoue, M., Takemoto, N., Hattori, T., Kato, K., and Kohsaka, S. 1994. Plasminogen binds specifically to alpha-enolase on rat neuronal plasma membrane. *J. Neurochem.* **63**(6), 2048-2057.

45. López-Alemany, R., Suelves, M., and Muñoz-Cánoves, P. 2003. Plasmin generation dependent on alpha-enolase-type plasminogen receptor is required for myogenesis. *Thromb. Haemost.* **90**(4), 724-733.

46. Kwon, M., MacLeod, T. J., Zhang, Y., and Waisman, D. M. 2005. S100A10, annexin A2, and annexin a2 heterotetramer as candidate plasminogen receptors. *Front. Biosci.* **10**, 300-325.

47. Wygrecka, M., Marsh, L. M., Morty, R. E., Henneke, I., Guenther, A., Lohmeyer, J., Markart, P., and Preissner, K. T. 2009. Enolase-1 promotes plasminogen-mediated recruitment of monocytes to the acutely inflamed lung. *Blood* **113**(22), 5588-5598.

48. O'Mullane, M. J., and Baker, M. S. 1998. Loss of cell viability dramatically elevates cell surface plasminogen binding and activation. *Exp. Cell Res.* **242**(1), 153-164.

49. Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J.-L., Castedo, M., Mignot, G., Panaretakis, T., Casares, N., Métivier, D., Larochette, N., van Endert, P., Ciccosanti, F., Piacentini, M., Zitvogel, L., and Kroemer, G. 2007. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* **13**(1), 54-61.

50. Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M., and Henson, P. M. 2005. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* **123**, 321-334.

51. Shui, W., Sheu, L., Liu, J., Smart, B., Petzold, C. J., Hsieh, T. Y., Pitcher, A., Keasling, J. D., and Bertozzi, C. R. (2008) *Proc. Natl. Acad. Sci. USA* **105**(44), 16952-16957.

52. Rogers, L. D., and Foster, L. J. (2007) *Proc. Natl. Acad. Sci. USA* **104**(47), 18520-18525.

53. Gu, S., Liu, Z., Pan, S., Jiang, Z., Lu, H., Amit, O., Bradbury, E. M., Hu, C.-A. A., and Chen, X. (2004) *Mol. Cell. Proteomics* **3**(10), 998-1008.

54. Sunaga, K., Takahashi, H., Chuang, D.-M., and Ishitani, R. (1995) *Neurosci. Lett.* **200**(2), 133-136.

55. Srere, P. A., and Knull, H. R. (1998) *Trends Biochem. Sci.* **23**(9), 319-320.

56. Singh, P., Salih, M., Leddy, J. J., and Tuana, B. S. (2004) *J. Biol. Chem.* **279**(34), 35176-35182.

57. Minaschek, G., Groschel-Stewart, U., Blum, S., and Bereiter-Hahn, J. (1992) *Eur. J. Cell Biol.* **58**(2), 418-428.

58. Campanella, M. E., Chu, H., and Low, P. S. (2005) *Proc. Natl. Acad. Sci. USA* **102**(7), 2402-2407.

59. Ishitani, R., and Chuang, D.-M. (1996) *Proc. Natl. Acad. Sci. USA* **93**(18), 9937-9941.

60. Eguchi, Y., Shimizu, S., and Tsujimoto, Y. (1997) *Cancer Res.* **57**, 1835-1840.

61. Leist, M., Single, B., Castoldi, A. F., Kühnle, S., and Nicotera, P. (1997) *J. Exp. Med.* **185**, 1481-1486.

62. Robey, R. B., and Hay, N. (2006) *Oncogene* **25**(34), 4683-4696.

63. Raje, C. I., Kumar, S., Harle, A., Nanda, J. S., and Raje, M. (2007) *J. Biol. Chem.* **282**(5), 3252-3261.

64. Manjili, M. H., Park, J., Facciponte, J. G., and Subjeck, J. R. (2005) *Immunobiology* **210**(5), 295-303.

65. de Freitas Balanco, J. M., Moreira, M. E. C., Bonomo, A., Bozza, P. T., Amarante-Mendes, G., Pirmez, C., and Barcinski, M. A. (2001) *Curr. Biol.* **11**, 1870-1873.

66. Barcinski, M. A., Moreira, M. E. C., de Freitas Balanco, J. M., Wanderley, J. L. M., and Bonomo, A. C. (2003) *Kinetoplastid Biol. Dis.* **2**, 6-7.

67. Sambrano, G. R., and Steinberg, D. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1396-1400.

68. Mills, J. C., Stone, N. L., Erhardt, J., and Pittman, R. N. (1998) *J. Cell Biol.* **140**, 627-636.

69. Coleman, M. L., Sahai, E. A., Yeo, M., Bosch, M., Dewar, A., and Olson, M. F. (2001) *Nat. Cell Biol.* **3**, 339-345.

70. Sebbagh, M., Renvoizé, C., Hamelin, J., Riché, N., Bertoglio, J., and Bréard, J. (2001) *Nat. Cell Biol.* **3**, 346-352.

71. Leverrier, Y., and Ridley, A. J. (2001) *Nat. Cell Biol.* **3**, E91-E93.

72. Felez, J., Chanquia, C. J., Fabregas, P., Plow, E. F., and Miles, L. A. (1993) *Blood* **82**(8), 2433-2441.

73. Miles, L. A., Hawley, S. B., Baik, N., Andronicos, N. M., Castellino, F. J., and Parmer, R. J. (2005) *Front. Biosci.* **10**, 1754-1762.

74. Lähteenmäki, K., Edelman, S., and Korhonen, T. K. (2005) *Trends Microbiol.* **13**(2), 79-85.

75. Plow, E. F., Herren, T., Redlitz, A., Miles, L. A., and Hoover-Plow, J. L. (1995) *FASEB J.* **9**(10), 939-945.

76. Odaka, C., Tanioka, M., and Itoh, T. (2005) *J. Immunol.* **174**(2), 846-853.

77. Falcone, D. J., Borth, W., Khan, K. M., and Hajjar, K. A. (2001) *Blood* **97**(3), 777-784.

78. Das, R., Burke, T., and Plow, E. F. (2007) *Blood* **110**(10), 3763-3772.

79. Herren, T., Burke, T. A., Das, R., and Plow, E. F. (2006) *Biochemistry* **45**(31), 9463-9474.

80. Hawley, S. B., Green, M. A., and Miles, L. A. (2000) *Thromb. Haemost.* **84**(5), 882-890.

81. Ling, Q., Jacovina, A. T., Deora, A., Febbraio, M., Simantov, R., Silverstein, R. L., Hempstead, B., Mark, W. H., and Hajjar, K. A. (2004) *J. Clin. Invest.* **113**(1), 38-48.

82. Cesarman, G. M., Guevara, C. A., and Hajjar, K. A. (1994) *J. Biol. Chem.* **269**(33), 21198-22203.

83. Feo, S., Arcuri, D., Piddini, E., Passantino, R., and Giallongo, A. (2000) *FEBS Lett.* **473**, 47-52.

84. Colell, A., Ricci, J.-E., Tait, S., Milasta, S., Maurer, U., Bouchier-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N. J., Li, C. W., Mari, B., Barbry, P., Newmeyer, D. D., Beere, H. M., and Green, D. R. (2007) *Cell* **129**(5), 983-997.

85. Tisdale, E. J. (2002) *J. Biol. Chem.* **277**(5), 3334-3341.

86. Glaser, P. E., Han, X., and Gross, R. W. (2002) *Proc. Natl. Acad. Sci. USA* **99**(22), 14104-14109.

87. Tisdale, E. J., Azizi, F., and Artalejo, C. R. (2009) *J. Biol. Chem.* **284**(9), 5876-5884.

88. Jeffery, C. J. (2009) *Mol. BioSyst.* **5**(4), 345-350.

89. Sabbatini, A., Dolcher, M. P., Marchini, B., Chimenti, D., Moscato, S., Pratesi, F., Bombardieri, S., and Migliorini, P. (1997) *Clin. Exp. Rheumatol.* **15**(6), 655-658.

90. Roozendaal, C., Zhao, M. H., Horst, G., Lockwood, C. M., Kleibeuker, J. H., Limburg, P. C., Nelis, G. F., and Kallenberg, C. G. M. (1998) *Clin. Exp. Immunol.* **112**(1), 10-16.

91. Pratesi, F., Moscato, S., Sabbatini, A., Chimenti, D., Bombardieri, S., and Migliorini, P. (2000) *J. Rheumatol.* **27**(1), 109-115.

92. Moscato, S., Pratesi, F., Sabbatini, A., Chimenti, D., Scavuzzo, M., Passatino, R., Bombardieri, S., Giallongo, A., and Migliorini, P. (2000) *Eur. J. Immunol.* **30**, 3575-3584.

93. Saulot, V., Vittecoq, O., Charlionet, R., Fardellone, P., Lange, C., Marvin, L., Machour, N., Le Loët, X., Gilbert, D., and Tron, F. (2002) *Arthritis Rheum.* **46**(5), 1196-1201.

94. Lee, K. H., Chung, H.-S., Kim, H. S., Oh, S.-H., Ha, M.-K., Baik, J.-H., Lee, S., and Bang, D. (2003) *Arthritis Rheum.* **48**(7), 2025-2035.

95. Fujii, A., Yoneda, M., Ito, T., Yamamura, O., Satomi, S., Higa, H., Kimura, A., Suzuki, M., Yamashita, M., Yuasa, T., Suzuki, H., and Kuriyama, M. (2005) *J. Neuroimmunol.* **162**(1-2), 130-136.

96. Xiang, Y., Sekine, T., Nakamura, H., Imajoh-Ohmi, S., Fukuda, H., Nishioka, K., and Kato, T. (2004) *Arthritis Rheum.* **50**(5), 1511-1521.

97. Sasajima, T., Watanabe, H., Sato, S., Sato, Y., and Ohira, H. (2006) *J. Neuroimmunol.* **181**(1-2), 150-156.

98. Oremek, G. M., Müller, R., Sapoutzis, N., and Wigand, R. (2003) *Anticancer Res.* **23**(2A), 1131-1134.

CHAPTER V

Subtle Alterations In Innate Apoptotic Immune Responsiveness With Age: Implications for Immunosenescence

A. INTRODUCTION

Aging in animals is associated with a paradoxical dysregulation of both the adaptive and the innate arms of the immune systems. This altered immune responsiveness, also called immunosenescence, is characterized by a diminished protective responsiveness to exogenous and infectious immunogens (1-7), and by persistent, elevated levels of cytokines and inflammatory mediators, even in the absence of an overt physiological stress (8-12, 66). Finally, immunosenescence is associated with elevated levels of autoimmune antibodies, and corresponding elevated and pathogenic reactivity to endogenous self-antigens (13, 14). The causative mechanisms underlying these aging-associated alterations have not been elucidated. It is striking that inflammatory responsiveness and tolerance are precisely those aspects of immune responsiveness known to be modulated as a consequence of apoptotic cell interactions (Innate Apoptotic Immunity; IAI).

Macrophages have been implicated as one population of cells whose function is altered in an aging-associated manner. Macrophages play a pivotal role both in innate and adaptive immunity. Their innate immune functions include the production of an array of inflammatory mediators (including NO species; see Chapter IV) and cytokines. Among these, tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) are notable. These cytokines stimulate cellular immune responses and induce the production of acute-phase protection against infection and also set a stage for general systemic inflammatory responses. TNF-α and IL-6, among other inflammatory cytokines, have been reported to be elevated in individuals as a function of advancing age (15, 16). It is notable that this chronic aging-associated elevation in inflammatory activity appears to be independent of overt pathogenic stimulation. For example, while IL-6 is rarely detected in young individuals in the absence of trauma or infection, it is commonly present

in the serum even of healthy older individuals (61, 63). Although no compelling studies point to defects with age in the antigen-presenting and co-stimulatory functions of macrophages and other accessory cells (58), the production by macrophages and other monocytes of proinflammatory cytokines clearly increases with age (59-63). Some of these aging-associated alterations of innate responsiveness have been recapitulated with isolated macrophage *in vitro* (5, 32, 33, 35, 64, 65).

Toll-like receptors (TLRs) are crucial components for detecting pathogenic molecules (or "Pathogen-associated molecular patterns"; PAMPs) and triggering a robust innate immune response (28-30). The engagement of TLRs, which are expressed at high levels in macrophages, allows for immediate antimicrobial activity and subsequent development of antigen-specific adaptive immune responses (29, 31). A number of studies have suggested that TLR surface expression and responsiveness decline in an aging-associated manner in macrophages (5, 6, 32-36).

When they lose appropriate function, nucleated cells are triggered to die in a physiological process (apoptosis) that occurs throughout life in multicellular organisms. Apoptotic cells are phagocytosed and eliminated rapidly by neighboring cells and recruited professional phagocytes, such as macrophages (17, 18). They also exert profound effects on the immune responsiveness of cells which they (or the vesicles ["bodies"] they shed) contact. The recognition of apoptotic cells (and apoptotic bodies) elicits the active suppression of inflammatory and other immune responses ("the calming touch of death"), termed Innate Apoptotic Immunity (IAI; refs. 19-27). It is not an overstatement to say that this represents the final step – and perhaps the ultimate objective - of the apoptotic program.

We speculated that an alteration of IAI in macrophages might underlie the phenomenon of aging-associated immunosenescence. In this report, we examined a variety of macrophage activities comprehensively, as a function of animal age. In particular, we evaluated macrophage responsiveness to TLR ligands, innate apoptotic immune responsiveness, as well as TLR cell surface expression, and phagocytic activity directed to apoptotic cells. Although we observed no profound aging-associated alterations in these intrinsic macrophage responses, we did find that macrophages from aged mice do exhibit subtle functional alterations, especially with regard to the plasticity with which they are able to respond adaptably.

Our studies concentrated on approaches that allowed analyses of macrophages on a single-cell level, so as to reveal potential heterogeneities within responder populations. In order to evaluate the responsiveness of macrophages to a variety of treatments and doses in a statistically significant manner and to minimize animal usage, we found it necessary technically to work with relatively large (i.e. $\sim 5 \times 10^6$) primary macrophage populations from single animals *ex vivo,* and to prepare replicate populations from independent animals of an age cohort. This is greater than the number of resident macrophages that can be recovered from any unmanipulated site of a single animal, necessitating the use of thioglycollate elicitation to boost the number of recovered cells. An alternative strategy, involving the differentiation *in vitro* of bone marrow-derived macrophages, obviates issues of aging (data not shown) and was abandoned as not appropriate for these studies. In later experiments, we were able to recover sufficient numbers of resident peritoneal and splenic macrophages for some analyses.

B. RESULTS

i. Single-cell analysis of aging-associated innate macrophage responsiveness.

We evaluated the aging-associated alterations in peritoneal (both elicited and resident) macrophage responsiveness. Macrophages were recovered from male and female C57BL/6 mice. The mean lifespan of these long-lived mice is about 26 months (see http://research.jax.org/faculty/harrison/ger1vi_LifeStudy1.html). We isolated macrophages from C57BL/6 mice at discreet times throughout adult life: 2 - 3 months ("Young"), 14 - 15 months ("Middle-Aged"), and 24 - 25 months ("Old") of age. As we have shown previously, the TLR-dependent induction of proinflammatory cytokine gene transcription is attenuated by macrophage interaction with apoptotic cells ("apoptotic targets") specifically (26). Indeed, while the transcription of genes encoding proinflammatory cytokines is repressed by apoptotic cell interactions, the interaction with apoptotic cells triggers the induction of anti-inflammatory gene transcription in macrophages (**Figure XXII**). Both of these responses occur even when protein synthesis is inhibited with cycloheximide, indicating that they are primary ("immediate-early") responses to apoptotic recognition, occurring without a requirement for intervening translation.

Our detailed characterization of aging-associated macrophage responsiveness relied on the cytofluorimetric analysis of intracellular cytokine levels in single cells. We chose this approach so as to be able to address potential heterogeneity within responder populations (thereby obviating confounding issues relating to the analysis of unfractionated cell populations that exhibit alterations in an aging-associated manner [see Discussion below]). For this work, we assessed intracellular levels of the proinflammatory cytokines Tumor Necrosis Factor–α (TNF-α), Interleukin–6 (IL-6), and Interferon–α (IFN-α). We also evaluated expression of the anti-inflammatory cytokine Interleukin–10 (IL-10).

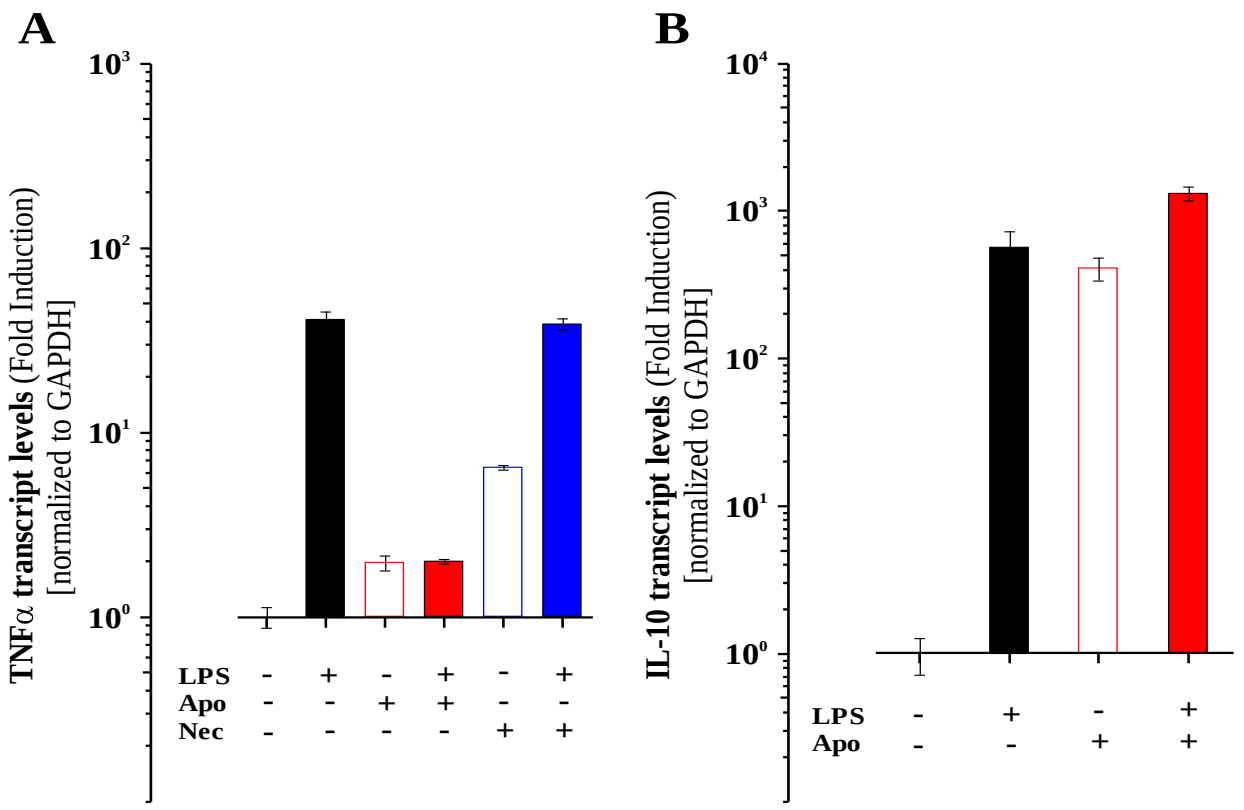


Figure XXII: Measures of macrophage responsiveness: The anti-inflammatory modulation exerted by apoptotic corpses is manifest on the level of cytokine gene transcripts within macrophages. Responses of elicited peritoneal macrophages from young mice to TLR-dependent stimulation and apoptotic cell interactions were measured by analyzing cytokine transcript levels by qRT-PCR. Elicited peritoneal Macrophages were incubated without (□□□) or with (■■■) 100ng/ml LPS in the presence of apoptotic targets (□■) or necrotic targets (□■) or without targets (□■) (targets were derived from Jurkat T cells; target to macrophage ratio of 6:1). Cells were lysed at 3 hr. post addition of targets, RNA was prepared, and macrophage-specific TNF-α (**A**) and IL-10 (**B**) transcripts were quantified by qRT-PCR analysis.

As shown in **Figure XXIII**, we observed that the TNF-α response of elicited peritoneal macrophages from young mice provided a robust measure of TLR responsiveness (in this case, TLR4-dependent responsiveness induced with bacterial lipopolysaccharide [LPS]) as well as apoptotic modulation. We have utilized this response principally in the analyses below. While IL-6 and IL-10 levels also were altered by apoptotic cells and TLR stimulation, consistent with previous results indicating induction of both by TLR agonists and apoptotic cell repression of IL-6 and induction of IL-10 (20, 67-71), those responses appeared very modest by intracellular staining (see below, **Figures XXV** and **XXXI**). Under the conditions employed, which facilitated effective intracellular staining of other cytokines, we were unable to detect significant intracellular IFN-α levels.

ii. Innate apoptotic immune responsiveness in macrophages is not altered substantially with age.

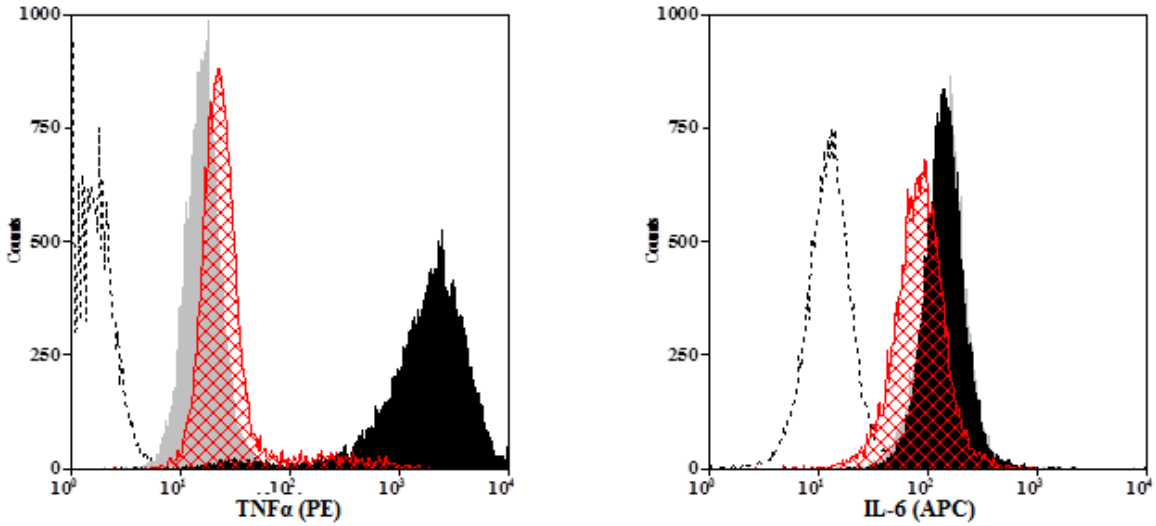
We examined the apoptotic cell-mediated regulation of intracellular levels of cytokines in LPS-treated elicited peritoneal macrophages. We compared the ability of elicited peritoneal macrophages from young and old mice to respond to different concentrations of apoptotic splenocytes ("apoptotic targets").

It was possible to quantify apoptotic suppression employing two distinct methods of analysis. Most simply, evaluating the mean fluorescence intensity (MFI) of, in this case, TNF-α staining for the entire macrophage ($F4/80^+$) population (55-57), we calculated apoptotic suppression as the diminution in the MFI for TNF-α staining of a treated sample, relative to the MFI for TNF-α staining of the maximally (LPS only) stimulated culture. All values were corrected for the "background" MFI of the unstimulated control culture. Thus,

Eq. 6.1: $\text{Apoptotic Suppression}_{MFI} = 1 - \{(MFI_{Sample} - MFI_{Untreated}) / (MFI_{LPS} - MFI_{Untreated})\}$

A – TNF-α

B – IL-6



C – IL-10

D – IFN-α

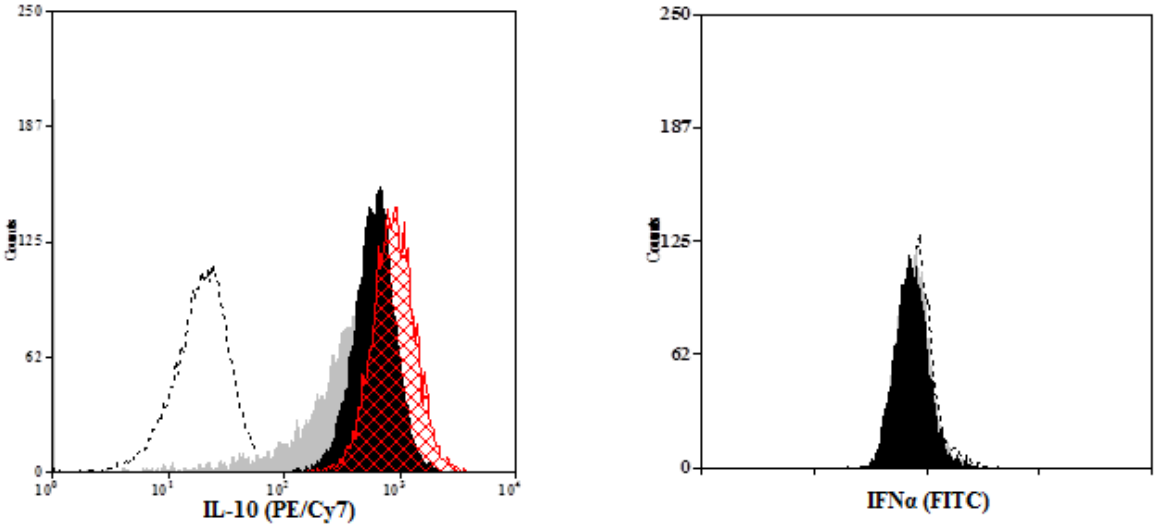


Figure XXIII: Measures of macrophage responsiveness: Cytofluorimetric analyses. Responses of elicited peritoneal macrophages from young mice to TLR-dependent stimulation and apoptotic cell interactions were measured by intracellular staining for cytokines. Elicited peritoneal Macrophages were incubated with or without LPS and apoptotic targets (target to macrophage ratio of 6:1) for 5 hr; Brefeldin A (5 µg / ml) was included during the last 3 hr. of incubation. Cells then were fixed, permeabilized, and stained. Intracellular levels of TNF-α (**A**), IL-6 (**B**), IL-10 (**C**) and IFN-α (**D**) are indicated for gated F4/80^{+} cells. In all panels, results are presented for unstimulated macrophages (gray, solid histogram), macrophages stimulated with LPS alone (black, solid histogram), macrophages stimulated with LPS and apoptotic targets (red, hatched histogram), and macrophages stained with an isotype control antibody (black, dashed line).

Alternatively, taking into account heterogeneity of responsiveness within the macrophage population, we calculated the fraction of $F4/80^+$ cells that were positive by intracellular TNF-α staining. For each experiment, we determined the MFI value for TNF-α staining that separated the brightest (~ 2-5%) of untreated $F4/80^+$ cells from the vast majority, and used that value as the cutoff for categorizing cells as TNF-α^+. With that cutoff, ~ 95-99% of the LPS-treated population was identified as TNF-α^+. We then calculated apoptotic suppression as the diminution in the fraction of TNF-α^+ macrophages in a treated sample, relative to the fraction of TNF-α^+ macrophages in the maximally stimulated culture. In this analysis, then,

Eq. 6.2: $\text{Apoptotic Suppression}_{\text{Fractional Diminution}} = 1 - \{(\text{TNF-}\alpha^+ \text{ cells})_{\text{Sample}} / (\text{TNF-}\alpha^+ \text{ cells})_{\text{LPS}}\}$

In both cases, the extent of apoptotic suppression was normalized to the unsuppressed (LPS-stimulated without apoptotic cells) condition. As is clear from the data presented below, both analyses (see **Figures XXIV**, **XXV**, **XXVIII**, **XXIX**) led to the same conclusions.

Data from representative experiments examining the suppression of intracellular levels of TNF-α in elicited peritoneal macrophages from young and old mice mediated by apoptotic cells are presented in **Figure XXIV - A** and **Figure XXIV - B** respectively. It is clear from these plots that elicited peritoneal macrophages from both young and old mice are essentially indistinguishable with respect to their dose-dependent responsiveness to apoptotic splenocytes, at least in terms of TNF-α expression.

Multiple experiments extended and confirmed these observations. Compiled data from independent experiments involving 5 young mice, 5 middle-aged mice, and 5 old mice demonstrate that no significant aging-associated differences exist in the apoptotic cell-mediated suppression of TNF-α expression in macrophages **Figure XXIV – C**. In addition, no gender-specific differences in responsiveness were observed (data not shown).

A – Apoptotic suppression in macrophages from young mice

B – Apoptotic suppression in macrophages from old mice

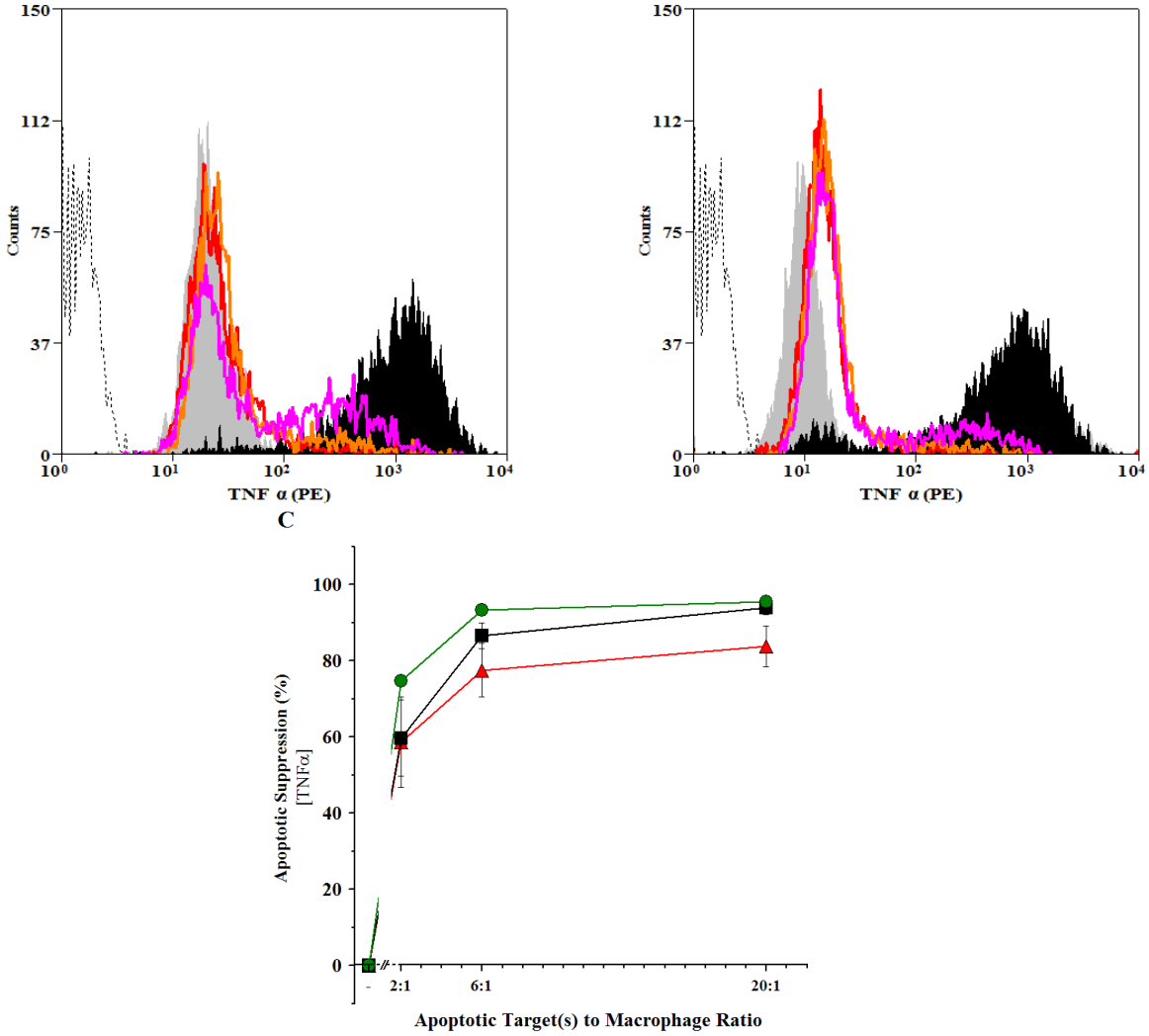


Figure XXIV: Apoptotic suppression of TNF-α in macrophages is not altered with age. Examples of cytofluorimetric analyses of individual experiments evaluating apoptotic suppression in elicited peritoneal F4/80$^+$ macrophages from young and old mice are presented in Panels **A** and **B**, respectively. Results are presented for unstimulated macrophages (gray, solid histogram), macrophages stimulated with LPS alone (black, solid histogram), macrophages stimulated with LPS and apoptotic cells (apoptotic target to macrophage ratio = 2:1 pink, open histogram; apoptotic target to macrophage ratio = 6:1 orange, open histogram; apoptotic target to macrophage ratio = 20:1 red, open histogram); and macrophages stained with an isotype control antibody (black, dashed line). Compiled data from elicited peritoneal macrophages from young (■), middle aged (●), and old (▲) mice, stimulated with LPS in the absence and in the presence of apoptotic splenocytes (from young mice), at the indicated ratios of apoptotic targets to macrophages, for 5 hr also is shown (**C**). Cells then were stained for TNF-α. Apoptotic suppression was calculated for gated F4/80$^+$ cells by fractional diminution analysis, as described in the text (Eq. 6.2). Macrophages from individual mice (n = 7 per age cohort) were handled separately. There was no statistical significance ($p > 0.05$ [P value = 0.2874]) between the age groups as seen by 2-way ANOVA between the age groups.

Notably, we observed no aging-associated differences in macrophage responsiveness to LPS stimulation (note **Figures XXIV – B** & **C**). This was surprising in light of previous reports (5, 32, 33, 35, g, h) that have suggested that TLR responsiveness in macrophages is impaired in an aging-associated manner. This contention prompted us to explore the issue of macrophage TLR responsiveness more comprehensively (see below: Chapter VI, section vii).

We extended our examination of apoptotic cell-mediated modulation of macrophage responsiveness with age by evaluating the effects on the expression of additional cytokines. Just as the suppression by apoptotic cells of TNF-α expression in macrophages was unaltered as a function of animal age, apoptotic cell suppression of IL-6 expression also was unaffected by age (data not shown). In contrast to the apoptotic suppression of proinflammatory cytokine expression, apoptotic cells induce the expression of anti-inflammatory cytokines, such as IL-10 (67, 69). We examined this apoptotic cell-mediated modulation of IL-10. While the IL-10 response of elicited peritoneal macrophages from young and old mice to apoptotic cells in the presence of LPS is modest, apoptotic cells are able to up-regulate IL-10 in elicited peritoneal macrophages from both young and old mice to similar levels (**Figure XXV**). Thus, by several criteria, macrophages appear to suffer no substantial alteration in their responsiveness to apoptotic cells with age.

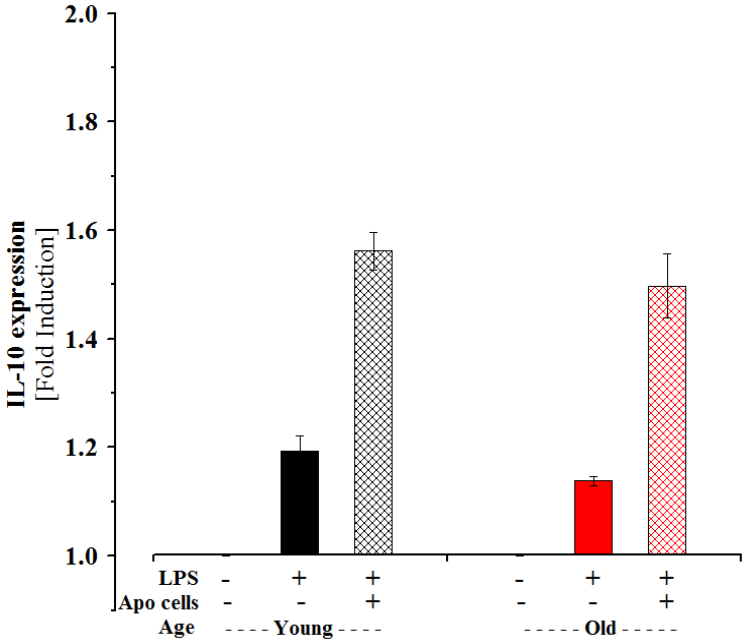


Figure XXV: Apoptotic cell-mediated up-regulation of IL-10 in macrophages is not altered with age. Elicited peritoneal macrophages from young (2 - 3 mo.) and old (24 -25 mo.) mice were treated without or with LPS in the absence (solid bars) and in the presence of (cross hatched bars) apoptotic splenocytes from young mice (at a ratio of apoptotic targets to macrophages of 20:1) for 5 hr. Cells then were stained for IL-10. Calculation of relative IL-10 expression was by MFI analysis of gated F4/80^{+} cells, normalized to unstimulated controls, as described in the text (Eq. 6.1). Macrophages from individual mice (n = 6 per age cohort) were handled separately.

iii. *Immunosuppressive activity is particular to apoptotic cells.*

Cell death is an ongoing process physiologically in metazoan organisms; those cell deaths occur pre-dominantly by apoptosis. Cells also die pathologically (necrotic cell death), however. Immune pathologies, including elevated levels of inflammatory cytokines, associated with immunosenescence might reflect immune responses altered by the interaction of macrophages with cells dying pathologically. On the other hand, previous work from our laboratory (20) has shown that necrotic cells do not suppress innate immune responsiveness of macrophages, such as the production of TNF-α and other cytokines, in response to TLR agonists, such as LPS.

We examined the consequences of the interaction of macrophages from young and old mice with necrotic cells, in comparison with apoptotic cells. In contrast to the anti-inflammatory effects that apoptotic cells exert (**Figures XXIV** and **XXV**), interactions with necrotic cells did not alter expression of TNF-α in macrophages, and this was the case for macrophages from young and old mice (**Figure XXVI**). Necrotic cell interactions also had no effect on the expression in macrophages of IL-6 and IL-10, independent of animal age (data not shown). Importantly, these data suggest that the selective discrimination that macrophages make among dying cells is not altered in an aging-associated manner (see next).

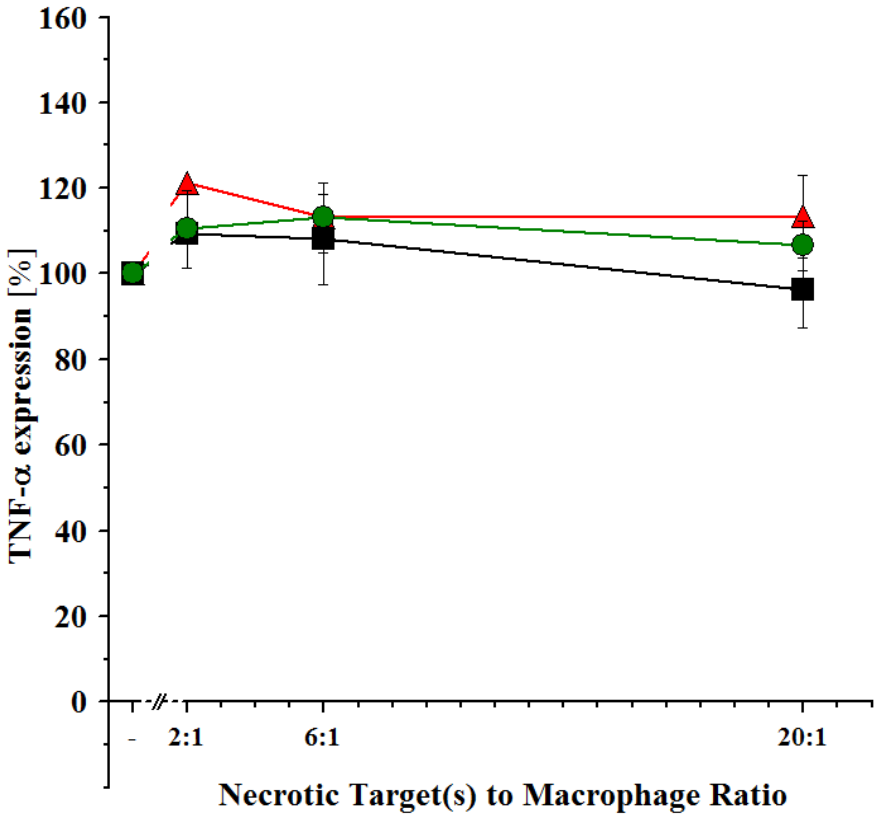


Figure XXVI: Interaction with necrotic cells does not alter macrophage responsiveness. Elicited peritoneal macrophages from young (■), middle aged (●), and old (▲) mice, were treated without or with LPS in the absence and in the presence of necrotic splenocytes (from young mice, at the indicated ratios of necrotic targets to macrophages) for 5 hr. Cells then were stained for TNF-α. Calculation of relative TNF-α expression was by MFI analysis of gated $F4/80^{+}$ cells, normalized to unstimulated controls, as described in the text (Eq. 6.1). Macrophages from individual mice (n = 6 per age cohort) were handled separately. There was no statistical significance ($p > 0.05$) between the age groups as seen by 2-way ANOVA between the age groups.

iv. Macrophage recognition of apoptotic targets, linked to phagocytosis, is not altered with age.

In addition to the modulation of immune responsiveness, recognition of apoptotic cells by macrophages leads to their phagocytosis. Other dead cells that do not modulate immune responsiveness, and pathogens which elicit innate immune responses, also are phagocytosed by macrophages, underscoring the independence of the processes of apoptotic immunomodulation and phagocytosis (26). Especially in this context, macrophages are sentinels for immune surveillance. Phagocytosis is essential for antigen presentation, often leading to robust adaptive immune responses. The clearance of apoptotic cells, in contrast, provides a continuous supply of self-peptides which, especially in the absence of pathogenic adjuvants, promotes self-tolerance.

Our analyses of innate apoptotic responsiveness indicated that recognition of apoptotic targets by macrophages was not altered with age. As an independent approach to evaluating apoptotic recognition, we analyzed apoptotic cell phagocytosis by macrophages as a function of age (**Figure XXVII**). Further, we imagined that an alteration in the phagocytosis of apoptotic cells could be responsible for some immunosenescence-associated autoimmune pathologies.

We observed that elicited peritoneal macrophages phagocytose apoptotic (**Figure XXVII - A**) as well as necrotic (**Figure XXVII - B**) targets comparably (see also 20). These recognition and phagocytic activities were equivalent in macrophages from young and old mice (**Figure XXVII - A** & **B**), indicating that neither macrophage phagocytic activity, nor the selective discrimination by macrophages among dying cells, is altered in an aging-associated manner.

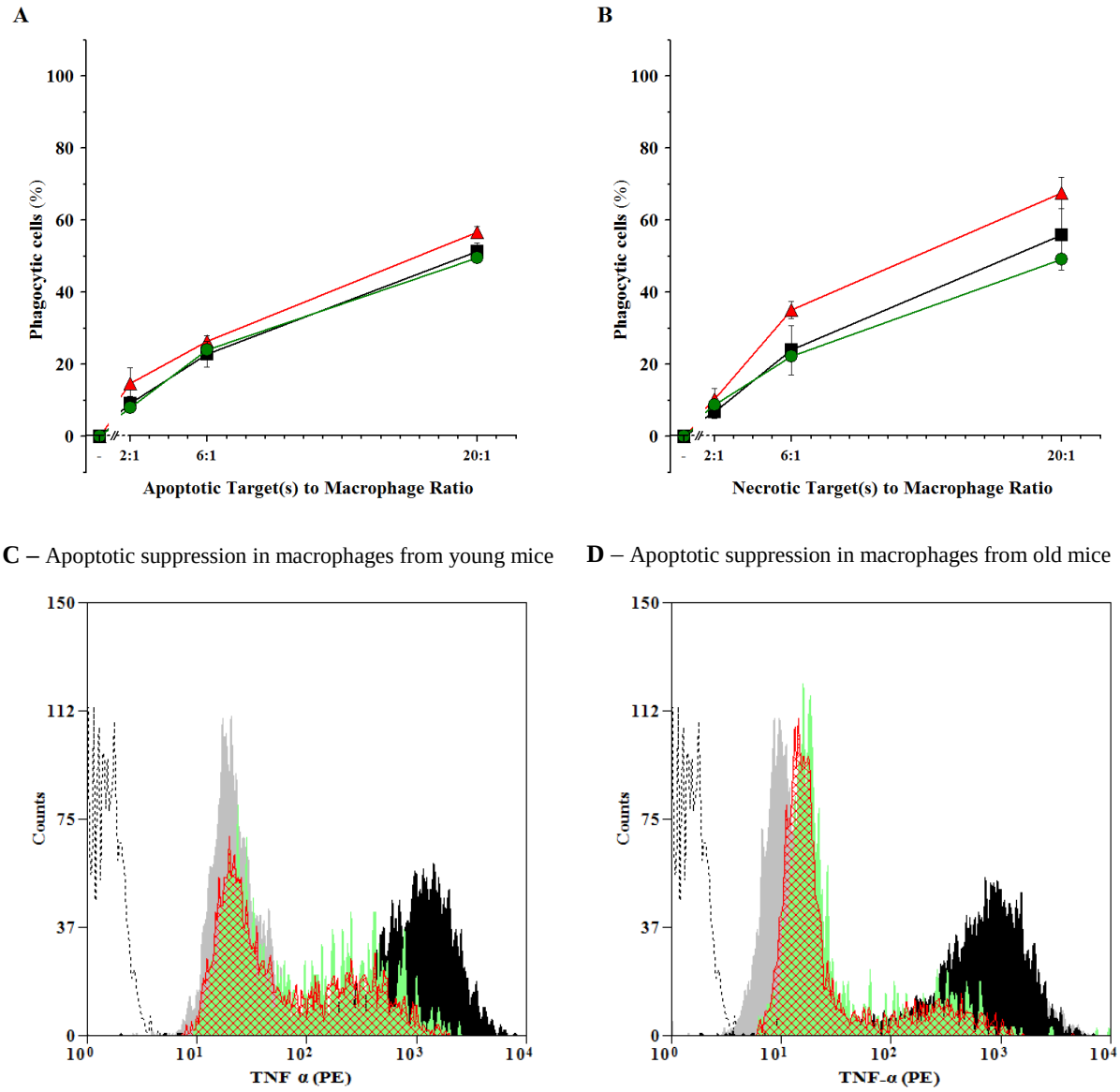


Figure XXVII: Macrophage recognition of apoptotic targets, linked to phagocytosis or immune modulation, is not altered with age. **A**, **B**) Elicited peritoneal macrophages from young (■), middle aged (●), and old (▲) mice, were incubated for 4 hr. with labeled CFSE-labeled apoptotic splenocytes (**A**) or necrotic splenocytes (**B**) at the indicated target to macrophage ratios. Phagocytosis was assessed cytofluorimetrically, as described in Chapter III. **C**, **D**) Cells were stimulated with LPS or not during the phagocytosis reaction, and then were stained for TNF-α expression. Apoptotic suppression and phagocytosis were evaluated in the same macrophages cytofluorimetrically. Results from TNF-α staining are shown for F4/80$^+$ cells from young (**C**) and old (**D**) mice: macrophages not treated with LPS (gray, solid histogram), macrophages stimulated with LPS alone (black, solid histogram), macrophages stimulated with LPS together with apoptotic targets (red, cross-hatched); macrophages stimulated with LPS that had phagocytosed apoptotic targets (green solid histogram); and macrophages stained with an isotype control antibody (black, dashed line). Macrophages from individual mice (n = 4 per age cohort) were handled separately. There was no statistical significance ($p > 0.05$) between the age groups as seen by 2-way ANOVA between the age groups.

Significantly, when we compared apoptotic cell-mediated suppression in macrophages that were incubated with apoptotic targets and in those that actually had phagocytosed apoptotic targets, we found that levels of TNF-α were reduced profoundly and equivalently in both populations, independent of age (**Figure XXVII - C** & **D**). This observation extends our previous findings that apoptotic cell-mediated suppression of inflammatory responses occurs in a process independent of apoptotic cell engulfment (26; see Chapter IV), and indicates that both processes are unaltered with age.

v. Apoptotic cell immunosuppressive activity is elevated slightly with age.

We have shown previously that the immunosuppressive activity of apoptotic cells arises during the process of apoptotic cell death in all cells, independent of the tissue type of origin (26, 27). Consistent with that work, we found in these experiments that apoptotic cells derived from thymocytes were as effective as apoptotic cells prepared from splenocytes with respect to their abilities to exert immunomodulatory effects on primary macrophages from animals of different ages (data not shown).

Just as we tested whether primary macrophages recovered from mice were altered as a function of animal age with respect to their responsiveness to [young] apoptotic cells, we wondered whether the ability of apoptotic cells to elicit immunosuppressive responses was subject to any aging-associated alteration. We therefore quantified apoptotic suppression in macrophages as a function of apoptotic target dose with apoptotic cells prepared from splenocytes recovered from young and old mice. As shown in **Figure XXVIII – A** & **B** (using TNF-α expression as the most robust readout for apoptotic suppression), we observed a significant difference in the effectiveness of apoptotic targets generated from old mice as compared with apoptotic targets prepared from young mice.

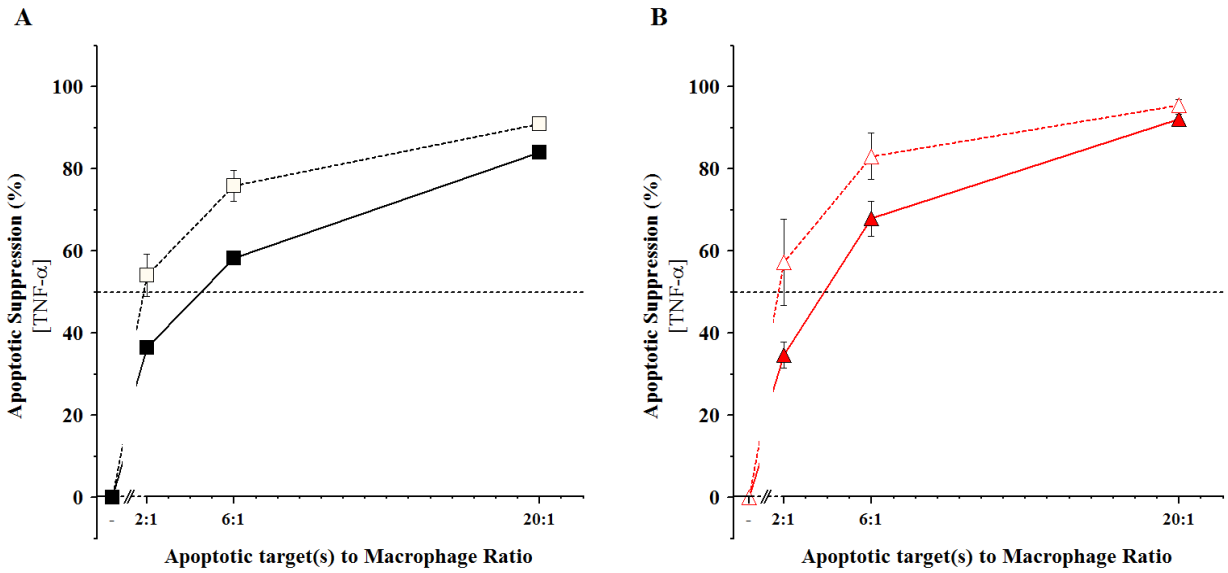


Figure XXVIII: Apoptotic targets derived from young and old mice behave similarly with regard to innate apoptotic modulation. Elicited peritoneal macrophages from young (**A**) and old (**B**) mice were treated without or with LPS in the absence and in the presence of apoptotic splenocytes from young (■, ▲) and old (□, △) mice (at the indicated ratios of apoptotic targets to macrophages) for 5 hr. Cells then were stained for TNF-α. Apoptotic suppression was calculated for gated F4/80^{+} cells by fractional diminution analysis, as described in the text (Eq. 6.2). Macrophages from individual mice (n = 4 per age cohort) were handled separately. P value for apoptotic suppression is < 0.01 between apoptotic splenocytes from young (■, ▲) and old (□, △) mice in elicited peritoneal macrophages from young (**A**) and old (**B**) mice as seen by 2-way ANOVA between the age groups.

Quantification of this unexpectedly enhanced activity suggests that, on a per cell basis, apoptotic cells from old animals have ~2 fold (?) more immunosuppressive activity than do apoptotic cells from young animals. Experiments were performed in a criss-cross fashion with responder macrophages from young (**Figure XXVIII – B)** and old (**Figure XXVIII – A**) mice, and the age-associated difference in immunosuppressive activity of apoptotic cells was observed consistently.

vi. The plasticity of macrophage responsiveness is reduced with age.

Macrophages are heterogeneous and flexibly responsive cells that respond nimbly to environmental signals. Indeed, it is their plasticity of responsiveness that enables them to serve so effectively as immune sentinels (40, 41). In tissues, mononuclear phagocytes respond to environmental cues (e.g., microbial products, damaged cells, activated lymphocytes) with the acquisition of distinct functional phenotypes. In response to various signals, macrophages may undergo classical, "M1" activation (stimulated by TLR ligands and IFN-γ) or alternative, "M2" activation; these states mirror the Th1 – Th2 polarization of T cells (37-39). We have shown that macrophages that interact with apoptotic cells, even in the presence of TLR agonists, acquire an anti-inflammatory phenotype. This is characterized by the up-regulation in anti-inflammatory mediators (such as TGF-β, IL-10, Arginase II, etc.) as well as a down-regulation in proinflammatory mediators (such as TNF-α, IL-6, IL-12, iNOS, etc.). This is similar to the M2 state (42, 43), contrasting with the M1-like state that results from simple TLR stimulation.

The lifetime of the anti-inflammatory state triggered by a single encounter with apoptotic cells has not been established. We speculated that the duration of this anti-inflammatory state could impact systemic inflammation, and that its fragility might promote the immune dysregulation associated with immunosenescence. Consequently, we evaluated the lifetime

("persistence") of the anti-inflammatory state in macrophages as a function of age by testing the ability of macrophages to recover responsiveness to TLR-dependent challenge at varying times after encounter with apoptotic targets.

Our data (**Figure XXIX**) indicate that the lifetime of the anti-inflammatory state triggered by apoptotic targets is relatively brief ($t_{½}$ = ~45 min in macrophages from young mice), and that this persistence is enhanced in macrophages from older animals ($t_{½}$ = 3 hrs. in macrophages from old mice).

vii. The expression and function of Toll-like receptors is not impaired with age.

In view of previous reports (5, 32, 33, 35, g, h) suggesting that TLR responsiveness in macrophages is impaired in an aging-associated manner, we were surprised that we observed no aging-associated differences in macrophage responsiveness to LPS stimulation (see **Figures XXIX**). Consequently, we chose to examine the issue of macrophage TLR responsiveness more comprehensively, utilizing a cytofluorimetric approach so as to be able to assess macrophage responsiveness on a single-cell level. We compared the abilities of elicited peritoneal macrophages from young and old mice to respond *ex vivo* to a variety of TLR stimuli: the TLR4 agonist LPS; heat-killed *Listeria monocytogenes* [HKLM] and Zymosan, TLR2 agonists; the synthetic acylated lipopeptides Pam2CSK4, an agonist for TLR2/6, and Pam3CSK4, an agonist for TLR1/2; the TLR7/8 agonist R848, and Flagellin, an agonist for TLR5.

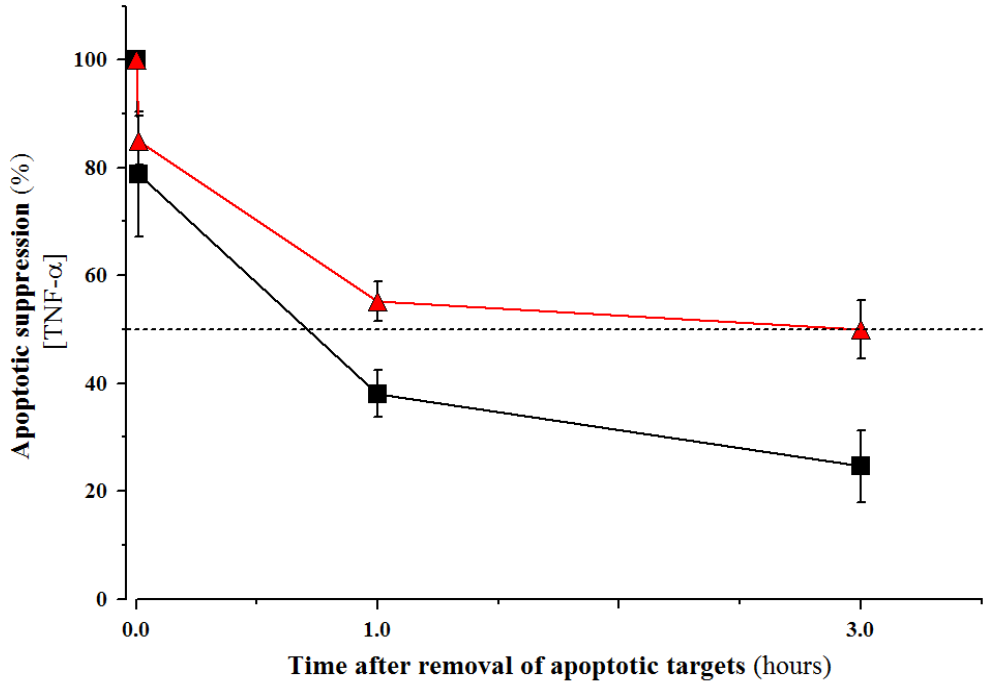


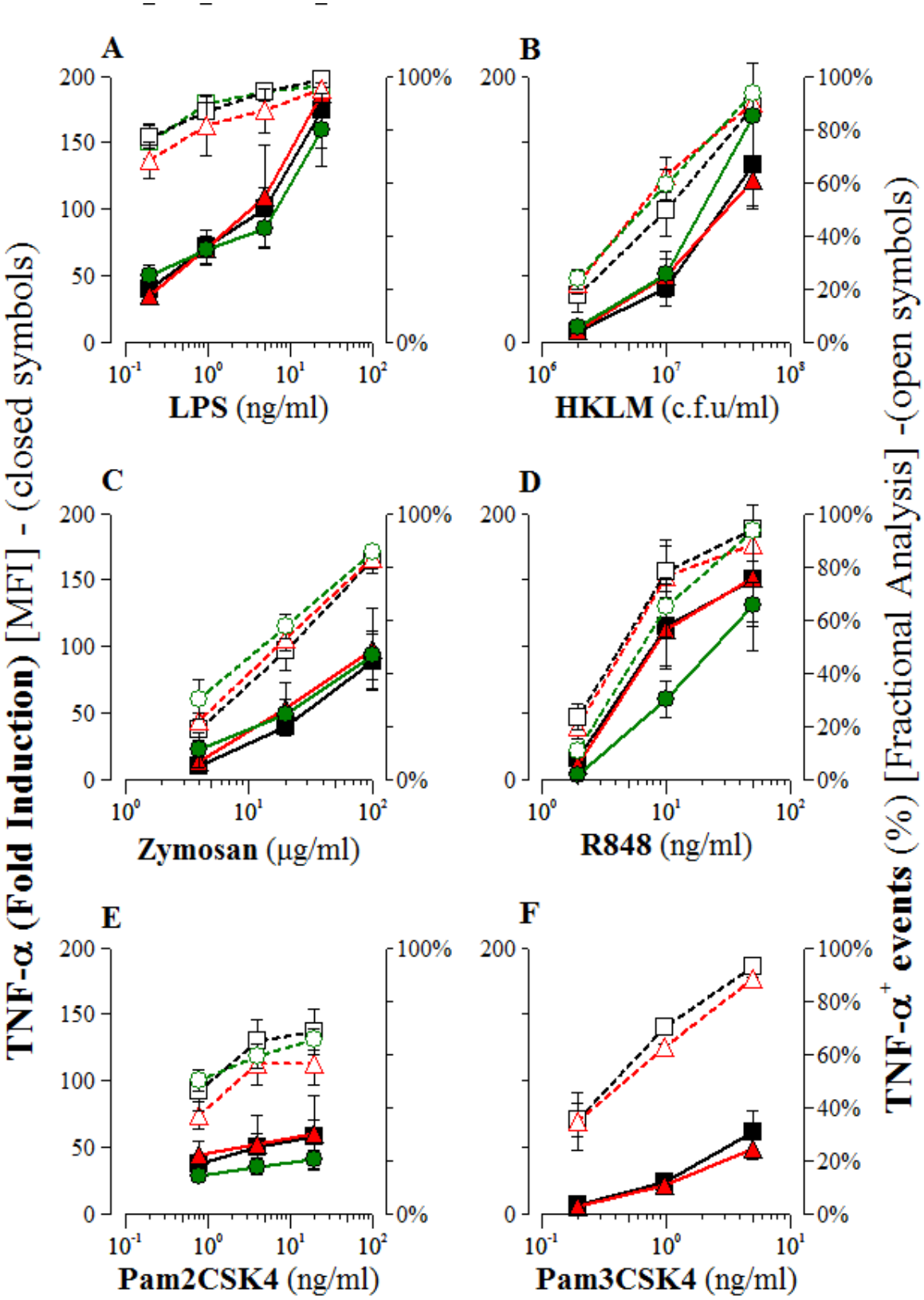
Figure XXIX: Aging-associated persistence of apoptotic suppression in macrophages. Elicited peritoneal macrophages from young (■) and old (▲) mice were treated without or with apoptotic splenocytes (at a ratio of apoptotic targets to macrophage of 6:1) for 16 hr. Macrophages then were washed to remove the target cells and stimulated with LPS at various times after target removal for 5 hr. Finally, cells were stained for TNF-α. Apoptotic suppression was calculated for gated F4/80$^+$ cells by fractional diminution analysis, as described in the text (Eq. 6.2). Macrophages from individual mice (n = 6 per age cohort) were handled separately. P value for aging-associated persistence of apoptotic suppression is < 0.01 (statistically significant) between the two age groups as seen by 2-way ANOVA.

Data from representative experiments examining intracellular levels of TNF-α in elicited peritoneal macrophages from young and old mice in response to HKLM stimulation are presented in **Figure XXX - G** and **Figure XXX - H**, respectively. It is evident from these plots that macrophages from young and old animals have very similar dose-dependent TNF-α responses, including comparable basal levels (see below and **Figure XXXII**). Compiled data from independent experiments involving multiple mice extended and confirmed these observations, demonstrating that no significant aging-associated differences exist in the TLR responsiveness of elicited peritoneal macrophages (**Figure XXX - A to F)**. We quantified TLR-dependent responsiveness by two distinct analytical methods, comparable to the approaches we took to the analysis of apoptotic suppression (above: Chapter IV, section ii), evaluating the mean fluorescence intensity (MFI) of cytokine (especially TNF-α) staining for the entire macrophage ($F4/80^+$) population (see Eq. 6.3), or by calculating the fraction of $F4/80^+$ cells that were positive by intracellular cytokine staining (see Eq 6.4).

Eq. 6.3: $\text{Fold Induction}_{MFI} = \{(MFI_{Sample} - MFI_{Untreated}) / MFI_{Untreated}\}$

Eq. 6.4: $\text{Population Response}_{\text{Fractional Analysis}} = \{(\text{TNF-}\alpha^+ \ F4/80^+ \text{ cells})_{Sample} / (F4/80^+ \text{ cells})_{Total}\}$

The compiled data document quantitatively that no significant differences distinguish TLR responsiveness of macrophages from young and old animals. It also is evident that the results of the two analytical approaches yield the same conclusions. Again, no gender-specific differences in macrophage responsiveness were observed (data not shown). We also observed no statistical differences in TLR responsiveness (as measured by TNF-α expression) in resident peritoneal macrophages, as well as splenic macrophages, from young and old mice, relative to parallel measurements in elicited peritoneal macrophages (data not shown).



(Figure XXX Cont…)

G –HKLM responsiveness in macrophages from young mice

H – HKLM responsiveness in macrophages from old mice

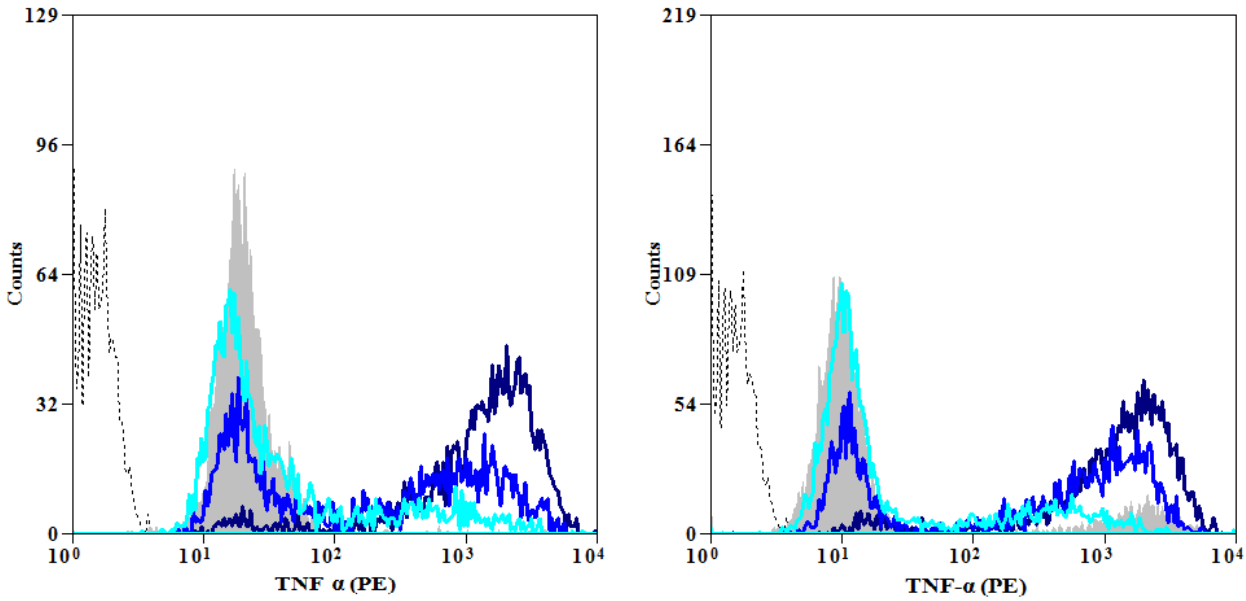


Figure XXX: TLR responsiveness in macrophages is unaltered with age. Examples of cytofluorimetric analyses of individual samples evaluating responsiveness to HKLM in F4/80$^+$ elicited peritoneal macrophages from young and old mice are presented in Panels **G** and **H**, respectively. Results are presented for unstimulated macrophages (light gray, solid histogram); macrophages stimulated with HKLM, 5×10^7 CFU / ml (dark blue, open histogram); macrophages stimulated with HKLM, 1×10^7 CFU / ml (blue, open histogram); macrophages stimulated with HKLM, 2×10^6 CFU / ml (aqua, open histogram); and macrophages stained with an isotype control antibody (black, dashed line). Compiled data from elicited peritoneal macrophages from young (■□), middle-aged (●○) and old (▲△) mice, treated with the TLR agonists LPS (**A**), HKLM (**B**), Zymosan (**C**), R848 (**D**), Pam2CSK4 (**E**), and Pam3CSK4 (**F**), at the indicated doses for 5 hr also is shown. After incubation, cells were stained for TNF-α. The responsiveness of gated F4/80$^+$ cells, as indicated by relative TNF-α expression ("fold induction" relative to unstimulated cells), calculated by MFI analysis, as described in the text (Eq. 6.3), is represented with filled symbols; population responsiveness, calculated by fractional analysis as in Eq. 6.4, is represented with open symbols. Macrophages from individual mice (young: n = 6; middle-aged: n = 7; old: n = 7) were handled separately. There was no statistical significance ($p > 0.05$) between the age groups for TLR responsiveness for any of the different agonists (**A-F**), as seen by 2-way ANOVA between the age groups.

As an independent measure of the age-associated TLR responsiveness of macrophages, we examined the effects of stimulation with a variety of TLR agonists on the expression of a distinct cytokine. While the magnitude of IL-6 responsiveness is decidedly less robust than that of TNF-α (as noted above: see **Figure XXIII**), induction of this cytokine provides a reliable measure of TLR function. Just as the induction of TLR-dependent TNF-α expression in macrophages was unaltered as a function of animal age, TLR-dependent IL-6 induction also was unaffected by age (**Figure XXXI**).

While we observed no appreciable differences in macrophage TLR responsiveness as a function of age, we did find a modest but significant elevation in basal intracellular levels of TNF-α and IL-6 in unmanipulated resident peritoneal macrophages from old mice as compared to young mice (**Figure XXXII-A**). These data are consonant with observations that aging is associated with elevated levels of cytokines and inflammatory mediators, even in the absence of any overt physiological stress (9-11). (No comparable differences were found with elicited peritoneal macrophages (**Figure XXXII-B**).

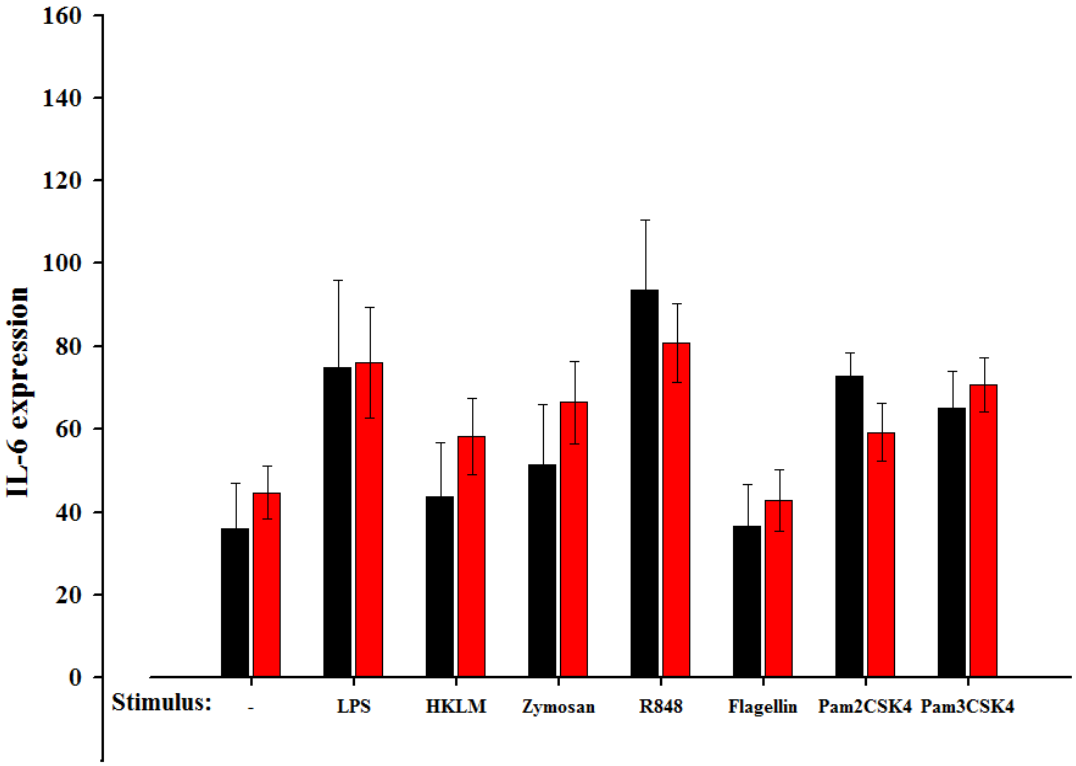


Figure XXXI: IL-6 production in macrophages also is unaltered by age. Elicited peritoneal macrophages from young (solid black bars) and old (solid red bars) mice were treated for 5 hr with the TLR agonists LPS (25 ng / ml), HKLM (5×10^7 CFU /ml), Zymosan (100 µg / ml), R848 (25 ng / ml), Flagellin (50 µg / ml), Pam2CSK4 (25 ng / ml), and Pam3CSK4 (250 ng/ ml). Cells then were stained for IL-6. Compiled data, represented as mean fluorescence intensity (MFI), is presented. Macrophages from individual mice (young: n = 6; old: n = 7) were handled separately. P value for aging-associated TLR responsiveness as measured by IL-6 production between the two age groups was not statistically significance (P value > 0.05) for any of the different TLR agonists tested, as seen by as seen by student T-test.

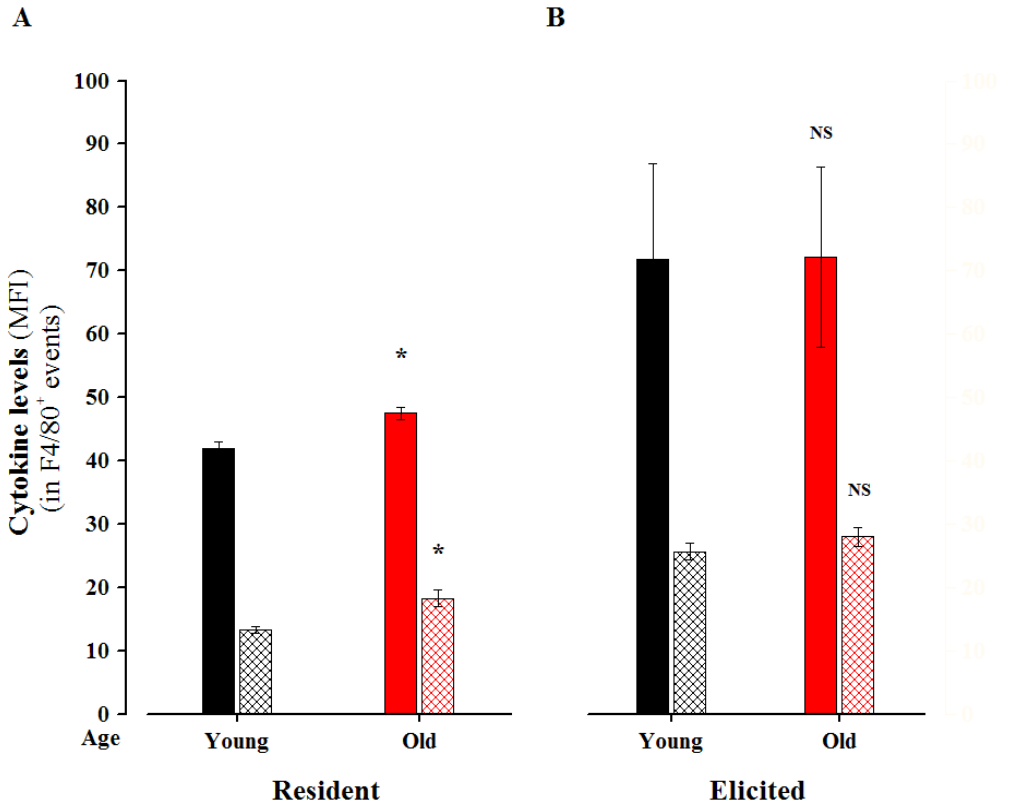


Figure XXXII: Basal cytokine levels in resident and elicited peritoneal macrophages. Resident (**A**) and elicited (**B**) peritoneal macrophages were recovered from young and old mice. Cells were stained immediately (without culturing) for TNF-α (solid bars) and IL-6 (hatched bars) levels. Compiled data, represented as the mean fluorescence intensity (MFI) for cytokine-specific staining of gated F4/80$^+$ cells, is presented. Macrophages from individual mice (resident [young and old]: n = 6 per age cohort; elicited [young and old]: n = 4 per age cohort] were handled separately. P value for basal cytokine levels in the resident peritoneal macrophages (**A**) as by TNF-α and IL-6 production is < 0.01 (* = statistically significant) between the two age groups as seen by student T-test. P value for basal cytokine levels in the elicited peritoneal macrophages (**B**) as by TNF-α and IL-6 production is > 0.01 (NS = not statistically significant) between the two age groups as seen by student T-test.

We had noticed that the yield of adherent F4/80$^+$ macrophages from the peritoneum appeared to decline with age, even as the total number of recovered cells increased. Consequently, we examined the fractional recovery of F4/80$^+$ macrophages in peritoneal lavage from both unmanipulated and thioglycollate-stimulated young and old mice. The diminution in the fraction of F4/80$^+$ cells with age was dramatic (**Figure XXXIII – A** & **B**). While the numbers of recovered macrophages from older animals was severely diminished, TLR expression on those cells (**Figure XXXIII – C** & **D**) was not reduced (and, in the case of resident macrophages, even was elevated; **Figure XXXIII – C**). These results contrast with previous conclusions reports regarding aging-associated macrophage TLR expression and function (32, 33, 35). It is worth noting that those previous conclusions, drawn from work analyzing cytokine and TLR expression in unfractionated peritoneal cell populations, may have been compromised by such aging-associated diminutions in the relevant population. We observed similar changes with age in the fractional representation of F4/80$^+$ macrophages among adherent splenic cells (data not shown).

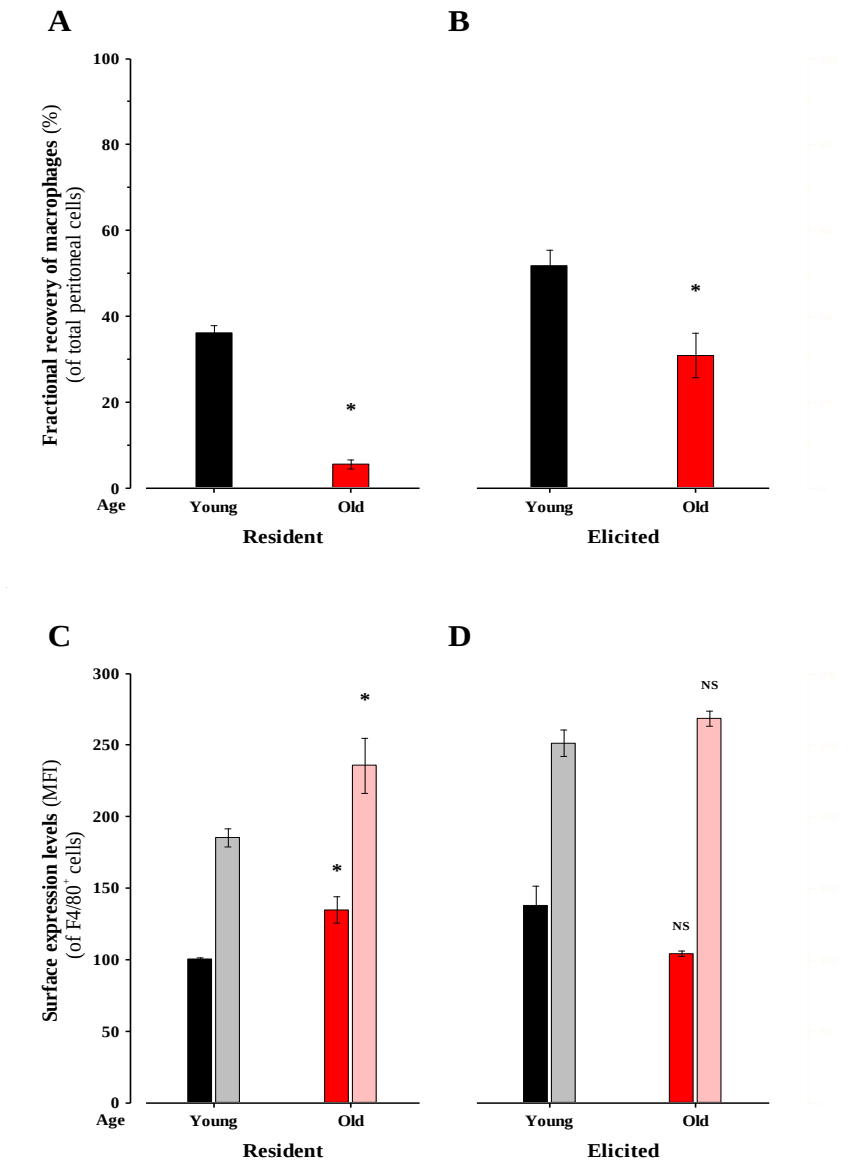


Figure XXXIII: Characterization of peritoneal macrophages. Peritoneal resident macrophages (**A**, **C**) and elicited peritoneal macrophages (**B**, **D**) were recovered from young and old mice. Cells were stained immediately (without culturing) for F4/80, TLR-2 (solid bars) and TLR-4 (hatched bars) levels. Macrophages from individual mice (resident [young and old]: n = 6 per age cohort; elicited [young and old]: n = 4 per age cohort] were handled separately. P value for fractional recovery of resident macrophages (**A**) and elicited macrophages (**B**) from the peritoneal cavity as by F4/80 staining is < 0.01 (* = statistically significant) between the two age groups as seen by student T-test. P value for basal TLR cell surface expression levels in the resident peritoneal macrophages (**C**) both for TLR-2 and TLR-4 expression is < 0.01 (* = statistically significant) between the two age groups as seen by student T-test. P value for basal TLR cell surface expression levels in the elicited peritoneal macrophages (**D**) both for TLR-2 and TLR-4 expression is > 0.01 (NS = not statistically significant) between the two age groups as seen by student T-test.

viii. Older mice exhibit other aging-associated alterations.

We were surprised that the alterations we observed in the responsiveness of macrophages from aged mice were subtle, in contrast to the pronounced aging-associated alterations that have been reported previously. Given the absence of dramatic differences in macrophage responsiveness with age, we were interested to evaluate whether the mice we studied exhibited other, well-characterized aging-associated alterations.

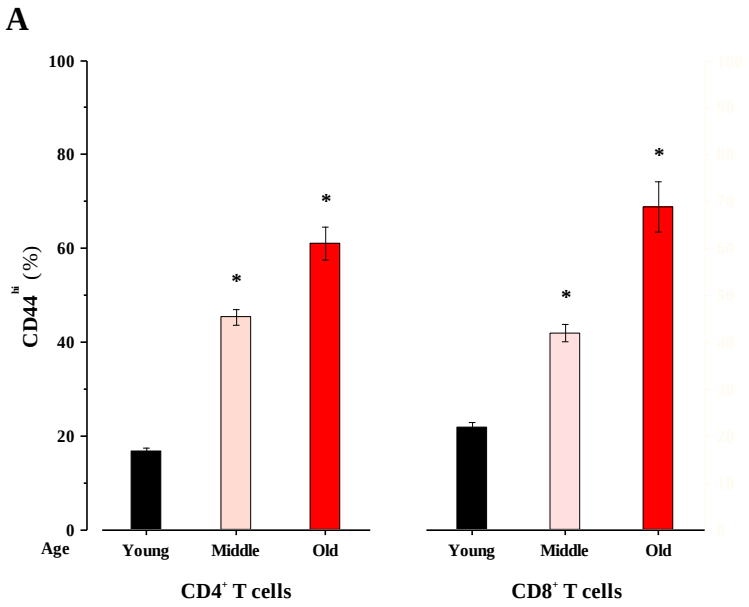
a. Expansion of previously-activated T lymphocytes with age

Alterations affecting T lymphocyte production and history are among the most dramatic aging-associated immune effects (*a*). With the decline in naïve T cell production resulting from thymic involution, a dramatic shift in the population of peripheral T lymphocytes toward a "previously activated" (memory-like) phenotype, marked by high levels of cell surface expression the glycoprotein CD44, ensues (72-74).

As expected, thymic involution was evident in our aged mice (data not shown). The fraction of T lymphocytes expressing high levels of cell surface CD44 ($CD44^{hi}$) also was greatly elevated with age (**Figure XXXIV**). Although the fractions of $CD4^+$ and $CD8^+$ T lymphocytes in the periphery did not change appreciably with age (data not shown), the expansion of the $CD44^{hi}$ subpopulation was particularly striking among $CD8^+$ T cells (**Figure XXXIV - C**).

b. Enhanced resistance with age to infection with Listeria monocytogenes

Previous work has documented that resistance to *Listeria monocytogenes* infection is enhanced unexpectedly in older mice as a consequence of the aging-associated decline in the abundance of $CD8\alpha^+$ dendritic cells, which serve as portals for *L. monocytogenes* entry. (45 - 49).



B – CD44 surface expression on $CD4^+$ splenic T cells

C – CD44 surface expression on $CD8^+$ splenic T cells

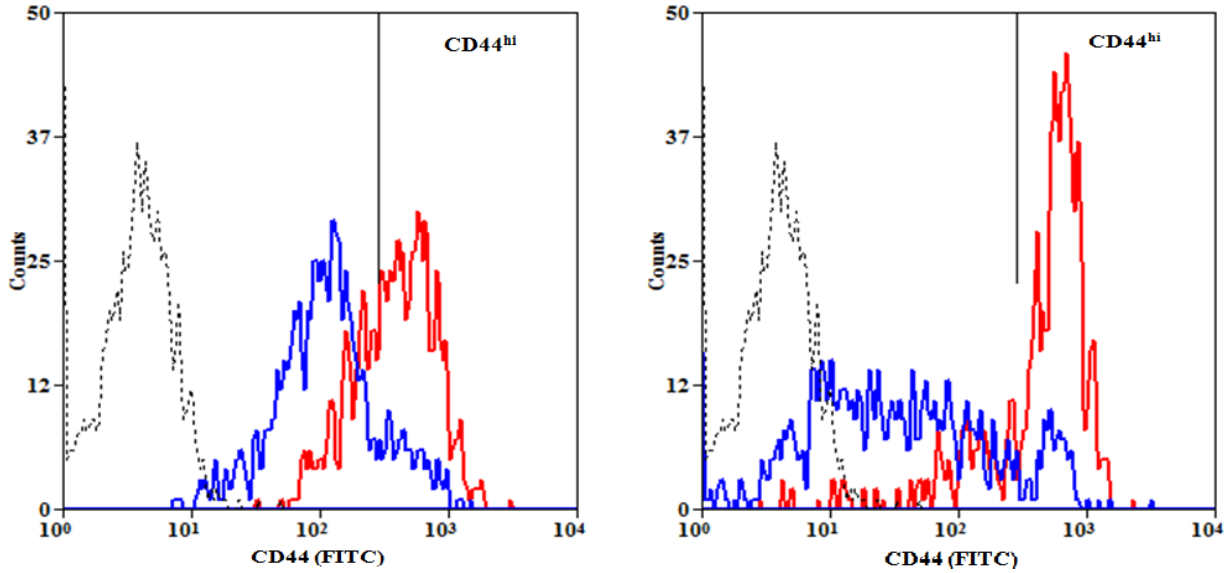


Figure XXXIV: Aged mice exhibit increased memory T cells population. CD44 expression on $CD4^+$ (**A**, left) and $CD8^+$ (**A**, right) T lymphocytes from the spleens of young (black solid bars), middle-aged (red hatched bars) and old (red solid bars) mice was evaluated cytofluorimetrically. The fractions of T lymphocytes expressing high cell surface levels of CD44 (see next) were tabulated. Splenocytes from individual mice (n = 4 per age cohort] were handled separately. Examples of cytofluorimetric analyses of individual samples evaluating CD44 surface expression on $CD4^+$ and $CD8^+$ T lymphocytes are presented in Panels **B** and **C**, respectively (dotted line – Isotype control; Blue line – 2 – 3 mo. mice; Red line – 23 - 24 mo. mice). P value for $CD44^{hi}$ cell surface expression levels on CD4+ and CD8+ T cells (**A**) is < 0.001 (* = statistically significant) between the both young and middle-aged groups, as well as between young and old age groups as seen by student T-test.

We confirmed this observation with our mice (**Figure XXXV**). An infectious dose of about 2×10^5 *L. monocytogenes* cells was lethal to young mice within three days, but not to old mice (although the infected old mice evinced signs of morbidity, including lethargy, at that time). Young mice survived with a five-fold lower infectious dose, although the resulting bacterial burdens were elevated about 50-fold relative to old mice receiving the higher infectious dose (**Figure XXXV**).

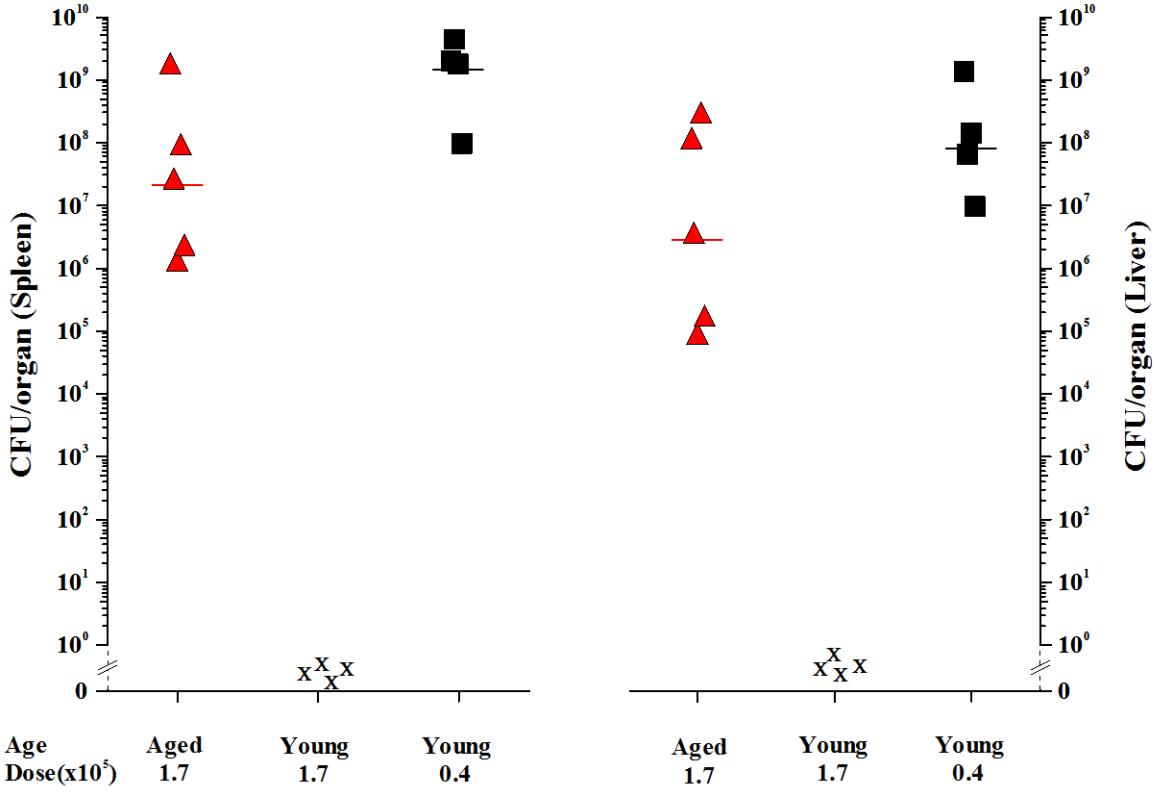


Figure XXXV: Alterations in systemic *Listeria monocytogenes* infection in aged mice. Mice were intravenously inoculated with *L. monocytogenes* at the indicated dosages. Three days post-infection, live animals were euthanized and bacterial burdens in liver and spleen were determined. Mean bacterial burdens from replicate determinations are indicated (line). Infected mice that had died are indicated (**x**).

C. DISCUSSION

We hypothesized that an aging-associated alteration in macrophage responsiveness, especially responsiveness to apoptotic cells (innate apoptotic immunity; IAI), might underlie the paradoxical alterations of immunosenescence, which is characterized by diminished protective responsiveness to exogenous and infectious immunogens (1-7), persistent, elevated levels of cytokines and inflammatory mediators, even in the absence of an overt physiological stress (8-12), and elevated levels of autoimmune antibodies and reactivity to endogenous self-antigens (13, 14). That innate apoptotic immune responses appear to regulate inflammatory responsiveness and self-tolerance motivates this hypothesis. Our focus on macrophages is based on their pivotal role as sentinels for immune surveillance at the nexus of innate and adaptive immunity.

Previous studies (5, 32, 33, 35) have suggested pronounced and dramatic alterations of macrophage function with age. For example, Renshaw *et al.* (35) reported that elicited peritoneal and splenic macrophages from older animals are substantially attenuated in their responses to TLR agonists, expressing much lower levels of TLRs, and secreting significantly lower levels of TNF-α and IL-6 in response to TLR stimulation. Boehmer *et al.* (32, 33) also reported aging-associated alterations in cytokine production from bulk cultures of elicited peritoneal and splenic macrophages stimulated via TLR4 (with LPS) or TLR2 (with zymosan), although they noted no differences in TLR2 and TLR4 expression with age on gated F4/80$^+$ cells.

To our surprise, we did not see substantial aging-associated alterations in TLR responsiveness and cytokine production. The robust and aging-independent responses of macrophages to TLR stimulation that we observed led us to evaluate macrophage responsiveness

to TLR agonists comprehensively. By examining F4/80$^+$ macrophages on the single-cell level, we found that there are no aging-associated macrophage alterations either in TLR responsiveness or in the cell surface expression of different TLRs. We did observe aging-associated changes in the representation of macrophage within recovered cell populations. In particular, we found a dramatic diminution in the fraction of mature macrophages (F4/80$^+$) within the peritoneal lavage, as well as the spleen, as a function of animal age. That the responsiveness of the recovered F4/80$^+$ macrophages specifically was unaltered with age, however, stands in striking contrast to the conclusions of those earlier reports. Our results also provide a perspective from which to reconsider those earlier studies.

Substantively, given the variable, and even minor, representation of macrophages within peritoneal and other cellular preparations, the assessment of responses, including secreted cytokine and specific mRNA determinations, in bulk populations not adjusted for variable macrophage content does not provide an accurate reflection of macrophage function specifically. This population variability underscores the value of single-cell analyses. It is clear that conclusions from previous studies regarding aging-associated macrophage functions drawn from work analyzing cytokine and TLR expression in whole, unfractionated populations (32, 33, 35) are compromised by aging-associated population changes. It is worth noting as well the value of using mice from a single source (and housed together). In our studies, macrophages from inbred mice of a single colony can be compared solely as a function of age. Other work (35) using mice from disparate colonies is further compromised by the introduction of variables independent of age.

Our data document that there are no aging-associated changes in the responsiveness of peritoneal macrophages. These data are consistent with studies in humans (53-54, 75-77) in

which, when examining responsiveness in mononuclear cell specifically, no aging-associated alterations were observed. We did observe modest increases in the basal levels of proinflammatory cytokine expression, concordant with the reported aging-associated elevation in circulating cytokine levels, even in the absence of any overt physiological stress (9-11). These data suggest that macrophages may be involved in this characteristic aspect of immunosenescence.

We also found that the ability of macrophages to interact with apoptotic cells, leading to IAI responsiveness as well as the phagocytosis of the apoptotic targets, is not altered dramatically with age. On the other hand, macrophage responsiveness to apoptotic cells is altered subtly with age. We found that in macrophages, the lifetime of the anti-inflammatory state triggered by encounter with apoptotic cells is prolonged with age. We take this observation to suggest that the ability of macrophages to respond nimbly ("macrophage plasticity") becomes limited in an aging-associated manner. Although we examined only the switch away from an anti-inflammatory state, we speculate that this loss of plasticity may relate as well to a reduced ability of macrophages to dampen a proinflammatory response, and may be causally linked to the hyper-inflammatory state associated with immunosenescence.

These results, highlighting nuanced aging-associated alterations in cellular behaviors, prompt a reconsideration of expectations for changes that may underlie immunosenescence. Aging-associated and immunosenescence-associated alterations in immune function observed organismally may be consequences of subtle effects of aging at the cellular level on the behavior of macrophages (among other cell populations), such as modest alterations in the plasticity of responsiveness. Further studies are needed to examine these behaviors in greater detail, as well as to examine IAI responsiveness in other phagocytic populations in an aging-associated manner,

including the characterization of antigen-presenting function and adaptive immune responsiveness to apoptotic cell-derived antigens.

Cited Literature for Chapter V

1. Shimada Y, Hayashi M, Nagasaka Y, Ohno-Iwashita Y, Inomata M. 2009. Age-associated up-regulation of a negative co-stimulatory receptor PD-1 in mouse CD4+ T cells. *Exp Gerontol.* **44(8)**:517-22.

2. Boldizsar F, Mikecz K, Glant TT. 2010. Immunosenescence and its potential modulation: lessons from mouse models. *Expert Rev Clin Immunol.* **6(3)**:353-7.

3. Stacy S, Krolick KA, Infante AJ, Kraig E. 2002. Immunological memory and late onset autoimmunity. *Mech Ageing Dev*. **123(8)**:975-85.

4. Tseng CW, Kyme PA, Arruda A, Ramanujan VK, Tawackoli W, Liu GY. 2012. Innate immune dysfunctions in aged mice facilitate the systemic dissemination of methicillin-resistant S. aureus. *PLoS One.* **7(7)**:e41454.

5. Plowden J, Renshaw-Hoelscher M, Engleman C, Katz J, Sambhara S. 2004. Innate immunity in aging: impact on macrophage function. *Aging Cell.* **3(4)**:161-7.

6. Cecílio CA, Costa EH, Simioni PU, Gabriel DL, Tamashiro WM. 2011. Aging alters the production of iNOS, arginase and cytokines in murine macrophages. *Braz J Med Biol Res.* **44(7)**:671-81.

7. Goldmann O, Lehne S, Medina E. 2010. Age-related susceptibility to Streptococcus pyogenes infection in mice: underlying immune dysfunction and strategy to enhance immunity. *J Pathol.* **220(5)**:521-9.

8. Ko F, Yu Q, Xue QL, Yao W, Brayton C, Yang H, Fedarko N, Walston J. 2012. Inflammation and mortality in a frail mouse model. *Age (Dordr).* **34(3)**:705-15.

9. Ye SM, Johnson RW. 1999. Increased interleukin-6 expression by microglia from brain of aged mice. *J Neuroimmunol.* **93(1-2)**:139-48.

10. Jeon H, Mun GI, Boo YC. 2012. Analysis of serum cytokine/chemokine profiles affected by aging and exercise in mice. *Cytokine.* **60(2)**:487-92.

11. Singh T, Newman AB. 2011. Inflammatory markers in population studies of aging. *Ageing Res Rev.* **10(3)**:319-29.

12. Pawelec G. 1999. Immunosenescence: impact in the young as well as the old? *Mech Ageing Dev.* **108(1)**:1-7.

13. Goronzy JJ, Weyand CM. 2012. Immune aging and autoimmunity. *Cell Mol Life Sci.* **69(10)**:1615-23.

14. Xavier RM, Yamauchi Y, Nakamura M, Tanigawa Y, Ishikura H, Tsunematsu T, Kobayashi S. 1995. Antinuclear antibodies in healthy aging people: a prospective study. *Mech Ageing Dev.* **78(2)**:145-54.

15. Brüünsgaard H, Pedersen BK. 2003. Age-related inflammatory cytokines and disease. *Immunol Allergy Clin North Am.* **23(1)**:15-39.

16. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci.* **908**:244-54.

17. Surh CD, Sprent J. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature.* **372(6501)**:100-3.

18. Vaux DL. 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci U S A.* **90(3)**:786-9.

19. Henson PM, Johnston RB Jr. 1987. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *J Clin Invest.* **79(3)**:669-74.

20. Cocco RE, Ucker DS. 2001. Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol Biol Cell.* **12(4)**:919-30.

21. Patel VA, Longacre A, Hsiao K, Fan H, Meng F, Mitchell JE, Rauch J, Ucker DS, Levine JS. 2006. Apoptotic cells, at all stages of the death process, trigger characteristic signaling events that are divergent from and dominant over those triggered by necrotic cells: Implications for the delayed clearance model of autoimmunity. *J Biol Chem.* **281(8)**:4663-70.

22. Gray M, Miles K, Salter D, Gray D, Savill J. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci* **104(35)**:14080-5.

23. Birge RB, Ucker DS. 2008. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ.* **15(7)**:1096-102.

24. Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, Tam PK, Savill J. 2008. Apoptotic cells protect mice against lipopolysaccharide-induced shock. *J Immunol.* **180(7)**:4978-85.

25. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. 1997. Immunosuppressive effects of apoptotic cells. *Nature.* **390(6658)**:350-1.

26. Cvetanovic M, Ucker DS. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol.* **172(2)**:880-9.

27. Cvetanovic M, Mitchell JE, Patel V, Avner BS, Su Y, van der Saag PT, Witte PL, Fiore S, Levine JS, Ucker DS. 2006. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. J Biol Chem. 281(29):20055-67.

28. Akira S, Takeda K. 2004. Toll-like receptor signalling. *Nat Rev Immunol.* **4(7)**:499-511.

29. Kawai T, Akira S. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity.* **34(5)**:637-50.

30. Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. *Annu Rev Immunol.* **21**:335-76.

31. Yamamoto M, Takeda K. 2010. Current views of toll-like receptor signaling pathways. *Gastroenterol Res Pract.* **2010**:240365.

32. Boehmer ED, Meehan MJ, Cutro BT, Kovacs EJ. 2005. Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. *Mech Ageing Dev.* **126(12)**:1305-13.

33. Boehmer ED, Goral J, Faunce DE, Kovacs EJ. 2004. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. *J Leukoc Biol.* **75(2)**:342-9.

34. Mahbub S, Deburghgraeve CR, Kovacs EJ. 2012. Advanced age impairs macrophage polarization. *J Interferon Cytokine Res.* **32(1)**:18-26.

35. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. 2002. Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol.* **169(9):**4697-701.

36. Yoon P, Keylock KT, Hartman ME, Freund GG, Woods JA. 2004. Macrophage hypo-responsiveness to interferon-gamma in aged mice is associated with impaired signaling through Jak-STAT. *Mech Ageing Dev.* **125(2)**:137-43.

37. Murray PJ, Wynn TA. 2011. Protective and pathogenic functions of macrophage subsets. **11(11)**:723-37.

38. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* **8(12)**:958-69.

39. Liu C, Li Y, Yu J, Feng L, Hou S, Liu Y, Guo M, Xie Y, Meng J, Zhang H, Xiao B, Ma C. 2013. Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. *PLoS One.* **8(2)**:e54841.

40. Sica A, Mantovani A. 2012. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest.* **122(3)**:787-95.

41. Biswas SK, Mantovani A. 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* **11(10)**:889-96.

42. Johann AM, Barra V, Kuhn AM, Weigert A, von Knethen A, Brüne B. 2007. Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production. *FASEB J.* **21(11)**:2704-12.

43. Andreas Weigert, Nico Tzieply, Andreas von Knethen, Axel M. Johann, Helmut Schmidt, Gerd Geisslinger, Bernhard Brüne. 2007. Tumor Cell Apoptosis Polarizes Macrophages—Role of Sphingosine-1-Phosphate. *Mol Biol Cell*. **18(10)**: 3810–3819.

44. Panda A, Qian F, Mohanty S, van Duin D, Newman FK, Zhang L, Chen S, Towle V, Belshe RB, Fikrig E, Allore HG, Montgomery RR, Shaw AC. 2010. Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. *J Immunol.* **184(5)**:2518-27.

45. Patel PJ. 1981. Aging and cellular defense mechanisms: age-related changes in resistance of mice to Listeria monocytogenes. *Infect Immun.* **32(2)**:557-62.

46. Li G, Smithey MJ, Rudd BD, Nikolich-Žugich J. 2012. Age-associated alterations in CD8α+ dendritic cells impair CD8 T-cell expansion in response to an intracellular bacterium. *Aging Cell*. **11(6)**:968-77.

47. Mitchell LM, Brzoza-Lewis KL, Henry CJ, Grayson JM, Westcott MM, Hiltbold EM. 2011. Distinct responses of splenic dendritic cell subsets to infection with Listeria

monocytogenes: maturation phenotype, level of infection, and T cell priming capacity ex vivo. *Cell Immunol.* **268(2)**:79-86.

48. Matsumoto T, Miake S, Mitsuyama M, Takeya K, Nomoto K. 1979. Augmented resistance to Listeria monocytogenes in mice at an early stage of aging. *J Clin Lab Immunol.* **2(1)**:55-8.

49. Matsumoto T, Miake S, Mitsuyama M, Takeya K, Nomoto K. 1982. Enhanced resistance to Listeria monocytogenes due to non-specifically activated macrophages in aged mice. *J Clin Lab Immunol.* **8(1)**:51-4.

50. Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* **26(4)**:239-57.

51. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest.* **83(3)**:865-75.

52. Devitt A, Marshall LJ. 2011. The innate immune system and the clearance of apoptotic cells. *J Leukoc Biol.* **90(3)**:447-57.

53. Ahluwalia N, Mastro AM, Ball R, Miles MP, Rajendra R, Handte G. 2001. Cytokine production by stimulated mononuclear cells did not change with aging in apparently healthy, well-nourished women. *Mech Ageing Dev.* **122(12)**:1269-79.

54. Beharka AA, Meydani M, Wu D, Leka LS, Meydani A, Meydani SN. 2001. Interleukin-6 production does not increase with age. *J Gerontol A Biol Sci Med Sci*. **56(2)**:B81-8.

55. McKnight AJ, Macfarlane AJ, Dri P, Turley L, Willis AC, Gordon S. 1996. Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family. *J Biol Chem.* **271(1)**:486-9.

56. Hirsch S, Austyn JM, Gordon S. 1981. Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J Exp Med.* **154(3)**:713-25.

57. Austyn JM, Gordon S. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol.* **11(10)**:805-15.

58. Miller RA. 1996. The aging immune system: primer and prospectus. *Science* **273**: 70-74.

59. Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, Monti D, Franceschi C, Paganelli R. 1993. I ncreased cytokine production in mononuclear cells of healthy elderly people. *European Journal of Immunology* **23**:2375-2378.

60. Riancho JA, Zarrabeitia MT, Amado JA, Olmos JM, Gonz lez-Macias J. 1994. Age-related differences in cytokine secretion. *Gerontology* **40**:8-12.

61. Spaulding CC, Walford RL, Effros RB. 1997. Calorie restriction inhibits the age-related dysregulation of the cytokines TNF-alpha and IL-6 in C3B10RF1 mice. *Mechanisms of Ageing and Development* **93**:87-94.

62. Lio D, D'Anna C, Gervasi F, Scola L, Potestio M, Di Lorenzo G, List • F, Colombo A, Candore G, Caruso C. 1998. Interleukin-12 release by mitogen-stimulated mononuclear cells in the elderly. *Mechanisms of Ageing and Development* **102**: 211-219.

63. Bruunsgaard H, Pedersen M, Pedersen BK. 2001. Aging and proinflammatory cytokines. *Current Opinion in Hematology* **8**:131-136.

64. Solana R, Pawelec G, Tarazona R. 2006. Aging and innate immunity. *Immunity* **24**:491-494.

65. Van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, Shaw AC. 2007. Age-associated defect in human TLR-1/2 function. *Journal of Immunolology* **178**:970-975.

66. Pawelec G, Solana R, Remarque E, Mariani E. 1998. Impact of aging on innate immunity. *Journal of Leukocyte Biology* **64**:703-712.

67. Chung EY, Liu J, Homma Y, Zhang Y, Brendolan A, Saggese M, Han J, Silverstein R, Selleri L, Ma X. 2007. Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. *Immunity* **27(6)**:952-64.

68. Tassiulas I, Park-Min KH, Hu Y, Kellerman L, Mevorach D, Ivashkiv LB. 2007. Apoptotic cells inhibit LPS-induced cytokine and chemokine production and IFN responses in macrophages. *Hum Immunol* **68(3)**:156-64.

69. Chung EY, Kim SJ, Ma XJ. 2006. Regulation of cytokine production during phagocytosis of apoptotic cells. *Cell Res* **16(2)**:154-61.

70. Zhang Y, Kim HJ, Yamamoto S, Kang X, Ma X. 2010. Regulation of interleukin-10 gene expression in macrophages engulfing apoptotic cells. *J Interferon Cytokine Res* **30(3)**:113-22.

71. Kim S, Chung EY, Ma X. 2005. Immunological consequences of macrophage-mediated clearance of apoptotic cells. *Cell Cycle* **4(2)**:231-4.

72. Lau LL, Jamieson BD, Somasundaram T, Ahmed R. 1994. Cytotoxic T-cell memory without antigen. *Nature* **369(6482)**:648-52.

73. Murali-Krishna K, Ahmed R. 2000. Cutting edge: naive T cells masquerading as memory cells. *J Immunol* **165(4)**:1733-7.

74. Vezys V, Yates A, Casey KA, Lanier G, Ahmed R, Antia R, Masopust D. 2009. Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* **457(7226)**:196-9.

75. Candore G, Di Lorenzo G, Melluso M, Cigna D, Colucci AT, Modica MA, Caruso C. 1993. gamma-Interferon, interleukin-4 and interleukin-6 in vitro production in old subjects. *Autoimmunity* **16(4)**:275-80.

76. Amadori A, Zanovello P, Cozzi E, Ciminale V, Borghesan F, Fagiolo U, Crepaldi G. 1988. Study of some early immunological parameters in aging humans. *Gerontology* **34(5-6)**:277-83.

77. Corberand JX, Laharrague PF, Fillola G. 1986. Neutrophils of healthy aged humans are normal. *Mech Ageing Dev* **36(1)**:57-63.

CHAPTER VI

PERSPECTIVE

A. Overview

I explored three distinct aspects of innate apoptotic immunity (IAI) in depth. These disparate avenues of investigation converged in surprising ways to shed new perspectives on issues of pathogenesis, especially relating to the subversion of IAI (apoptotic mimicry), and on aging-associated immune alterations generally.

My exploration of the apoptotic regulation of nitric oxide (NO) production (Chapter III), beyond documenting the complex transcriptional regulation that attenuates NO synthesis both by diminishing expression of NO synthetic genes and up-regulating the expression of genes involved in alternate utilization of precursors, highlighted the important role of IAI in NO regulation and the value to pathogens of subverting IAI to enhance pathogenesis.

Studies to characterize the conserved molecular determinants on the surface of apoptotic cells that trigger innate apoptotic immune responses (Chapter IV) led to the identification of the externalization of glycolytic enzyme molecules as a common and early aspect of cell death in different cell types triggered to die with distinct suicidal stimuli. These results, too, underscored the connection between IAI and pathogenesis. Numerous bacterial and protozoan pathogens present glycolytic enzyme molecules on their surface. It has been argued previously that the presence of these surface molecules enhances pathogenesis by serving as binding and activation sites for plasminogen, our data suggest a radically different interpretation, relating to the suppression of host immunity. This work also poses questions regarding the relationship between cellular metabolism (especially glycolysis) and the robustness of immune suppression.

Finally, my investigation of IAI responsiveness as a function of animal age (Chapter V) revealed the absence of gross aging-associated deficits, either in IAI or in TLR responsiveness. On the other hand, my studies did point to nuanced aging-associated effects, which may serve to

alter profoundly immune status with age. This is, subtle changes, as distinct from absolute deficits, in responsiveness may underlay the complex aging-associated alterations of immunosenescence. For example, the modest increases in the basal levels of pro-inflammatory cytokine expression which I observed could represent a consequence of a diminished capacity of cells from older animals to dampen previous inflammatory responses (the loss of "plasticity").

Independently, previous work has emphasized the phenomenon of glycation as an aging-associated pathology, especially given its potential role in promoting inflammation. Together with my Chapter IV work, accenting the connection between glycolysis and IAI, this promotes renewed interest in the role of glycolytic intermediates in glycation. Although we observed that surface glycolytic enzyme molecules lacked enzymatic activity, the possibility exists that low levels of activity could contribute significantly to surface glycation. It may be worthwhile to revisit this issue in greater detail. Still, of greatest significance, I found that the immunosuppressive effect of apoptotic cells persists independently of animal age, and that it is dominant to any aging-associated pro-inflammatory effects (including glycation).

B. Recognition of apoptotic cells

During infection, there is a massive influx of inflammatory cells to the site of infection resulting in a robust immune response. The infected site is now in a state of inflammation which is soon resolved under normal physiological conditions. John Hunter, a Scottish surgeon in 1794, stated "Inflammation in itself is not to be considered as a disease but as a salutary operation consequent to some violence or some disease" (1). The acute inflammation state is soon followed by a resolution of inflammation, usually assisted by the help of anti-inflammatory mediators, as well as cell death (typically apoptosis) followed by clearance of inflammatory cells. More recently, it has come to our understanding that one more mechanism for resolution

of inflammation is the recognition of these apoptotic inflammatory cells by neighboring cells (31-33). The interaction of neighboring macrophages (amongst other cell types) with apoptotic cells then pushes them towards an "M2" phenotype; a term used for macrophages which have an anti-inflammatory phenotypic state, resulting in immune regulation and wound healing.

The physiological role of apoptosis in the resolution of inflammation encompasses - the initiation of apoptosis, the engulfment by professional phagocytes and finally the initiation of signaling events in the interacting cells triggering a dampening of proinflammatory signals and resolution of residual inflammation (2, 34-37). It is likely that any abnormalities and interferences in this immunological balance might trigger complications, including those of chronic inflammatory lung disease and atherosclerosis (3-7).

C. Apoptosis and apoptotic mimicry as an immune subversion strategy of microbial pathogens

I have mentioned above and described in detail in Chapter III that apoptotic cells are actively able to attenuate and suppress inflammatory responses. I also described and discussed in Chapter IV our novel findings of the externalization of glycolytic enzyme molecules on the surface of apoptotic cells. It is interesting that many pathogens rely on evading host immune responses by utilizing many mechanisms to either directly evade detection by the host immune system or by actively suppressing an active inflammation against itself. Thus, the immunomodulatory nature of apoptotic cells in itself is a prime target for pathogens to target and mimic for immune evasion, and indeed pathogen throughout the course of evolution have devised such strategies.

Indeed, there is a growing list of pathogens that induce apoptosis, resulting in their growth advantage in the hosts and heightened infectivity, both Gram-positive (*Bacillus anthracis,*

Streptococcus pneumonia, *Listeria monocytogenes*, *Clostridium difficile*, etc.); Gram-negative (*Yersinia pseudotuberculosis*, *Staphylococcus aureus*, etc.) bacterial species; as well as other pathogens like *Coxiella burnetii*, *Leishmania amazonensis*, and *Trypanosoma cruzi* (8-23). In all of these cases, pathogens either directly (mimicking apoptotic cells by exposure of glycolytic enzyme molecules (and other molecules externalized during apoptosis) or indirectly (by triggering and causing apoptosis in the infected organs, thereby calming inflammation and allowing the pathogen to establish a niche) utilize the evolutionarily conserved process of apoptosis and its immunomodulatory effect to increase replication and infectivity making the host susceptibility to infection.

Most of the systems that have been examined thus far, while counterintuitive, have shown a correlation between apoptosis and mimicry of apoptotic cells with bacterial pathogenesis. While these systems allow us to appreciate the importance of apoptosis in a timely manner, they are yet to provide definitively a direct link between apoptosis, apoptotic immunomodulation and bacterial pathogenesis. And indeed, when we try to skew the immune system *in vivo* by introducing apoptotic cells to mice infected with *L.monocytogenes* we see an increased susceptibility to the host to infection (**Figure XXXVI**).

It is clear that apoptosis is an important component of the immune system: necessary and beneficial in scenarios of resolution and normal homeostasis, and detrimental to the organism in cases of active pathogenic infections. We propose that apoptotic mimicry and induction of apoptosis by pathogens have co-evolved as a mechanism for modulating the immune response in order for the pathogen to gain a selective advantage during infection in the host. The interplay between host programs of apoptosis, apoptotic induction by pathogens and apoptotic mimicry by pathogens is of importance and something we would like to study in the future.

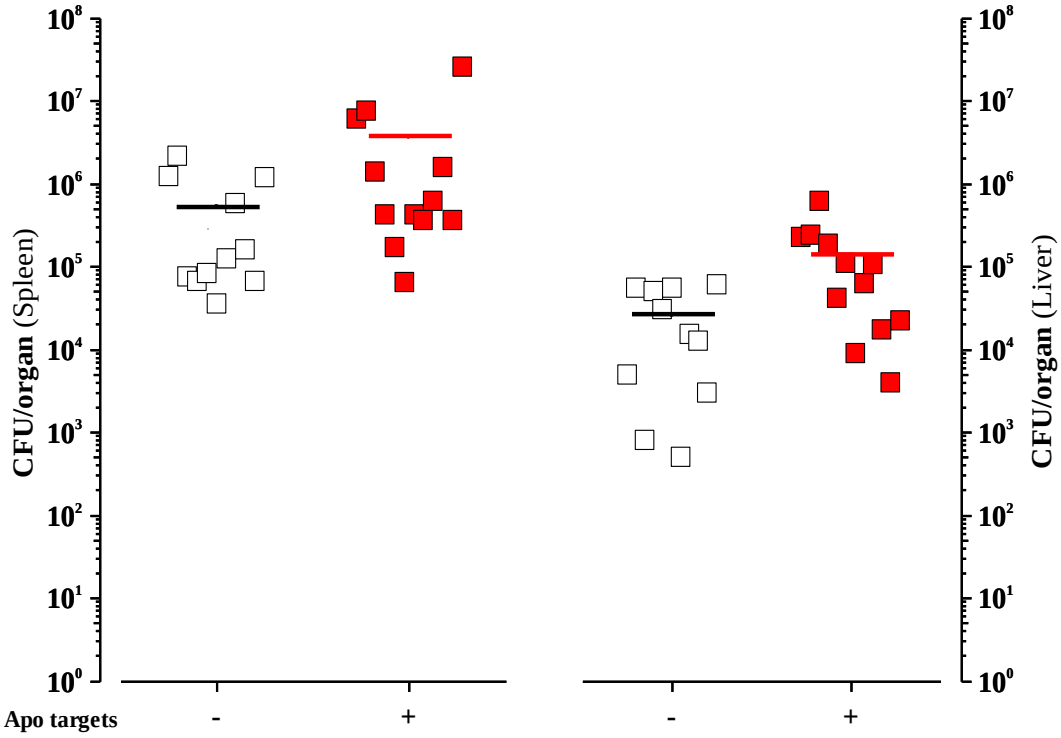


Figure XXXVI: ***in-vivo*** **setting to study apoptotic immunomodulation.** Mice were inoculated intravenously with 2×10^7 apoptotic splenocytes in 200 µl saline (or saline alone as a control) and then challenged with 1×10^4 CFU of *L. monocytogenes* ("Lmo") after 24 hr. (also inoculated intravenously). Bacterial burdens were determined in liver and spleen three days post-infection.

D. Clinical applications of Innate Apoptotic Immunity

It is now being well recognized in the field of transplantation that phenomenon of apoptotic cell clearance is immunomodulatory. The most common uses of apoptotic cells in a clinical setting have been in the field of transplantation immunology, for example in the application termed “photopheresis”. One of the ultimate goals in transplantation is to induce a state of donor-specific tolerance to reduce graft rejection. Utilizing the phenomenon of apoptotic cells down-modulation of proinflammatory functions of DC and macrophages and the tolerogenic interaction between apoptotic cells and antigen presenting cells, it has been shown in murine experimental systems and in humans, to help against transplant rejection (24-26, 30). In studying the mechanism behind generating tolerance against graft, a group recently reported that administration of apoptotic cells inhibits the activity of Th_{17} cells via down-regulation of inflammatory cytokine production, thereby potentially providing a lack of responsiveness towards graft derived antigens.

E. Conclusion

There is an increased recognition for the importance of apoptotic-cell mediated regulation in physiological and patho-physiological conditions. A detailed mechanistic understanding how apoptotic cells influence immunity is worthwhile, as it potentially can play a role in many medicinal as well as therapeutic approaches targeting these signaling pathways.

Cited Literature for Chapter VI

1. Serhan CN, Savill J. 2005. Resolution of inflammation: the beginning programs the end. *Nat Immunol.* **6(12)**:1191-7.

2. Weigert A, Jennewein C, Brüne B. 2009. The liaison between apoptotic cells and macrophages--the end programs the beginning. *Biol Chem.* **390(5-6)**:379-90.

3. Liang CP, Han S, Senokuchi T, Tall AR. 2007. The macrophage at the crossroads of insulin resistance and atherosclerosis. *Circ Res*. **100(11)**:1546-55.

4. Vandivier RW, Henson PM, Douglas IS. 2006. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest.* **129(6)**:1673-82.

5. McCubbrey AL, Curtis JL. 2013. Efferocytosis and lung disease. *Chest.* **143(6)**:1750-7.

6. Opferman JT, Korsmeyer SJ. 2003. Apoptosis in the development and maintenance of the immune system. *Nat Immunol*. **4(5)**:410-5.

7. Morimoto K, Janssen WJ, Terada M. 2012. Defective efferocytosis by alveolar macrophages in IPF patients. *Respir Med.* **106(12)**:1800-3.

8. Kemp K, Bruunsgaard H, Skinhøj P, Klarlund Pedersen B. 2002. Pneumococcal infections in humans are associated with increased apoptosis and trafficking of type 1 cytokine-producing T cells. *Infect Immun*. **70(9)**:5019-25.

9. Braun JS, Sublett JE, Freyer D, Mitchell TJ, Cleveland JL, Tuomanen EI, Weber JR. 2002. Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. *J Clin Invest*. **109(1)**:19-27.

10. Popov SG, Villasmil R, Bernardi J, Grene E, Cardwell J, Popova T, Wu A, Alibek D, Bailey C, Alibek K. 2002. Effect of Bacillus anthracis lethal toxin on human peripheral blood mononuclear cells. *FEBS Lett.* **527(1-3)**:211-5.

11. Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K, Jänicke RU. 2001. alpha-Toxin is a mediator of Staphylococcus aureus-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J Cell Biol.* **155(4)**:637-48.

12. Jonas D, Walev I, Berger T, Liebetrau M, Palmer M, Bhakdi S. 1994. Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect Immun.* **62(4)**:1304-12.

13. Mahida YR, Galvin A, Makh S, Hyde S, Sanfilippo L, Borriello SP, Sewell HF. 1998. Effect of Clostridium difficile toxin A on human colonic lamina propria cells: early loss of macrophages followed by T-cell apoptosis. *Infect Immun*. **66(11)**:5462-9.

14. Monack DM, Mecsas J, Bouley D, Falkow S. 1998. Yersinia-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. *J Exp Med.* **188(11)**:2127-37.

15. Ulett, G.C. and Adderson, E.E. 2006. Regulation of apoptosis by Grampositive bacteria: mechanistic diversity and consequences for immunity. *Curr. Immunol. Rev*. **2**:119–141

16. de Freitas Balanco JM, Moreira ME, Bonomo A, Bozza PT, Amarante-Mendes G, Pirmez C, Barcinski MA. 2001. Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. *Curr Biol.* **11(23)**:1870-3.

17. Wanderley JL, Moreira ME, Benjamin A, Bonomo AC, Barcinski MA. 2006. Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of Leishmania (L) amazonensis in mammalian hosts. *J Immunol*. **176(3)**:1834-9.

18. Zamboni DS, Rabinovitch M. 2004. Phagocytosis of apoptotic cells increases the susceptibility of macrophages to infection with Coxiella burnetii phase II through down-modulation of nitric oxide production. *Infect Immun.* **72(4)**:2075-80.

19. Benoit M, Ghigo E, Capo C, Raoult D, Mege JL. 2008. The uptake of apoptotic cells drives Coxiella burnetii replication and macrophage polarization: a model for Q fever endocarditis. *PLoS Pathog*. **4(5)**:e1000066.

20. Carrero JA, Unanue ER. 2006. Lymphocyte apoptosis as an immune subversion strategy of microbial pathogens. *Trends Immunol.* **27(11)**:497-503.

21. Carrero JA, Calderon B, Unanue ER. 2006. Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes. *J Exp Med.* **203(4)**:933-40.

22. Carrero JA, Unanue ER. 2007. Impact of lymphocyte apoptosis on the innate immune stages of infection. *Immunol Res.* **38(1-3)**:333-41.

23. Carrero JA, Unanue ER. 2012. Mechanisms and immunological effects of apoptosis caused by Listeria monocytogenes. *Adv Immunol*. **113**:157-74.

24. Morelli AE, Larregina AT. 2010. Apoptotic cell-based therapies against transplant rejection: role of recipient's dendritic cells. *Apoptosis.* **15(9)**:1083-97.

25. Morelli AE. 2006. The immune regulatory effect of apoptotic cells and exosomes on dendritic cells: its impact on transplantation. *Am J Transplant.* 6(2):254-61.

26. Saas P, Gaugler B, Perruche S. 2010. Intravenous apoptotic cell infusion as a cell-based therapy toward improving hematopoietic cell transplantation outcome. *Ann N Y Acad Sci.* **1209**:118-26.

27. Zhou F, Lauretti E, di Meco A, Ciric B, Gonnella P, Zhang GX, Rostami A. 2013. Intravenous transfer of apoptotic cell-treated dendritic cells leads to immune tolerance by blocking Th17 cell activity. *Immunobiology*. **218(8)**:1069-76.

28. da Costa TB, Sardinha LR, Larocca R, Peron JP, Rizzo LV. 2011. Allogeneic apoptotic thymocyte-stimulated dendritic cells expand functional regulatory T cells. *Immunology* **133**:123–132.

29. Gleisner, M.A., Rosemblatt, M., Fierro, J.A., Bono, M.R. 2011. Delivery of alloantigens via apoptotic cells generates dendritic cells with an immature tolerogenic phenotype. *Transplant. Proc.* **43:**2325–2333.

30. McCubbrey AL, Curtis JL. 2013. Efferocytosis and lung disease. *Chest*. **143(6)**:1750-7.

31. Mukaro VR, Hodge S. 2011. Airway clearance of apoptotic cells in COPD. *Curr Drug Targets.* **12(4)**:460-8.

32. Medeiros AI, Serezani CH, Lee SP, Peters-Golden M. 2009. Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling. *J Exp Med.* **206(1)**:61-8.

33. Liacos C, Konstadoulakis MM, Economou V, Katsaragakis S, Chatzigianni E, Georgiadis GG, Prekates A, Karampinis A, Bramis J. 2008. Increased apoptosis in the alveolar microenvironment of the healthy human lung. *J Surg Res.* **145(2)**:186-91.

34. Dockrell DH, Marriott HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, Whyte MK. 2003. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol.* **171(10)**:5380-8.

35. Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, van Rooijen N, van der Poll T. 2003. Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. *Am J Respir Crit Care Med.* **167(2)**:171-9.

36. Ishii Y, Hashimoto K, Nomura A, Sakamoto T, Uchida Y, Ohtsuka M, Hasegawa S, Sagai M. 1998. Elimination of neutrophils by apoptosis during the resolution of acute pulmonary inflammation in rats. *Lung.* **176(2)**:89-98.

37. Cox G, Crossley J, Xing Z. 1995. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. *Am J Respir Cell Mol Biol.* **12(2)**:232-7.

VITA

EDUCATION

Ph.D. (anticipated), Microbiology and Immunology (2013)
Department of Microbiology and Immunology, College of Medicine
University of Illinois, Chicago (UIC)

M. S., Biological Sciences with concentration in Biotechnology (2007)
Department of Molecular Biology and Biochemistry, School of Biological Sciences
University of California, Irvine (UCI)

B. Tech., Biotechnology (2005)
School of Biosciences and Technology
Vellore Institute of Technology (VIT), India

PUBLICATIONS

Ucker DS, Jain MR, **Pattabiraman G**, Palasiewicz K, Birge RB, Li H. (2012). Externalized glycolytic enzymes are novel, conserved, and early biomarkers of apoptosis. *J. Biol. Chem.* **287**(13):10325-10343.

Patel VA, Feng L, Lee DJ, Massenburg D, **Pattabiraman G**, Antoni A, Schwartz JH, Lieberthal W, Rauch J, Ucker DS, Levine JS. (2012). Recognition-dependent signaling events in response to apoptotic targets inhibit epithelial cell viability by multiple mechanisms: implications for non-immune tissue homeostasis. *J. Biol. Chem.* **287**(17):13761-13777.

Pattabiraman G, Lidstone EA, Palasiewich K, Cunningham BT, Ucker DS. Innate apoptotic cell recognition by viable cells: analysis using photonic crystal biosensors. (*Manuscript in preparation: expected submission Oct'13*)

Pattabiraman G, Galvin J, Palasiewicz K, Ucker DS. Nuanced alterations in innate apoptotic immune responses with age implicate the dynamics of macrophage responsiveness as a significant parameter in immunosenescence. (*Manuscript in preparation: expected submission Dec'13*)

Pattabiraman G, Palasiewicz K, Ucker DS. Toll-like receptor function of macrophages is not impaired with increasing age. (*Manuscript in preparation*)

POSTERS

Pattabiraman G, Hagen KL, and Ucker DS. Macrophages respond homogeneously to apoptotic cell-mediated inflammatory suppression: cytofluorimetric analysis of macrophage pro-inflammatory cytokine expression. *Great Lakes International Imaging and Flow Cytometry Association (GLIIFCA) meeting (2009)*

Pattabiraman G, Lidstone EA, Palasiewicz K, Cunningham BT, and Ucker DS. An innovative biosensor-based approach to assess specific apoptotic recognition. *Ninth Annual Chicago Biomedical Consortium (CBC) Symposium (2011)*

Pattabiraman G, and Ucker DS. Apoptotic cells attenuate NO production in macrophages through complex transcriptional regulation of iNOS and Arginase II. *UIC College of Medicine Research Forum (2011)*

ACADEMIC HONORS AND AWARDS

VIT Ignite Scholarship (2001-2004) – Awarded to top 1% students for academic excellence
Vellore Institute of Technology (VIT), India.

1st Place Award – Flow Cytometry Competition *(2009)*
Research Resources Center (Flow Cytometry Service), University of Illinois at Chicago

Outstanding Poster Award / Travel Stipend Award – *GLIIFCA meeting (2009)*